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Electrical conductivity as an indicator of damage due to low temperatures in beans leaves

Condutividade elétrica como indicador de danos por temperaturas baixas em folhas de feijão

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Abstract

The electrical conductivity test indirectly evaluates cell membrane disorganization by quantifying the electrolytes released into the water after tissue imbibing. The objective of this work was to evaluate methodological variations in the electrical conductivity test, for it to serve as an indicator of low temperature-induced damages and estimate the cold tolerance of bean plants. Cultivar IPR Uirapuru plants were subjected to minimum temperatures of 4 °C, 2 °C, 0 °C, -1 °C, -2 °C, -3 °C, and -4 °C for 1 h in a growth chamber under controlled conditions. After the treatment period, the response of plants to cold stress was evaluated by determination of the total protein content, and catalase (CAT) and ascorbate peroxidase (APX) enzymatic activities, and evaluation of photosystem II (Fm/Fv) efficiency and leaf anatomy. These results were compared with those obtained in the electrical conductivity test, which was performed in plants under cold stress as well as under a non-stress environment, with 2, 4, 6, and 8 leaf discs immersed in 30 mL of distilled water for 24 h in BOD, at temperatures of 25 °C, 30 °C, and 35°C. Analysis of variance was performed using a completely randomized design, and for electrical conductivity, a number of discs × cold stress temperature combinations were used for each soak temperature. The averages were compared using the Turkey's test at 5% and 10% probability. Pearson correlation coefficient (r) between the conductivity averages and other cold stress evaluation data was also performed. The results showed a marked reduction in the ratio (Fv/Fm) only in the treatments at -3 °C and -4°C, which indicated tissue death. At temperatures below 0°C, there was a collapse of the leaf blade tissues, and it was not possible to differentiate the palisade parenchyma from the spongy parenchyma in the treatments at -2°C, -3°C, and -4°C. There was an increase in the protein content since the temperature -3°C. The enzyme activity of CAT decrease at -4°C whereas that of APX increased. In the electrical conductivity test, there was a significant interaction between soak temperature and the number of discs, and an increase in conductivity of the solution with a decrease in temperature was verified in several treatments, among which, the combination that best correlated with the other tests was 25°C with six leaf discs. It was concluded that the electrical conductivity test presents results similar to those obtained from other physiological, biochemical, and anatomical tests, and therefore, it can be used to evaluate the damage caused by low temperatures in bean plants.

Key words: Oxidative stress. Cold. Enzymes. Membrane integrity.

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Resumo

O teste de condutividade elétrica avalia de forma indireta a desorganização da membrana celular, pela quantificação dos eletrólitos liberados na água após a embebição dos tecidos. Assim, o objetivo do trabalho foi avaliar as variações de metodologias no teste de condutividade elétrica como indicador de danos por temperaturas baixas para estimar a tolerância ao frio de plantas de feijão preto. Dessa forma, as plantas da cultivar IPR Uirapuru foram submetidas às temperaturas mínimas de 4ºC, 2ºC, 0ºC, -1ºC, -2ºC, -3ºC e -4ºC, por uma hora, em câmara de crescimento sob condições controladas. Após esse período, a resposta das plantas ao estresse por frio foi avaliada pela determinação do teor de proteínas totais, atividade enzimática da catalase (CAT) e da ascorbato peroxidase (APX), além da avaliação da eficiência do fotossistema II (Fv/Fm) e da anatomia foliar. Esses resultados foram comparados com os obtidos no teste de condutividade elétrica o qual foi realizado nas plantas após o estresse frio e em plantas não estressadas (ambiente) com 2, 4, 6 e 8 discos foliares imersos em 30 ml de água destilada, mantidos por 24 horas em B.O.D nas temperaturas de 25 ºC, 30 ºC e 35 ºC. A análise de variância foi realizada em delineamento inteiramente casualizado, e para condutividade elétrica utilizou-se fatorial número de discos x temperatura de estresse ao frio para cada temperatura de embebição separadamente. As médias foram comparadas pelo teste Tukey a 5 e 10% de probabilidade. Realizou-se a correlação de Pearson (r) entre as médias de condutividade e os demais testes de avaliação do estresse por frio. Nos resultados observou-se acentuada redução da relação (Fv/Fm) apenas nos tratamentos -3°C e -4°C indicando morte dos tecidos. Para temperaturas inferiores a 0°C houve colapso dos tecidos do limbo foliar, não sendo possível diferenciar o parênquima paliçádico do parênquima esponjoso nos tratamentos -2, -3 e -4 °C. Nas análises bioquímicas houve aumento da proteína a partir da temperatura -3ºC, a enzima CAT teve sua atividade diminuída a - 4ºC, e a APX apresentou aumento da atividade enzimática a partir de 0ºC. No teste de condutividade elétrica houve interação entre temperatura de embebição e o número de discos, e verificou-se aumento da condutividade da solução com a diminuição da temperatura em diversos tratamentos, e dentre eles a combinação que melhor correlacionou com os demais testes foi 25°C com 6 discos foliares. O teste de condutividade elétrica apresenta resultados semelhantes aos obtidos com os demais testes fisiológicos, bioquímicos e anatômicos, podendo ser utilizado para avaliação de danos causados por temperaturas baixas em plantas de feijão.

