Australian Journal of Crop Science AJCS

AJCS 13(06):881-888 (2019) ISSN:1835-2707
doi: 10.21475/ajcs.19.13.06.p1593

Assessment of cold stress in avocado cultivars based on visual, physiological and biochemical criteria

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Abstract

Low temperatures affect avocado plants and their response varies across genetic variants. The objective of this study was to assess cold stress of avocado cultivars based on visual, physiological, and biochemical criteria for evaluating freezing injury in these plants. The avocado cultivars 'Geada,' 'Fortuna,' 'Fuerte,' 'Quintal,' 'Margarida,' and 'Primavera' were subjected to temperatures of −2.5, −4.0, −5.0, and −6.0 °C for 1 h in a growth chamber. Their responses to cold stress were evaluated on the basis of visual damage score, photosynthetic rate, water pressure potential, protein content and enzymatic activity. Moreover, the experimental design was factorial and completely randomized with four replicates of each cultivar. An analysis of variance of the results was performed and the means were compared using Tukey's post-hoc test ($p < 0.05$). Evaluations based on the plants water pressure potential, total protein content, catalase activity, photosynthetic rate, and visual damage score were efficient in estimating the effects of cold stress in avocado plants. Additionally, photosynthesis and visual damage score were significantly correlated in all evaluations, enabling classification of the cultivars in terms of response to cold stress. We found that Fuerte was the most tolerant cultivar followed by Geada. The Primavera was the most sensitive cultivar.

Keywords: ascorbate peroxidase; catalase; frost; leaf damage; *Persea americana* Mill; photosynthesis.

Introduction

The avocado (*Persea americana* Mill) is an important crop worldwide, as it produces fruits with a high-energy value, little carbohydrate content, and oils rich in β-sitosterol and oleic acid, with potential for use in the food and cosmetic industries (Salgado et al., 2008). The ability of its oil to be utilized as a source of renewable biofuel has been studied, because of its high quality, physicochemical properties, and compatibility with other raw materials. In addition, it may be possible to extract from its core two main raw materials for producing biodiesel—pulp oil and ethyl alcohol (Menezes et al., 2010; Knothe, 2013).

Avocado originated in Mexico. Nevertheless, nowadays it is cultivated in tropical and subtropical regions all over the world. Hybrids whose adaptations to growth conditions vary considerably have been produced from three races of avocados with different thermal requirements: West Indian, Guatemalan and Mexican (Gardiazabal, 2004; Tapia et al., 2008; Tkachuk, 2009).

Many plant species develop specific mechanisms for survival in environments with prolonged periods of freezing. Cold tolerance or resistance may be genetic, but it also depends on the age of the plant (Saini et al., 2018).

Young avocado plants are very sensitive to intense solar radiation and frost, therefore, requiring adequate protection. In the vegetative phase, severe damage may occur, and during flowering such injuries cause a reduction in fruit set, with negative effects on yield. When frost occurs on adult plants under good field conditions (i.e., deep, fertile, and well-drained soils), plants show higher tolerance (Koller, 1984; Mindêllo Neto et al., 2004; Dgadr, 2010).

At the foliar level, low temperature damage in tropical and subtropical plants occurs due to the freezing of intercellular spaces, which causes the rapid dehydration of the cells. Moreover, the adhesion of the ice crystals and their rapid growth causes the rupture of the cellular structures and death of the leaves (Pierce, 2001; Snyder and Melo Abreu, 2005).

Plants react to environmental stress by closing the stomata, decreasing CO_2 diffusion, and consequently, decreasing photosynthetic rate and biomass accumulation. In addition, chemical and enzymatic reactions are restricted under cold stress (Wang et al., 2009; Distelbarth et al., 2013).

