Antioxidant activity and physiological bases of recovery from cold stress

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ABSTRACT

- Tolerance to low temperatures is an important trait considering that the sunflower production area is expanding to marginal regions with suboptimal growing conditions and the increasing requirement of early sowing to maximize the growing season in countries such as the Mediterranean Area, North America, India and Argentina. The present study of the response of sunflower to low temperatures focused on the primary responses of young plants to 72 h treatment at 5°C with the aim of detecting regulatory mechanisms induced at this early stage.
- Studying the antioxidant activity and physiological bases involved in recovery from cold stress in sunflower (*Helianthus annus* L.) seedlings may allow these characteristics to be used in breeding programs aimed at selecting varieties of sunflower adapted to stress from suboptimal temperatures.
- The purpose of this research was to establish the recovery from cold stress in terms of the activity of the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), and its relationship with the physiological response of two sunflower hybrids to the contrasting response to cold stress.
- Prior studies in the Plant Physiology Laboratory (FCA-UNC) identified two sunflower hybrids with contrasting response to cold at the germination stage: sensitive the hybrid Pampero (PM) and tolerant the hybrid Sierra Alto Oleico (SAO). Ten-day old seedlings of commercial Pampero (PM) and Sierra Alto Oleico (SAO) hybrids were placed in cold storage for 72 hours at 5°C, and cold stress recovery was assessed in terms of the following variables: Level of Damage to Cell Membranes through the content of malondialdehyde (MDA), Antioxidant Enzyme Activity: Superoxide Dismutase (SOD) and Catalase (CAT), and Chlorophyll Content at 0, 24, 48 and 72 hours after exposure to cold. In addition, Total Plant Dry Mass and Leaf area were determined per plant. A split-split plot experimental design was used for the trials, where the main plot factor is the Genotype, that of the subplot is the corresponding treatment, and that of the sub-subplot is the sampling time. Each treatment had n = 30 individuals per genotype, with three independent replicates. For statistical analysis, the INFOSTAT Professional 2010 program was used. The information generated was subjected to analysis of variance and the DGC means comparison test with $p<0.05$.
- The response to cold stress was greater in the SAO than the PM hybrid, suggesting that the former possesses repair mechanisms at the cell level which are activated more quickly in response to low temperature. This coordination and the activity levels of the SOD and CAT enzymes found in this study in Sierra Alto Oleico match the lower level of cell damage (expressed in lower MDA levels), compared to Pampero. * Higher antioxidant activity and lower MDA levels allow sunflower plants to keep their photosynthesizing apparatus active, maintaining the functionality of chlorophyll for dry matter production, and leaf area during the early stages of growth, after exposure to cold stress.
- This research helps to explain for the first time in sunflower crops the physiological mechanisms and the activity of the main antioxidant defense enzymes (SOD and CAT) involved in recovery from cold stress in contrasting response sunflower genotypes. All the variables described here can be used as criteria for screening cold-stress tolerant sunflower hybrids.

Key words: genotypes, low temperature, early sowing, physiological traits, oxidative stress, antioxidant defense.

INTRODUCTION

The capacity of sunflower plants to recover after a period of low temperatures is a subject little studied. Tolerance to low temperatures is an important feature for the crop, bearing in mind that the area of sunflower production is expanding to marginal regions with suboptimal growing conditions. The study of the antioxidant activity and physiological bases involved in recovery from cold stress in sunflower seedlings (Helianthus annus L.) can enable these characteristics to be used in breeding programs for selection of genotypes adapted to the cold.

Otegui and Lopez Pereira (2006) state that the damage in sunflower leaves, produced by cold during early phases, delays crop growth. There are physiological traits such as the chlorophyll content and the plant dry weight which are positively associated with tolerance to cold stress. A study in sunflower by Hewezi et al. (2006) showed that low temperatures alter plant morphology due to changes produced in some physiological mechanisms, such as protein and sugar biosynthesis. They noted a decrease in the expression of genes that code for glutamate and for sugar (sucrose, fructose and mannose) synthesis, compounds that are involved in carbon fixation. There is a marked difference in plant development in those genotypes that are cold-tolerant because these recover photosynthetic activity earlier than the sensitive genotypes. This increased photosynthetic activity in low temperature conditions means greater production of sugars that could act as a cryoprotection mechanism. These results provide important elements in the differentiation of sensitive genotypes and tolerant genotypes.

