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Physiological and Biochemical Contrasting Responses Associated with Growth Performances in Sunflower Seedlings after a Cold Stress

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Research Article

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ABSTRACT

In the climate change context, extreme events have become more frequents. Spring is the sunflower sowing season. Late frosts events or several days of low temperatures often occur causing delays in crop growth and even losses in plant density per hectare. Tolerance to low temperatures is an important trait considering that the sunflower production area is expanding to marginal regions with suboptimal growing conditions. Furthermore, an early sowing practice to maximize the sunflower growing period is commonly implemented in Argentina and in others countries. An interesting approach to identifying tolerant genotypes in breeding programs is exploring biochemical and physiological traits involved in growth recovery after cold exposure in sunflower seedlings. In this research, the physiological and biochemical responses of two contrasting sunflower genotypes were evaluated. Commercial hybrids Pampero (PM) and Sierra (SA) were exposed to cold treatment at 5°C for 96 hours. Growth performance, malondialdehyde content, superoxide dismutase and catalase enzyme activities, chlorophyll content and electrolyte leakage were assessed at 0, 24, 48 and 72 hours after the cold treatment. Different patterns between genotypes were observed. The sensitive genotype PM showed higher oxidative damage, lower membrane stability, reduced catalase and reduced superoxide dismutase activities, in contrast with the tolerant genotype. Moreover, tolerant genotype SA showed a great capacity to recover the chlorophyll content and restart growth while the sensitive genotype showed only a slight increase in chlorophyll content and a clear delay in growth.

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Keywords: Sunflower seedlings, Cold-stress; Recovery; Growth; Physiological response

ABBREVIATIONS

APX: Ascorbate Peroxidase; CAT: Catalase; CT; Cold Treatment; DNA, Deoxyribonucleic Acid; DWL: Dry Weight of Leaves; DWS: Dry Weight of Stem; DWR, Dry Weight of Roots; GR: Glutathione Reductase; HO: High Oleic; H_2O_2: Hydrogen Peroxide; MDA: Malondialdehyde; PM; Pampero Genotype; ROS: Reacting Oxygen Species; SA: Sierra Genotype; SOD: Superoxide Dismutase; TDW, Total Dry Weight.

INTRODUCTION

Sunflower (*Helianthus annuus L.*) is an oilseed crop widely spread throughout the world. It is mainly used for nutritional and cosmetic oils production. In Argentina, the area cultivable with sunflower covers around two million hectares. Planted area and average yields per hectare of the latest campaigns denotes a displacement of the sunflower growing areas towards the west of the Pampas region and the Chaco area, in addition to their consolidation in the southeast region of Buenos Aires [1]. There is great genotypic variability and commercial hybrids adapted to different environmental conditions. Particularly in Argentina, the use of High-Oleic (HO) sunflowers has expanded. The sunflower oil obtained from HO cultivars is one of the most appreciated oils on the market. An oil with a high concentration of omega 9 fatty acids is produced from seeds HO sunflower, which does not contain trans fats and has a low proportion of saturated fatty acids compared to others [2]. High-oleic cultivars are generally less sensitive to cold temperatures, and therefore have more stable oleic acid concentrations than traditional ones [3]. It has been identified genotypes with different germinative responses to cold temperatures. These genotypes were commercial cultivars: hybrid Sierra HO and Pampero, which have shown to be tolerant and sensitive to cold, respectively [4]. The average sunflower cycle comprises 125 days depending on thermal sums, genotypes, sowing dates, latitude and availability of water and nutrients [5]. Sunflower base-growth temperature is around 8°C. Sunflower is a summer crop, planting should occur as early as possible in the spring season to avoid crop critical period exposure to water deficit and heat stress in the hotter summer months [6-8]. But this early sowing management practice to maximize seed and oil yield often expose seedlings to cold temperatures. It is important to increase sunflower cold-tolerance in the early stages of growth and development to allow early successful sowing [9].