Furthermore, conditions that limit CO_2 fixation lead to accumulation of reactive oxygen species (ROS), namely, hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and superoxide (O_2^-), which react with membrane lipids, degenerating the cell (Asada, 2006; Mohammadian et al., 2012). Avocados have several antioxidant mechanisms for

eliminating ROS, and the enzymatic antioxidants are highly efficient in catalyzing and regenerating molecules that act in this process. These include superoxide dismutase, catalase (CAT), peroxidase, and ascorbate peroxidase (APX) (Sofo et al., 2005; Saini et al., 2018).

According to Mohammadian et al. (2012), cold tolerance in plants is primarily related to the activity of antioxidants, such as ROS scavengers, to combat oxidative stress. In conjunction with other physiological mechanisms, the efficiency of the antioxidant system increases plant stress tolerance (Scandalios, 2005). However, there have been only a few studies comparing the activity of antioxidant enzymes in avocado cultivars exposed to cold stress.

Thus, the objective of this study was to evaluate cold stress response in avocado cultivars, through visual assessment of the evidence of cold damage and the plants' physiological and biochemical responses to cold stress.

Results and Discussion

Photosynthesis evaluation 1, 7, and 21 days after cold stress treatments

The net photosynthetic rate of plants 1 day after the cold stress treatments (Table 1) varied with the different temperatures and cultivars. At a minimum temperature of −2.5 °C, there were not significant differences between the treated plants and the control group. However, at −4 °C, Fuerte had a higher net photosynthetic rate than Margarida and Primavera, but it was not significantly different from that in the others.

All cultivars analyzed resisted exposure to low temperatures of up to −4 °C, except for Primavera, which showed a marked reduction in net photosynthesis at this level of stress. Only the cultivars Fuerte and Geada presented green leaves and photosynthetic activity when exposed to −5 °C, although there was a sensible drop in the value of liquid photosynthesis in both cultivars.

After 7 and 21 days, we found that there was no significant difference among all cultivars in terms of physiological responses to exposure to a minimum temperature of −2.5 °C (Table 2). However, at −4 °C, there was a significant decrease in photosynthesis, evident first in Primavera. At −5 °C, the cultivars Fortuna, Margarida, and Primavera experienced a damaged photosynthetic system, whereas Fuerte and Geada showed evidence of photosynthesis after 7 days of recovery, and Quintal did only after 21 days.

Fuerte was photosynthetically superior to the other cultivars and did not differ from the control after each treatment, and thus it could be considered tolerant to temperatures as low as −5 °C. Notably, exposure to −6 °C caused severe damage to all cultivars.

The reduction in the photosynthetic capabilities of avocado cultivars after cold stress is a consequence of the damage caused by the low temperatures. This limits photosynthetic activity, leading to a decrease in the assimilation of CO_2, which can be attributed to the closure of the stomata, photoinhibition, and changes in the levels of transcription and expression of photosynthetic enzymes (Allen and Ort, 2001; Yamori et al., 2012; Taiz et al., 2015). In this study, there was a decrease in the photosynthetic rate after 1 day of exposure to cold, highlighting Fuerte as superior to the other cultivars in terms of cold stress tolerance at a temperature of −5 °C or higher.

Several studies have found that plants experience reduced photosynthetic activity when exposed to cold temperatures. For example, Xiaochuang et al. (2017) found that rice plants subjected to N application before treatments at low temperatures had a significant reduction in their photosynthetic rates. Similarly, Dalmannsdottir et al. (2017) found that photosynthetic activity in perennial ryegrass (*Lolium perenne*) and timothy (*Phleum pratense*) was reduced after exposure to low temperatures, under natural conditions in autumn. Moreover, Oliveira and Peñuelas (2004) found the same in plants of *Cistus albidus* L. and *Quercus ilex* exposed to natural winter conditions in the Mediterranean and greenhouse conditions.

Cell pressure potential

The pressure potential also suggested a statistically significant interaction between temperatures and cultivars. The potential pressure at temperatures up to −4 °C was positive, and Fortuna had the lowest value (Table 3).