The stress leads to damage at cell membrane level that is shown through the content of malondialdehyde (MDA) and also in traits associated with growth. These parameters have been used as selection criteria in different genotypes of *Chloris gayana* K. and *Cenchrus ciliaris* L under conditions of high temperature, salinity and drought (Griffa et al., 2010, Lanza Castelli et al., 2010, Luna et al., 2000), but there is no information regarding the use of these indicators for screening sunflower genotypes tolerant to cold stress.

In abiotic stress conditions, reactive oxygen species (ROS) are generated by metabolic processes, acting as change signals that regulate the expression of genes (Pei et al., 2000; Desikan et al., 2004; Pastori and Foyer., 2002; Mittler et al., 2004, Shin and Schachtman., 2004) and ion channel activity (Foreman et al., 2003). In higher plants, excessive production of ROS is an intrinsic feature of a stressed metabolism under various types of stress. Response mechanisms to stress are complemented by the production of antioxidant enzymes. These are a group involved in the elimination of ROS. The main anti-stress enzymes are: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). Insufficient elimination of ROS produces oxidative stress, which is characterized by harmful reactions of ROS with biologically important macromolecules such as proteins, lipids and DNA, and this can cause cell damage (Inze and Van Montagu, 1995). Based on this analysis, the following working hypothesis was posed: *"The genotypes tolerant to cold stress in sunflower (Helianthus agnus L.) have a high activity of antioxidant enzymes (SOD and CAT) which reduces cell damage, maintaining a constant level of chlorophyll and increase in dry matter"*

The aim of this research was to study the antioxidant activity and the physiological bases involved in recovery from cold stress in sunflower (*Helianthus annus L.*), linking the activity of antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) with the level of cell damage (MDA), chlorophyll content, increased dry matter and leaf area in genotypes with contrasting responses to cold during the initial stages.

MATERIALS AND METHODS

Commercial hybrids selected were Pampero (PM) and Sierra Alto Oleico (SAO), because these are traditional in the sunflower farming areas in Argentina. In previous studies in the Laboratory of Plant Physiology, Faculty of Agricultural Sciences of the UNC, these hybrids showed a differential response to cold (Fabio *et al*., 2010 communication to Congress): SAO tolerant and PM sensitive to cold.

For this research, commercial hybrid PM and SAO seeds were sown in sterile fine sand in aluminum trays, 11 cm deep by 9 cm in diameter, and placed in a seed germination chamber at 20-30°C, and 16-hour light 8-hour dark photoperiod for 10 DDS (ISTA, 2010). After the 10 days growth in germination chamber, a group of seedlings of each genotype were placed in a cold chamber at a temperature of 5ºC with a photoperiod of constant light of 3600 MJ/day for 4 days (PMF and SAOF). Two other groups of seedlings continued growing under optimal conditions in germination chamber, to act as control groups (PMC and SAOC).

Determination of antioxidant activity and oxidative damage: The antioxidant activity determinations were performed on seedlings of each genotype (cotyledons, leaves and stems) at 0, 24 and 72 hs after 4 days of cold exposure and the activity of the superoxide dismutase (SOD) enzyme was quantified in USOD.mg protein according to the Beauchamp and Fridovich technique (1973); the activity of the enzyme catalase (CAT) µmol H2O2.min.mg protein according to Aebi (1984), and the malondialdehyde (MDA) content mmol/.mg fresh weight using the techniques described by Heath and Parker (1968), Dhidsa et al. (1981) and Hodges et al. (1999). CAT and

SOD activity were expressed as specific activity relative to total protein content, determined according to the measurement technique described by Bradford (1976) and Sedmark and Grosseberg (1977). The same measurements were performed in controls.