Low temperature represents one of the most harmful abiotic stressors affecting temperate plants [10]. Cold stress or low temperature exposure, can decrease plant metabolism activity and even impose severe yield penalty on crop plants [11,12]. The damage caused by low temperatures during sunflower seedling stage slows down crop growth. Some traits, such as plant chlorophyll content and dry weight, have been positively associated with cold-stress tolerance in sunflower crop. Hewezi et al. demonstrated that low temperatures modify plant morphology through changes in some physiological mechanisms, such as biosynthesis of proteins and sugars; they also reported a decrease in the expression of genes encoding glutamate and synthesis of sugars (sucrose, fructose and mannose),

which are compounds involved in carbon fixation [13]. One way to evaluate cooling tolerance is through measuring physiological, biochemical and growth parameters that allow to identify contrasting responses and integrate relationships with a better development and growth of the seedling [14]. The most common physiological parameters used are the loss of membrane electrolytes and the content of chlorophyll, since they are highly affected by stress and are highly correlated with growth. Loss of stability of cell membranes due to low temperatures in turn produces a reduction in their capacity to maintain intracellular content. The transfer of intracellular content to the extracellular medium has been frequently reported. A high percentage value means a substantial ion loss through membranes, which become unstable when affected by cold. The relative loss of electrolytes is usually measured to evaluate cell damage caused by chilling, both *via* low positive and low negative temperatures [15-17].

On the other hand, cold temperatures generate oxidative stress in plants by accumulating reactive oxygen species (ROS) and the regulation of oxidative metabolism is analyzed by the oxidative damage and the antioxidant defense. Oxidative stress is a common phenomenon involved in abiotic and biotic stress in plants. Under stress conditions, such as extreme high or low temperatures, ROS are generated through metabolic processes, acting as change signals regulating gene expression and ion channel activity [18-21]. ROS including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), are one of the earliest known biochemical responses of eukaryotic cells to abiotic stresses. ROS are produced mostly in chloroplasts, mitochondria, and peroxisomes [22]. In higher plants, increased ROS production is a characteristic inherent to a stressed metabolism under diverse stress types [23]. Stress response mechanisms are accompanied by the production of antioxidant enzymes involved in ROS degradation. The main anti-stress enzymes are Superoxide Dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX), and Glutathione Reductase (GR) [24]. Inadequate ROS degradation causes oxidative stress, characterized by damaging ROS reactions with biologically important macromolecules such as proteins, lipids and DNA, likely producing cell damage [25]. Oxidative stress causes damage at the cellular membrane level *via* lipid peroxidation, which is manifested in Malondialdehyde (MDA) content. Plant growth morphological traits, such as height, dry weight and fresh weight, are also reduced under stress conditions [26]. These parameters have been used as selection criteria in genotypes of *Chloris gayana* K. and *Cenchrus ciliaris L*. under high temperatures, salinity and drought conditions [27-29]. However, there is no information about the use of these indicators to characterize cold-tolerant sunflower genotypes.

Studying physiological and biochemical responses to low temperature might allow to identify tolerant materials [30-33]. The objective of this work was to evaluate the physiological and biochemical responses of two contrasting sunflower genotypes in their cold-tolerance after being exposed to a period of cold stress, in order to find relationships with growth performance in the seedling stage.

MATERIALS AND METHODS

Plant material and growth conditions

Two commercial hybrids of sunflower were used: Pampero (PM) and Sierra High-Oleic (SA). These materials were identified as cold-tolerant (SA) and cold-sensitive (PM) respectively in a previous work. Seeds of these hybrids were sown in pots (11 cm in height and 9cm in diameter) containing sterilized fine sand as substrate. After sowing, pots were watered periodically and kept for 15 days in a growth chamber at 30ºC day/20ºC night with a photoperiod of 16hs light/8 hs darkness. When seedlings had 2 leaves expanded the treatments were imposed.