At −5 °C, only Fuerte presented potential with values above zero, indicating that its cells were still intact. The other cultivars presented null values, suggesting tissue damage and cell wall rupture. All cultivars were damaged after exposure to a temperature of −6 °C. With the freezing caused by low temperatures, the rupture of the cells and leaf structures caused dehydration and death of the tissues, causing a loss of turgor (Pierce, 2001).

Morphological criteria: visual damage scores

Based on the visual damage scores evaluated after 21 days of cold stress, it was possible to verify marked losses in all cultivars after exposure to a −5 °C temperature treatment, whereas exposure to −4 °C produced more prominent symptoms in Primavera than in the other cultivars (Table 4).

The cultivar Fuerte experienced less damage than that by Fortuna, Quintal, and Primavera, but not less than that by Geada and Margarida. The cultivars did not significantly differ in terms of severe damage experienced following exposure to −6 °C.

Visual evaluations have been widely used in the assessment of cold damage in coffee plants both in the field and under controlled conditions (Caramori et al., 2002).

The avocado cultivars Geada and Quintal showed intermediate tolerance to cold stress, followed by Fortuna and Margarida. Primavera has proven to be the most sensitive cultivar, and thus its use should be recommended only in areas with minimal periods of frost.

Other studies have also observed that genetic differences may play a role in cold tolerance of plants. Soares et al. (2002) who observed the behavior of six-year-old avocado cultivars under cold conditions in the field with a minimum air temperature of −2.8 °C found that Fuerte, Jumbo, Ermor, and Solano experienced a lower level of injury in the crown. They concluded that the origin of the breed of the cultivar influence its ability to tolerate cold temperatures.

Table 1. Photosynthetic rate (µmol m^{-2} s^{-1}) of avocado plants submitted to minimum temperatures during 1 hour and control under environmental conditions – assessment 1 day after the treatments.

Cultivars	Temperatures (°C)				
	Control	−2.5	−4.0	−5.0	−6.0
Geada	9.77 Aa*	8.64 Aa	7.72 Aab	2.14 Bb	0.00 Ba
Fuerte	12.76 Aa	6.46 Ba	11.54 Aa	6.73 Ba	0.00 Ca
Fortuna	9.77 Aa	5.21 Ba	9.30 ABab	0.00 Cb	0.00 Ca
Quintal	8.37 Aa	7.18 Aa	8.59 Aab	0.00 Bb	0.00 Ba
Margarida	10.42 Aa	5.64 Ba	6.78 ABbc	0.00 Cb	0.00 Ca
Primavera	9.54 Aa	6.65 ABa	3.18 BCc	0.00 Cb	0.00 Ca
C.V. (%)			35.57		

*Means followed by the same uppercase in the row and lowercase in the column do not differ from each other according to the Tukey's post-hoc test at a 5% probability level. CV = Coefficient of variation.

Fig 1. Visual score of damage in avocado after cold stress (1 = No damage, 2 = Leaves with slight damage, 3 = Leaves with moderate damage, 4 = Leaves with severe damage, and 5 = Death).

Table 2. Photosynthetic rate (µmol m^{-2} s^{-1}) of avocado plants submitted to minimum temperatures during 1 hour and control under environmental conditions – assessment 7 and 21 days after the treatments.

Cultivars	Temperature (°C)			
	−2.5	−4.0	−5.0	−6.0
	7 days			
Geada	6.87 Aa*	7.27 Aab	1.77 Bb	0.00 Ba
Fuerte	9.13 Aa	7.44 Aab	8.23 Aa	0.00 Ba
Fortuna	7.60 Aa	5.06 Aab	0.00 Bb	0.00 Ba
Quintal	8.77 Aa	7.32 Aab	0.00 Bb	0.00 Ba
Margarida	7.14 Aa	7.80 Aa	0.00 Bb	0.00 Ba
Primavera	6.91 Aa	4.30 Ab	0.00 Bb	0.00 Ba
C.V. (%)	35.77			
	21 days			
Geada	10.96 Aa*	7.72 Aab	3.06 Bb	0.00 Ba
Fuerte	8.68 Aa	11.54 Aa	8.13 Aa	0.00 Ba
Fortuna	6.31 Aa	9.30 Aa	0.00 Bb	0.00 Ba
Quintal	8.82 Aa	8.59 Aa	1.76 Bb	0.00 Ba
Margarida	10.20 Aa	6.78 Aab	0.00 Bb	0.00 Ba
Primavera	8.57 Aa	3.18 Bb	0.00 Bb	0.00 Ba
C.V. (%)	42.67			