Determination of physiological variables: Chlorophyll was measured by extraction of segments in a boiling solution of 80% ethanol with spectrophotometer at an absorbance of 640 nm (Tetley and Thimann 1974) at 0, 24, 48 and 72 h after exposure to cold and expressed in µgr.ml. At the end of the assay, leaf area (cm2.pl) and total dry weight (mg.pl) were measured in the plants.

Statistical analysis: For testing, a split-plot experimental design was used with 3 replications, where the main plot factor is the genotype, that of the subplot the corresponding treatment, and that of the sub-sub-plot, the time of sampling. Each treatment had n=30 individuals per genotype, with three independent replicates. For statistical analysis, the INFOSTAT Professional 2010 program was used (Di Rienzo *et al* 2010; FCA-UNC, Argentina). The information generated was subjected to analysis of variance and DGC means comparison test with $p<0.05$.

RESULTS AND DISCUSSION

ANTIOXIDANT ACTIVITY AND OXIDATIVE DAMAGE:

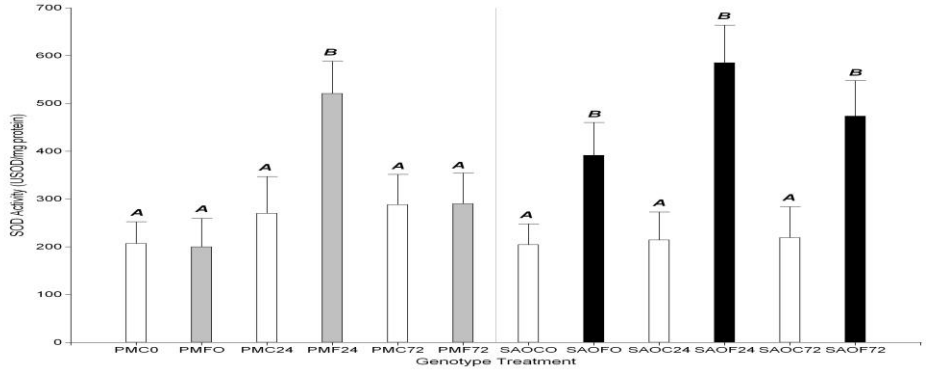

*Fig 1: Activity of the Superoxide dismutase (SOD) enzyme during the cold stress recovery period at 0, 24 and 72 hours after exposure to low temperature of 5°C. PMC: Pampero Control, PMF: Pampero Cold, SAOC:Sierra Alto Oleico Control, SAOF: Sierra Alto Oleico Cold.*Identical letters indicate there is no significant difference between treatments p<0.05*

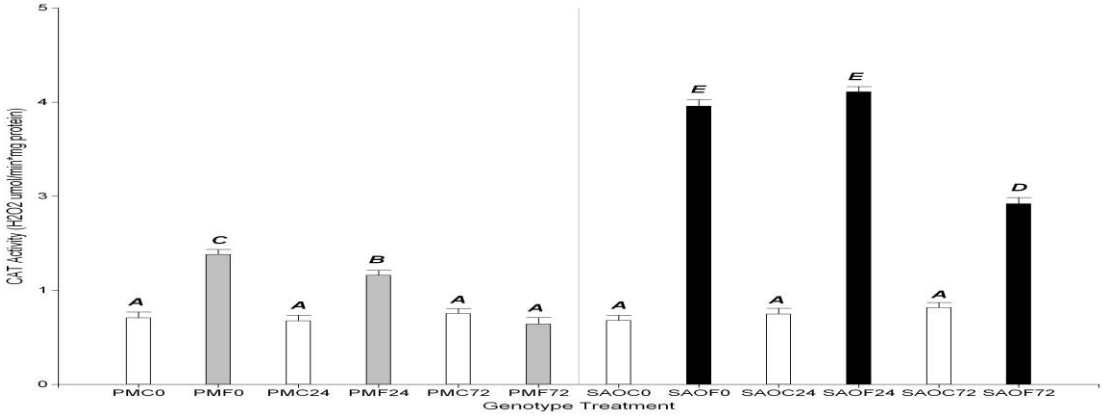

*Fig 2: Activity of the catalase (CAT) enzyme during the cold stress recovery period at 0, 24 and 72 hours after exposure to low temperature of 5°C. PMC: Pampero Control, PMF: Pampero Cold, SAOC: Sierra Alto Oleico Control, SAOF: Sierra Alto Oleico Cold. * Identical letters indicate there is no significant difference between treatments p<0.05*