Treatments

The control treatment (C) consists of 40 pots of each sunflower hybrid kept in the growth chamber with the same conditions previously descripted. On the other hand, the cold treatment (CT) was established by placing 40 pots in a chamber with a constant temperature of 5°C for 96 hours, with a photoperiod of 16 h light/8 h darkness. After the cold treatment, the cooled pots were returned to the growth chamber in controlled treatment conditions and were kept for 25 days. Leaf samples were collected from 8 plants, per sample moment, at 0, 24 and 72 hours after cold treatment. This samples were used to measure physiological and biochemical parameters. Growth characters were measured collecting 8 plants per hybrid per treatment at 15 and 25 days at the end of assay (30 and 40 days after sowing).

Biochemical measurements

Antioxidant enzyme activities (SOD and CAT) and oxidative damage (MDA content) were quantified at 0, 24 and 72 hours using 100 mg of leaves per sample. Lipid peroxidation in leaves was measured by assessing MDA content, as described by Heath and Packer [34]. To determinate antioxidant enzyme activities, the samples were processed following the protocol described by Tommasino et al. SOD activity was estimated according to the method described by Beauchamp and Fridovich [35]. Enzymatic activity was expressed as SOD units (USOD) per milligram of protein. Protein content in the enzyme extracts was quantified according to the Bradford method. CAT activity was measured through the consumption of H_2O_2 measured by the absorbance at 240 nm [36]. One unit of CAT activity was defined as the amount of enzyme required for catalyzing the conversion of 1 µmol H_2O_2 into water per minute. Results were expressed as µmol H_2O_2 extinct per minute per milligram of protein.

Physiological measurements

Electrolyte leakage: six leaf discs/plant (20 mm in diameter) were collected from both treatments at 0, 24, and 72 h after the end of the exposure to low temperature, washed and submerged in 35 ml of deionized water. Then, discs were incubated at 25°C for 24 h and initial conductivity (initial Ω) in the bathing water was determined using a conductivity meter. The discs were autoclaved at 100°C for 30 minutes to measure conductivity of injured tissues (maximum Ω). Electrolyte leakage was calculated as the percentage of TM=(initial Ω /maximum Ω) x 100, following Murray, Cape and Fowler [37].

Chlorophyll content: Chlorophyll content was measured by analyzing extracts obtained by boiling in an 80% ethanol solution using a UV-120 spectrophotometer (Shimadzu) at 645/652 y 663 nm and following the technique described by Wintermans and Mots [38].

Growth performance evaluation

At 10 and 25 days after ending the cold-treatment, total dry weight (TDW) was measured using 8 plants per each hybrid and treatment. Collected samples were dried out in a stove at 60°C to constant weight. TDW was differentiated into parts, by Leaves Dry Weight (LDW), Stem Dry Weight (SDW) and Roots Dry Weight (RDW).

Statistical analysis

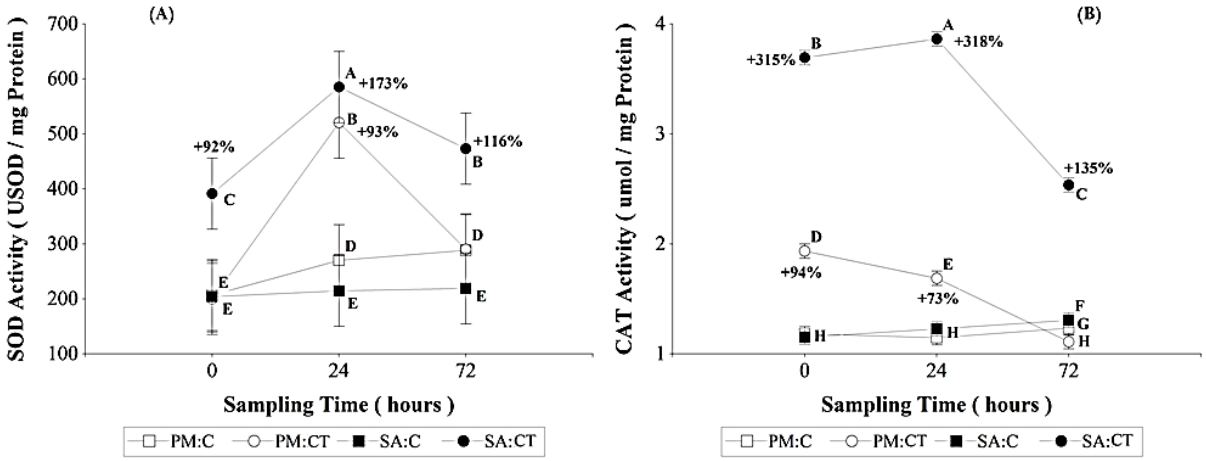
A completely randomized design was carried out with three repetitions per treatment per genotype for the determination of all variables. For comparisons between average values, general linear mixed models were used