* Means followed by the same uppercase in the row and lowercase in the column do not differ from each other according to the Tukey's post-hoc test at the 5% probability level. CV = Coefficient of variation.

Table 3. Pressure potential (MPa) values of avocado plants submitted to minimum temperatures during 1 hour and control under environmental conditions.

Cultivars	Temperature (°C)				
	Control	−2.5	−4.0	−5.0	−6.0
Geada	0.82 Aa*	0.13 Ba	0.79 Aab	−0.34 BCb	−0.57 Cb
Fuerte	0.58 Aba	0.36 Ba	0.93 Aab	0.38 Ba	−0.36 Cab
Fortuna	0.51 Aa	0.21 ABa	0.59 Ab	−0.25 Bb	−0.22 Bab
Quintal	0.52 Aba	0.41 BCa	1.00 Aab	−0.22 Db	−0.13 CDa
Margarida	0.65 Ba	0.34 BCa	1.26 Aa	−0.26 Db	−0.11 CDab
Primavera	0.63 Aba	0.46 Ba	1.00 Aab	−0.28 Cb	−0.24 Cab
C.V. (%)	2.19 **				

* Means followed by the same capital letter in the row and lowercase in the column do not differ from each other according to the Tukey's post-hoc test at the 5% probability level. ** The values were transformed for analysis of variance in $\sqrt{x+5}$, but the actual values are presented. C.V. = Coefficient of variation.

Table 4. Visual damage score of avocado plants submitted to minimum temperatures during 1 hour and control under environmental conditions – assessment 21 days after the treatments.

Cultivars	Temperature (°C)				
	Control	−2.5	−4.0	−5.0	−6.0
Geada	1.00 Aa*	1.00 Aa	1.75 Aab	4.00 Bab	5.00 Ca
Fuerte	1.00 Aa	1.00 Aa	1.00 Aa	3.25 Ba	4.75 Ca
Fortuna	1.00 Aa	1.25 Aa	1.00 Aa	4.25 Bb	5.00 Ba
Quintal	1.00 Aa	1.00 Aa	1.00 Aa	4.50 Bb	5.00 Ba
Margarida	1.00 Aa	1.00 Aa	1.00 Aa	4.00 Bab	5.00 Ba
Primavera	1.00 Aa	1.00 Aa	2.50 Bb	4.75 Cb	5.00 Ca
C.V. (%)	22.23				

* Means followed by the same capital letter in the row and lowercase in the column do not differ from each other by the Tukey test at the 5% probability level. C.V. = Coefficient of variation.

Table 5. Protein content (mg protein g FM^{-1}) of the leaves of avocado plants submitted to minimum temperatures during 1 hour and control under environmental conditions.

Cultivars	Temperature (°C)				
	Control	−2.5	−4.0	−5.0	−6.0
Geada	3.46 Ab*	2.90 Aa	1.69 Bb	1.82 Bb	2.08 Ba
Fuerte	4.94 Aa	3.37 Ba	4.96 Aa	2.73 Bb	2.37 Ba
Fortuna	3.51Ab	3.03 Aa	1.96 Bb	1.98 Bb	1.80 Ba
Quintal	3.05 Ab	2.92 Aa	3.65 Aa	2.02 Ab	2.45 Aa
Margarida	4.47 Aa	3.23 Aa	2.49 Ab	3.89 Aa	3.47 Aa
Primavera	2.54 Ab	3.76 Aa	3.05 Ab	1.57 Bb	2.62 Aa
C.V. (%)	30.37				

* Means followed by the same capital letter in the row and lowercase in the column do not differ from each other according to the Tukey's test at the 5% probability level. CV = Coefficient of variation.