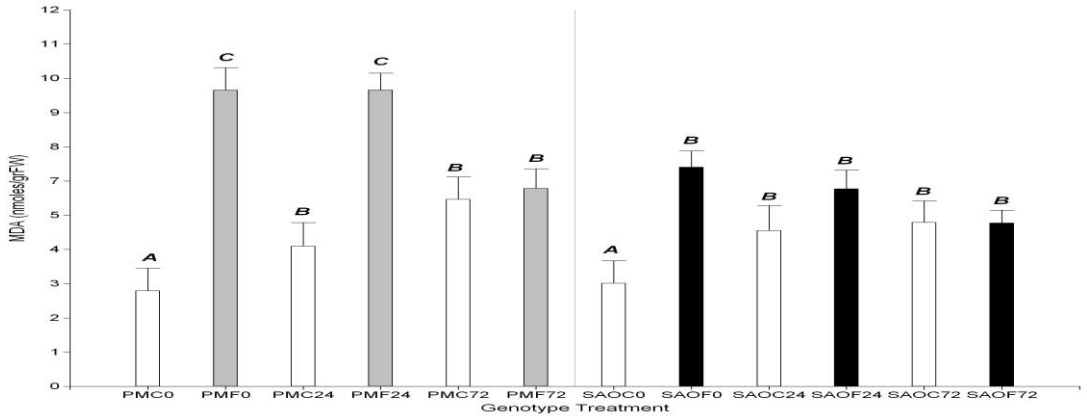

Fig 3: Damage to Cell Membranes quantified as of Malondialdehyde (MDA) content during the cold stress recovery period in sunflower hybrids of contrasting response. White bars: MDA of the control, gray bars: MDA of PM; black bars: MDA of SAO. PMC: Pampero Control, PMF: Pampero Cold, SAOC: Sierra Alto Oleico Control, SAOF: Sierra Alto Oleico Cold. * *Identical letters indicate there is no significant difference between treatments p<0.05*

PHYSIOLOGICAL VARIABLES:

Table 1: Chlorophyll content (µgr.ml) after exposure to suboptimal temperatures of 5ºC at different moments. PMC: Pampero Control, PMF: Pampero Cold, SAOC: Sierra Alto Oleico Control, SAOF: Sierra Alto Oleico Cold.

	CHLOROPHYLL CONTENT (µgr.ml)			
Hs.	**PMC**	**PMF**	**SAOC**	**SAOF**
0	**1.922 b**	**1.143 a**	**1.231 a**	**0.829 a**
24	**2.305 b**	**1.626 a**	**1.344 a**	**1.212 a**
48	**1.637a**	**1.866 a**	**1.290 a**	**1.239 a**
72	**1.637 a**	**1.865 a**	**1.289 a**	**1.239 a**

* Identical letters in the same row indicate there is no significant difference between treatments p<0.05

Table 2: Total Biomass (Total Plant Dry Mass) and Total Leaf Area of sunflower hybrid seedlings of contrasting response to cold after being subjected to temperatures of 5°C for 4 days.