and ANOVA was applied. For MDA, enzymes (CAT and SOD), chlorophyll content and electrolyte leakage, three-way interactions were applied between variables (e.g. genotype × treatment × time of measurement). Fisher test at 5% level of significance ($P \leq 0.05$) was performed using the InfoStat statistical software [39]. The standard error was plotted in all figures. In all measured parameters, the changes (in terms of percent value) between treatments were calculated using the following formula: Percent change (%)=[(Xc−Xs)/Xc) × 100], where Xc denote the mean value of control plants and Xs the mean value of stressed plants.

RESULTS

In both antioxidant enzyme activities (SOD and CAT) significant differences for three-way interactions were observed ($P<0.05$). For both genotypes (PM and SA), enzyme activities observed after Cold Treatment (CT) were higher than in Control treatment (C). Moreover, contrasting responses were observed between genotypes, where SA genotype exhibited high increased values than PM genotype. In the SA genotype, it was observed that SOD enzyme activity increased and remained at a high level during the 3 days after the cold treatment. On the other hand, the genotype (PM) only showed an increase in SOD activity at 24 hours after CT as shown in Figure 1.

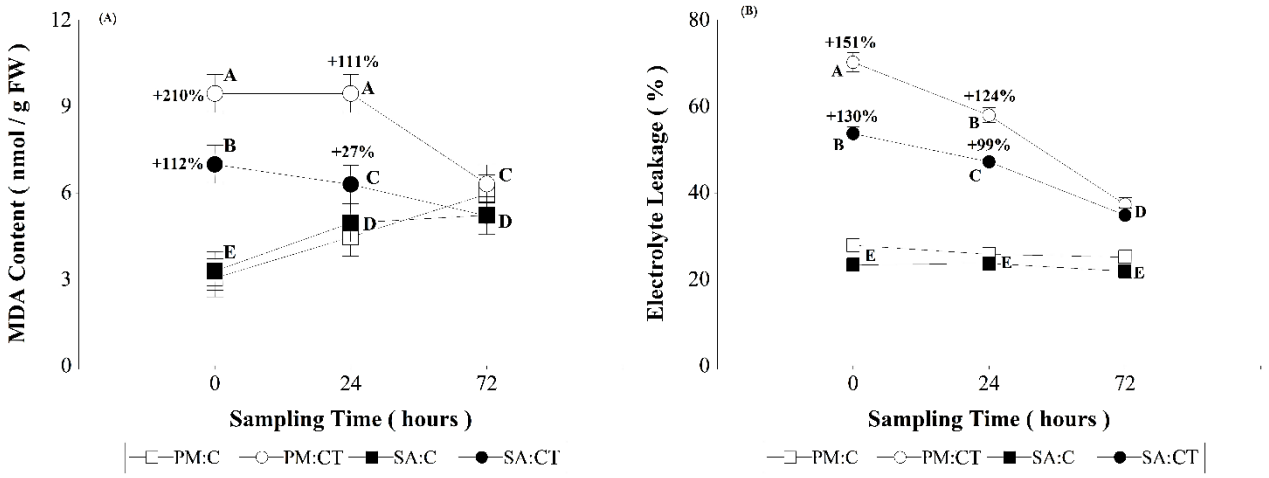
Figure 1. Enzyme activities in sunflower genotypes (SA) and (PM) under Control condition (C) and Cold treatment (CT). A) Superoxide dismutase activity (SOD). B) Catalase activity (CAT). Different letters denote significant differences ($P \leq 0.05$). Error bars indicate standard error (n=8). Percentage values indicates the increase observed in cold treatment with respect to the control treatment for each genotype.