Table 6. Activity of catalase (CAT) (mmol H_2O_2 min^{-1} mg FM^{-1}) and ascorbate peroxidase (APX) (UA min^{-1} mg FM^{-1}) of the leaves of avocado plants submitted to minimum temperatures during 1 hour and control under environmental conditions.

Cultivars	Temperature (°C)					
	Control	−2.5	−4.0	−5.0	−6.0	Mean
	Catalase (CAT)					
Geada	0.67	0.58	0.41	0.58	0.47	0.54 a
Fuerte	0.81	0.80	0.62	0.37	0.27	0.57 a
Fortuna	0.88	0.54	0.45	0.41	0.36	0.53 a
Quintal	0.54	0.48	0.44	0.36	0.43	0.45 a
Margarida	0.77	0.67	0.37	0.54	0.54	0.58 a
Primavera	0.73	0.90	0.70	0.27	0.63	0.64 a
Mean	0.73 A	0.63 AB	0.53 BC	0.42 C	0.45 C	
C.V. (%)	32.76					
	Ascorbate peroxidase (APX)					
Geada	2.30 Ab*	3.72 Ab	2.74 Ab	2.25 Ab	2.50 Ab	
Fuerte	7.21 Aa	7.28 Aa	8.96 Aa	5.63 Aa	2.45 Bb	
Fortuna	4.69 Ab	6.48 Aa	3.14 Aa	3.46 Ab	3.43 Ab	
Quintal	3.96 Bb	8.45 Aa	10.04 Aa	2.70 Bb	5.08 Bb	
Margarida	8.01 Aa	8.28 Aa	8.46 Aa	8.44 Aa	8.39 Aa	
Primavera	2.25 Ab	3.07 Ab	3.02 Ab	1.67 Ab	2.27 Ab	
C.V. (%)	39.24					

* Means followed by the same capital letter in the row and lowercase in the column do not differ from each other according to Tukey's post-hoc test at the 5% probability level. CV = Coefficient of variation.

Table 7. Pearson correlation coefficients (r) between the evaluated variables, considering (A) minimum temperatures and (B) avocado cultivars. Prot - protein; CAT-catalase; APX - ascorbate peroxidase; A1d – photosynthesis after 1 day; A7d - photosynthesis after 7 days; A21d - photosynthesis after 21 days; Visual - visual damage score; Ψ Pressure - pressure potential.

A	Correlations (r) for Temperature −°C						
	CAT	Prot	APX	A 1 d	A 7d	A 21d	Visual
Prot	0.98*						
APX	0.45^{ns}	0.52^{ns}					
A1d	0.90*	0.93*	0.65^{ns}				
A7d	0.96*	0.96*	0.65^{ns}	0.98*			
A21d	0.91*	0.92*	0.74^{ns}	0.97*	0.99*		
Visual	−0.86*	−0.88*	−0.81*	−0.96*	−0.96*	−0.99*	
Ψ Pressure	0.63^{ns}	0.72^{ns}	0.74^{ns}	0.90*	0.82*	0.85*	−0.87*
B	Correlations (r) for Cultivars						
	CAT	Prot	APX	A 1d	A 7d	A 21d	Visual
Prot	0.21^{ns}						
APX	-0.26^{ns}	0.80*					
A1d	-0.12^{ns}	0.47^{ns}	0.22^{ns}				
A7d	-0.14^{ns}	0.67^{ns}	0.42^{ns}	0.95*			
A21d	-0.26^{ns}	0.49^{ns}	0.31^{ns}	0.97*	0.96*		
Visual	0.36^{ns}	-0.53^{ns}	-0.56^{ns}	−0.84*	−0.83*	−0.89*	
Ψ Pressure	0.25^{ns}	0.89*	0.67^{ns}	0.15^{ns}	0.43^{ns}	0.20^{ns}	-0.14^{ns}

*Significant at 5% probability; ^{ns} not significant

Biochemical criteria

Protein content varied among cultivars and in response to temperature, because the plants responded to cold stress in different ways (Guy, 1990) (Table 5).The protein content was significantly reduced in cultivars Geada and Fortuna and in Fuerte, Quintal, and Primavera when exposed to temperatures below −4 °C and −5 °C, respectively. The protein content of Margarida remained unchanged in response to low temperatures.