Treatment	**Total Dry Weight (mg.pl)**	**Leaf Area per plant cm2.pl**
PMC: Pampero Control	**58.01 b**	**2.6073 a**
PMF: Pampero Cold	**42.7 a**	**2.6237 a**
SAOC: Sierra Alto Oleico Control	**71.1 b**	**3.0207 b**
SAOF: Sierra Alto Oleico Cold	**59.2 a**	**2.402 a**

* Identical letters in the same row indicate there is no difference between treatments p <0.05

DISCUSSION:

The results showed a relationship between the antioxidant enzymes, SOD and CAT, with the level of cell damage (MDA), chlorophyll content and total dry weight during the cold stress recovery period in genotypes of contrasting response. The results of this research show that the SAO genotype shows higher SOD and CAT antioxidant activity (Fig. 1 and Fig 2), which enables less membrane damage, expressed by the content of MDA (Fig. 3). The balance between SOD and CAT is crucial in determining the relative stationary concentration of superoxide radicals and hydrogen peroxide (Milter et al., 2004). The activity of the superoxide dismutase enzyme proved extremely sensitive to low temperature and this phenomenon was related to the protection of chloroplasts in both genotypes. This response is similar to results obtained in *Chloris gayana* K. and *Cenchrus ciliaris* L. which have shown that stress-tolerant plants are usually equipped with effective antioxidant defense systems (Jagtap and Bhargava, 1995). Recent evidence indicates a relationship between increased SOD, APX, CAT, DHAR and GR activity under salt-stress conditions and other stresses such as temperature and drought, with the increase being even more significant in the species and varieties that are tolerant to these stresses (Hernández et al., 2001; Sairam

et al., 2004). Transgenic plants that produce excessive antioxidant enzymes, e.g., superoxide dismutase and glutathione reductase, have also been associated with improved tolerance to stress (Allen et al., 1997; Aono et al., 1995). The accumulation of certain metabolites and changes in the activity of antioxidant enzymes such as catalase and peroxidase may be related to the potential of a plant to counteract the harmful effect of abiotic stress in different physiological and morphological processes (Fuentes, 2008)

In relation to MDA content, results in megathermal herbage have shown the presence of malondialdehyde (MDA), a product of membrane lipid peroxidation, as a precise criterion for the selection of genotypes tolerant to stress. For example, in *Chloris gayana* K. and *Cenchrus ciliaris* L genotypes, tolerant to salt stress, a lower level of MDA was observed in the leaf content (Luna et al., 2000, 2002; Lanza Castelli et al., 2010; Griffa et al., 2010). The results obtained indicate that the cause of the higher MDA content in the PM genotype is due, among other things, to the accumulation of hydrogen peroxide generated by stress. The low SOD activity, which slowly dissociates hydrogen peroxide, is associated with a moderate CAT activity, which would result in an increase in membrane lipid peroxidation resulting in increased MDA content. The SAO genotype, which presented better regulation of oxidative stress, also showed constant chlorophyll content (Table 1), and increased production of dry weight than the PM genotype (Table No. 2), after cold stress, showing a more rapid recovery from cold compared to PM. These results suggest that in sunflower the carboxylation capacity of RuBisCo is dependent on the kinetic properties of the enzyme and on electron transport, which is significantly reduced in cold-sensitive genotypes, leading to a reduction in net photosynthesis. Similarly, the chlorophyll content was a sensitive indicator of effects in the electron transport chain. Cold induces numerous physiological and biochemical alterations at cellular level: it stimulates the respiratory rate, reduces photosynthesis, alters the production of energy, increases membrane permeability, inactivates some enzymes and alters the cell structure (Inze and Van Montagu, 1995). These results agree with those of Otegui and Lopez Pereira (2006) that the damage in sunflower leaves produced by cold during early phases, delays crop growth. The results in relation to leaf area showed no significant differences in the growth stage analyzed.

CONCLUSIONS

- The cold-stress tolerant sunflower genotype has a quick recovery due to increased antioxidant activity and lower MDA levels that enables it to maintain its photosynthetic apparatus active, with constant levels of chlorophyll for dry matter production.
- This research helps explain for the first time in the sunflower crop the physiological mechanisms and the activity of key antioxidant defense enzymes (SOD and CAT) involved in recovery from cold stress in genotypes of contrasting response. All variables described here can be used as criteria for selecting cold stress tolerant sunflower hybrids.

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