For CAT activity, it was increased in both genotypes after cold treatment. However, SA genotype showed CAT activity values strongly higher than observed in PM genotype.

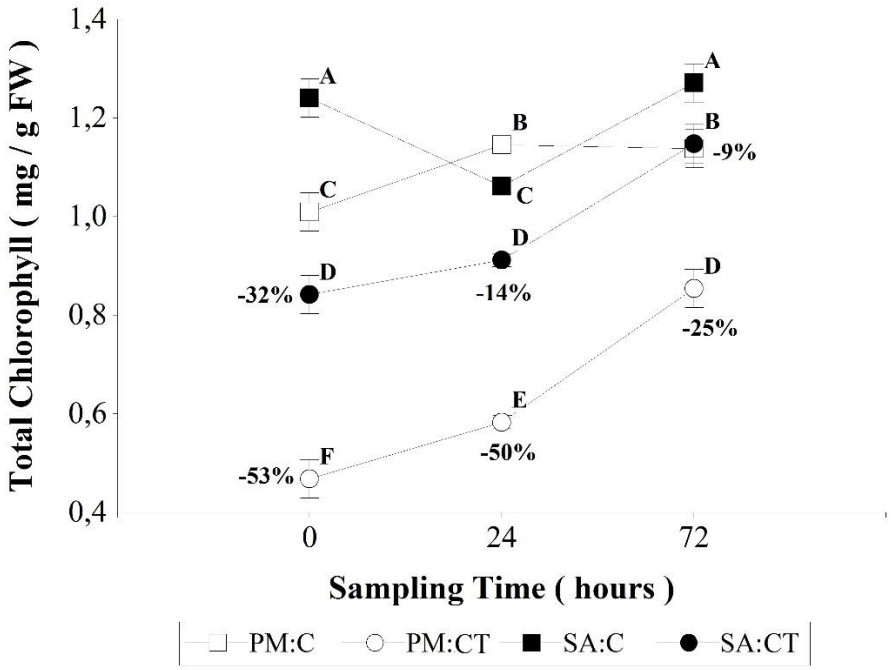
After cold treatment, high oxidative damage values quantified by malondialdehyde content were observed in both genotypes. MDA content was higher in PM genotype (210% over control) than SA genotype (112% over control). By the other hand, a similar response was observed in the electrolyte leakage parameter. The PM genotype showed high electrolyte leakage (151% and 124% over control value) and the SA genotype exhibited lower electrolyte leakage (130% and 99% over control) as shown in Figure 2.

Figure 2. Oxidative damage and membrane stability measured on (SA) and (PM) genotypes under control condition (C) and cold treatment (CT). A) Malondialdehyde content (MDA). B) Electrolyte leakage (EL). Different letters denote significant differences ($P \leq 0.05$). Error bars indicate standard error (n=8). Percentage values indicate the increase observed in cold treatment with respect to the control treatment for each genotype.