By increasing their protein content, cells of plants attempt to lower their freezing point, thereby protecting the cellular organelles and plasma membrane from damage caused by cold (Kratsh and Wise, 2000). The increasing protein content in response to decreasing temperatures indicates that a species can tolerate low temperatures without disrupting the normal functioning of the cells, since only undamaged cells are able to produce proteins (Taiz et al., 2015). However, none of the avocado cultivars tested exhibited this behavior, suggesting that this was not a strategy used to promote cold tolerance.

In terms of CAT enzymatic activity, there was no statistically significant interaction between cultivars and temperature (Table 6). The temperature averages differed from each other, and the highest values were observed in the control group. Moreover, CAT activity decreased with increasing low temperature stress, suggesting that CAT was being consumed to counteract the increase in ROS production in response to stress (Guy, 1990).

Similarly, several other authors have found changes in CAT activity in response to low temperature stress; MacRae and Ferguson (1985) believed that in *Pisum sativum*, *Vigna radiata*, *Cucumis sativus,* and *Passiflora* spp., the decrease in CAT activity might have been a result of the inability of damaged peroxisome membranes to transport CAT precursors to the peroxisome. On the other hand, Matsumura et al. (2002) found that at low temperatures, the activity of the CAT enzymes in tolerant transgenic rice was four times higher than in non-transgenic plants exposed to cold stress.

The enzymatic activity of APX significantly differed among the cultivars and temperatures tested (Table 6). The cultivars Geada, Fortuna, Margarida, and Primavera showed no differences in APX activity among the different temperatures. In Fuerte, there were no differences in APX levels with temperatures as low as −5 °C; however, APX levels decreased at −6 °C, when the cells were already damaged.

Cultivar Quintal experienced an increase in enzymatic activity at −2.5 and −4.0 °C, differently from the others. This increase prevented the accumulation of H_2O_2 and consequent cellular damage, suggesting that this enzyme may play an important role in plant responses to oxidative stress related to temperature decreases. Similarly, MacRae and Ferguson (1985) found that the APX pathway was an alternative enzymatic system for preventing the accumulation of H_2O_2.

Willekens et al. (1995) suggested that when CAT decreases, the APX activity might increase, this way it can partially compensate for the loss of CAT activity. Thus, they concluded that the plants' capacity to control H_2O_2 levels is one of the factors that contribute to develop their ability to resist various stresses.

There is a great diversity of responses to antioxidant enzymes reported in the literature, in species evaluated under nutritional and vegetative conditions. Cultivars that are tolerant to low temperatures usually increase the activity of their antioxidant enzymes, as observed by Zhang et al. (2011, 2012) in bananas, Floriani et al. (2011) in eucalyptus, and Souza et al. (2014) in *Xanthosoma sagittifolium*. These responses may be dependent on the enzymes evaluated.

The application of boron on strawberries caused the increased activity of CAT, peroxidase, and superoxide dismutase, thereby reducing damage caused by low temperatures (Gunes et al., 2016). The beneficial effects of potassium fertilization on increasing cold tolerance are reported in several studies, such as in the tropical forests of Mexico (Gomes-Ruiz et al., 2016) and in grapes (Sarikhani et al., 2014). In cucumbers, Li et al. (2011) found that there was an increase in the activity of antioxidant enzymes following the application of cinnamic acid before treatment with low temperatures. Similarly, Hamed et al. (2011) reported an increased activity of oxidant enzymes associated with mycorrhizal activity, conferring a greater tolerance to cold temperatures in tomato plants.

In contrast, in many species an increase in cold tolerance is not related to enzymatic activities, for example in *Arabidopsis thaliana* (Distelbarth et al., 2013). According to Guy (1990), plant enzymes and proteins can be divided into two classes: those sensitive to low temperatures and those inactivated by low temperatures.

Correlation analysis

To estimate cold stress in avocado plants at the different temperatures tested in our study, we highlighted the evaluations of photosynthesis, visual damage score, CAT activity, and total protein content. Notably, those tests were significantly correlated with each other. Therefore, they were efficient to estimate the level of stress caused by low temperatures in avocado plants (Table 7).

When the tests were compared among each other, all photosynthesis evaluations were significantly correlated with the visual damage score. Thus, these tests were also able to discern the cold stress responses of the cultivars studied.

Our results suggest that the visual damage score was efficient for verifying cold damage in avocado plants, as it clearly highlighted Fuerte, which is of Mexican origin, as the most tolerant avocado cultivar. Also, the cultivar Fuerte experienced the least amount of damage to its photosynthetic and enzymatic system.

Materials and methods

Plant materials

The experiment was conducted at the Agronomic Institute of Paraná (IAPAR) in Londrina, PR, Brazil. The assessments comprised six avocado cultivars: Geada, Fortuna, Fuerte, Quintal, Margarida, and Primavera. Their plantlets were produced by cuttings, and then, grown in nurseries until they reached an average height of approximately 1 m. Afterwards, four pots of each cultivar were selected for low

temperature treatments, and a group of untreated plants was kept in the greenhouse as a control.

Cold Stress Treatments

The simulation of low temperatures was performed in a growth chamber (Commercial S.S. Scientific, Londrina, Paraná), in the Laboratory of Environmental Simulation at the Ecophysiology sector of IAPAR. The plants were conditioned in the test area inside the chamber for a 24 h acclimation period (beginning at noon) at a minimum temperature of 5 °C, relative humidity of 60%, and 12:12 h light-dark cycle. After acclimation, the temperature was reduced linearly, reaching the minimum at approximately 18:00 h. The minimum temperature was maintained for 1 h, after which it was elevated until it reached 13 °C, which took 6 h. The chamber was programmed to reproduce, approximately, the natural thermal conditions of real frost. Relative humidity increased with decreases in temperature, as expected, reaching values close to saturation at the lowest temperatures (Silva et al., 2004). Groups of four plants of the same cultivars were submitted for 24 hours, as explained above, to the one of the following minimum temperatures: −2.5, −4.0, −5.0, and −6.0 °C.
After each treatment, the plants were removed from the chamber, and leaf samples were collected for biochemical testing. Subsequently, the plants were transferred to the nursery where physiological and visual evaluations were performed.

Physiological evaluations

Photosynthesis was evaluated after 1, 7, and 21 days of cold stress using an IRGA portable infrared gas analyzer (model LCpro-SD, ADC BioScientific Limited, Hertfordshire). The leaves were exposed to artificial light with an incident photosynthetic radiation of 869 µmol m^{-2} s^{-1}.
The pressure potential was determined a day after the low temperature treatments, using two leaf discs with an area of 2.04 cm^2 each, collected from leaves located in the middle third of the plants. They were placed for 24 h in thermocouple psychrometers (C-30, Wescor, Inc.) and connected to a datalogger (CR-7, Campbell Scientific, Inc). The microvoltage supplied by the system was converted to water potential (in MPa), based on the previous calibration of the sensors with solutions of sodium chloride, thereby obtaining the total water potential in the leaf. After 24 h, the psychrometers were submersed in liquid nitrogen, and the pressure potential was set to zero. After that, the sensors registered the osmotic potential. Then, the pressure potential was obtained from the difference between the total water potential and the osmotic potential.

Visual damage scores

The injuries observed in the leaves and trunk of the plants were visually evaluated 21 days after the low temperature treatments. Based on these visual evidences, (Caramori et al., 2002), we assigned scores from 1 to 5; 1 = No damage, 2 = Leaves with slight damage, 3 = Leaves with moderate damage, 4 = Leaves with severe damage, and 5 = Death (Fig 1).