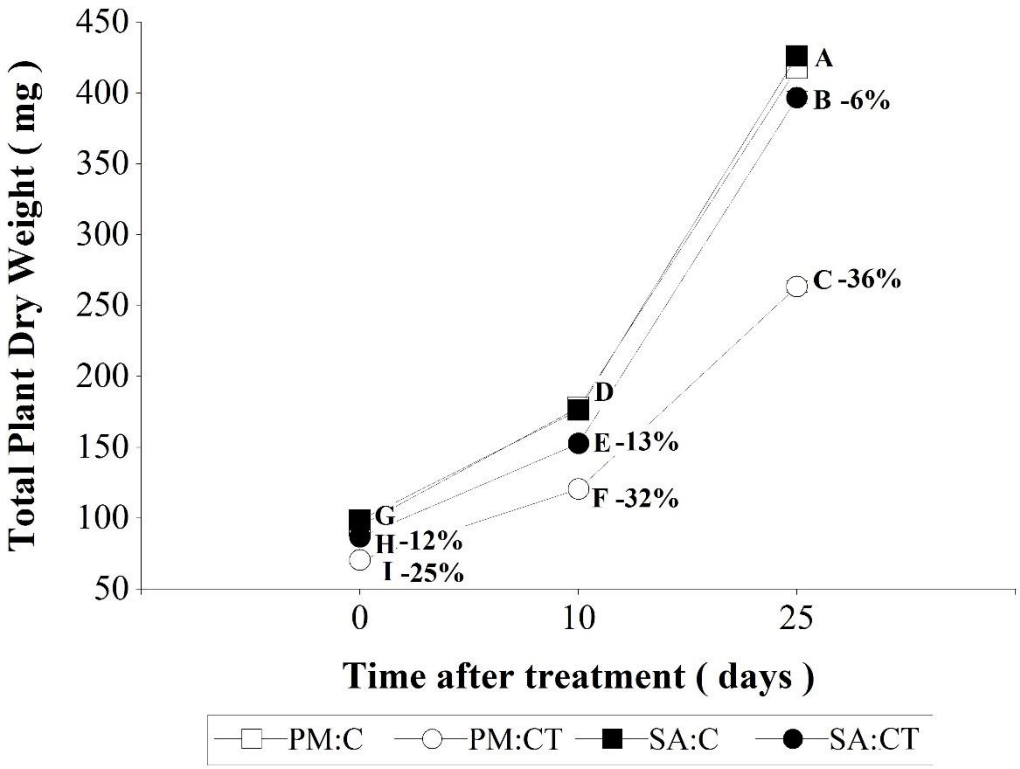
After cold treatment, total chlorophyll content decreased in both genotypes. The PM genotype showed the lowest values (53% less content than their control). 72th h after cold treatment, both genotypes had enhanced the chlorophyll content, the SA genotype showing the highest values related to control treatment (9% less than control at 72 h) as shown in Figure 3.

Figure 3. Total chlorophyll content measured on (SA) and (PM) genotypes under control condition (C) and cold treatment (CT). Different letters denote significant differences ($P \leq 0.05$). Error bars indicate standard error (n=8). Percentage values indicate the decrease observed in cold treatment respect to the control treatment for each genotype.



For growth performance, both genotypes presented lower total dry weight than control treatment. At the end of cold treatment, SA genotype exhibited 12% less TDW than control condition, but this difference were smaller 25 days later (9% less). In contrast, the PM genotype showed 25% loss of TDW and this difference was greater 25 days later (36%). Therefore, the SA genotype presented a capacity to tolerate and recover from cold treatment while the PM genotype was more affected and delayed in its growth. Similar patterns were observed in dry weight of leaves (DWL), dry weight of stem (DWS) and dry weight of roots (DWR) (data not showed) as shown in Figure 4.

Figure 4. Total plant dry weight measured on (SA) and (PM) genotypes under control condition (C) and cold treatment (CT). Different letters denote significant differences ($P \leq 0.05$). Error bars indicate standard error (n=8). Percentage values indicate the decrease observed in cold treatment respect to the control treatment for each genotype.