Biochemical evaluations

The leaves samples collected were stored in a −80 °C freezer until further analyses. To obtain the crude extract of the samples, 0.25 g of leaf tissue was soaked in liquid nitrogen and in 5 ml of 50 mM potassium phosphate buffer (pH 7.0), to which 4% (w / v) polyvinylpyrrolidone were added, and then, the mixture was centrifuged (6955 g) for 10 min at 4 °C. After that, the supernatants were transferred to 2 ml Eppendorf tubes and kept in a freezer at −14 °C until further analyses, which were performed in duplicates.
A Bradford assay (1976) was used to quantify the total proteins in the crude extracts. This assay is based on the color change of Coomassie Brilliant Blue G-250 reagent when bound to proteins. Moreover, the calibration curve of the reagent was performed using bovine serum albumin (0–15 µg $µL^{-1}$) as the standard. The concentration of total proteins was calculated by comparing the readings of the materials with those from the standard curve, and it was expressed in mg of protein per g of fresh matter (FM) (mg protein g^{-1} FM).
The activity of the enzyme APX was based on the methodology of Nakano and Asada (1981) with modifications for the species. The enzyme APX catalyzes the reduction of H_2O_2 through the oxidation of ascorbate. The reaction solution consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM H_2O_2, 0.5 mM ascorbate, and 100 µL of the plant crude extract. Furthermore, the readings were performed in a spectrophotometer at 290 nm, after the addition of H_2O_2 in the buffer containing ascorbate and the enzymatic extract, every 15 s for 2 min of the reaction. The activity of the enzyme was calculated using a molar extinction coefficient of 2.8 mM cm^{-1}. These values were expressed in units of activity per minute per milligram of fresh matter (UA min^{-1} mg FM^{-1}); an enzymatic activity unit represents by the amount of enzyme that catalyzes the oxidation of a micromole of ascorbate.
The activity of CAT was determined by the catalytic decomposition of H_2O_2, which was monitored by spectrophotometry at 240 nm (Peixoto et al., 1999). The reaction solution consisted of 50 mM potassium phosphate buffer (pH 7.0) and 12.5 mM H_2O_2. The assay began with the addition of 50 µL of the plant crude extract in a 3 mL quartz cuvette. A reading at 240 nm was taken immediately after addition of the extract and every 30 s after that for 4 min of the reaction. The difference in absorbance (ΔA240) was multiplied by the molar extinction coefficient of H_2O_2 (36 M^{-1} cm^{-1}) to obtain the activity of the enzyme expressed in mmol H_2O_2 min^{-1} mg FM.

Statistical analysis

The experiment was completely randomized with a factorial design, consisting of six cultivars and five minimum temperatures, with four replicates composed of one plant each. An analysis of variance was conducted on the data, and the means were compared using Tukey's post-hoc test at a 5% probability level. Subsequently, Pearson's simple correlation coefficients (r) were calculated for all combinations of the evaluation tests, where the significance of the r values was determined using a t-test at a 5% probability level.

Conclusion

In this study, we found that the cultivar 'Fuerte' was the most tolerant to temperatures as low as −5 °C. However, exposure to temperatures of −6 °C was lethal for all avocado cultivars studied. Thus, it is possible to state with confidence that the cultivar Fuerte was the most tolerant to cold, because it experienced the least amount of damage to its photosynthetic and enzymatic systems. Furthermore, evaluations of visual damage, photosynthesis, protein content, and peroxidase activity provided consistent results to quantify cold tolerance in avocado cultivars. Additionally, the pressure potential could be used as an indicator of cell damage in plants exposed to lethal temperatures.

Acknowledgements

The authors would like to acknowledge Coordination for the Improvement of Higher Education Personnel - CAPES for supporting this study. We are thankful to IAPAR for the equipment and facilities to conduct this study.

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