DISCUSSION

In the present work, physiological, biochemical and growth parameters were measured to evaluate the response in two sunflower genotypes after a cold treatment. Plants have developed various morphological, cellular, physiological, biochemical and molecular adaptations to preserve themselves in abiotic stress situations [40]. To analyze the pattern of antioxidant defenses, we measured some key antioxidant enzymes activities, like total SOD and CAT activities [41]. In this work, higher SOD and CAT activities were observed in the tolerant sunflower genotype while other authors, using different plant species, have reported significantly decreased SOD activity in sensitive cultivars and increases in tolerant ones [42]. For both genotypes (PM and SA), enzymes activities observed after cold treatment (CT) were higher than in control treatment (C). Moreover, contrasting responses were observed in both genotypes, where SA genotype exhibited higher increase values than PM genotype. This result indicates that cold causes changes in the oxidative metabolism and the regulation of the antioxidant system even several days after the stress has occurred. Different results have been reported on stresses effects on CAT activities such as induction, reduction or stable CAT activities under stress [43-46]. However, in our current study, total CAT activity highly increased in the tolerant genotype, and also increased in the sensitive genotype but at a lower level. Nevertheless, differential responses in CAT activities were observed between the studied sunflower genotypes.

Several investigations report a better regulation of oxidative damage observed in tolerant genotypes when compared to susceptible ones. MDA has been considered as an indicator of oxidative damage in various crops and it has been used as a suitable indicator for tolerant genotype selection [47]. In our study, different contents of MDA were found between evaluated genotypes. The tolerant genotype (SA) showed lower oxidative damage than the susceptible genotype (PM). A similar pattern was observed in the electrolyte leakage parameter. Furthermore, these two parameters have a direct relation, because MDA content increases as result of membranes lipid peroxidation

and electrolyte leakage rises as result of membrane damage. This indicates the PM genotype was the most affected by cold treatment and SA genotype showed a capacity to control the oxidative damage.

Changes in total chlorophyll content have been reported when plants were exposed to different types of abiotic stresses [48-50]. In our work, decreased content in total chlorophyll was observed in both genotypes after cold exposure. The tolerant genotype (SA) showed a great ability to recover the chlorophyll content after the cold treatment while the sensitive genotype (PM) showed lower content and less ability to get over the cold stress.

Regarding growth performance, temperature influences most plant processes, including photosynthesis, transpiration, respiration, germination, growth and flowering. All metabolic processes are slowed down by cold [51]. It is well known that cold stress has a profound impact on plant morphologies, causing growth repression and reduced yields. Many works focus in evaluating growth during stress to observe tolerance responses, but in the present work the focus was to evaluate the growth performance after a cold-stress period, to observe the seedlings recovery behavior. Our results indicate that the tolerant genotype was less affected when exposed to cold treatment and had a great capacity to recover in terms of growth parameters after cold treatment. In contrast, the sensitive genotype was more affected and showed a clear growth delay.

CONCLUSION

Cold temperature greatly disrupts oxidative metabolism and generates biochemical, physiological and growth changes in sunflower seedlings. After cold treatment, oxidative metabolism was involved in a better behavior of the tolerant genotype during stress treatment and the recovery period. The sensitive genotype, in contrast, showed higher oxidative damage, lower membrane stability, reduced catalase and superoxide dismutase activity. Moreover, the tolerant genotype showed a great ability to recover the chlorophyll content and reestablish growth parameters in the recovery period while the sensitive genotype showed only a small recovery in chlorophyll content and a clear delay in growth. The assay implemented here can be an important tool to characterize genotypes in a sunflower breeding program, which could allow to develop strategies for selection of tolerant genotypes with better growth performance at sowing stage in cold conditions.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

Ernesta Fabio conducted the assays in controlled conditions, collected seedling samples, carried out growth measurements and determinations of physiological traits. Exequiel Tommasino carried out biochemical measurements and analyzed statically data. All three authors contributed to writing the manuscript.

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