Palavras-chave: Estresse oxidativo. Frio. Enzimas. Integridade de membranas.

Introduction

Brazil is the largest producer and consumer of common bean (*Phaseolus vulgaris* L.) in the world, with the state of Paraná being the largest producer of this legume. In addition, people in the Southern region of Brazil have been traditional bean consumers of the black tegument (ZABOT et al., 2014). As a result, any biotic or abiotic factors that negatively affect the cultivation of black beans can harm the consumers by virtue of rise in prices and/or lack of product in the market.

Among the abiotic factors that affect the physiology of plants, temperature is a prominent one, and bean plants are particularly sensitive to thermal variations in the environment (LUO, 2011). This variability is a key determinant of yield for the bean plant, as it directly affects its development during different phenological stages. The range 18-30 **°C** is considered optimal for bean plants (FILGUEIRA, 2008), and when grown in the winter season, they can be adversely affected by low temperatures, as the crop is susceptible to frost at temperatures below -3 **°C** or -4 **°C**.

Low temperature causes cell damage in plants, because the liquid present in the intracellular space freezes, causing membrane ruptures by compression and irreversible extravasation of the cytoplasm (SANGHERA et al., 2011). These leached electrolytes can be quantified by evaluating the electrical conductivity of the imbibition solution. This involves quantification of the electrolytes released by the leaf in the imbibition

water, as this would be proportional to the degree of disorganization of the plasma membrane (TAIZ; ZEIGER, 2017).

In addition, different components of the photosynthetic system are affected when the plants are exposed to low temperatures (TAIZ; ZEIGER, 2017). In the case of bean plants, the decrease in photosynthesis is attributed to the decrease in stomatal conductance along with other factors such as photo-assimilated partition (PEISKER; TICHÁ, 1991; PEÑA-VALDIVIA et al., 1994).

For evaluating the damage caused by low temperatures, chlorophyll fluorescence is a widely used method that helps observe the functioning of the photosynthetic apparatus. Emitted by photosystem II, it is an efficient tool to measure the response of plants to stress (ENNAHLI; EARL, 2005; NAUMANN et al., 2008; MASSACCI et al., 2008; CALATAYUD et al., 2008; FLOWERS et al., 2008; STIRBET; GOVINDJEE, 2011). The main parameter used to characterize the fluorescence is the Fv/Fm ratio, which indicates the photochemical efficiency of photosystem II. Normally, this ratio decreases in stressed plants (KRAUSE; WEIS, 1991).

Plants have also developed a variety of responses to extreme temperatures that minimize damage and promote the maintenance of cell balance (KOTAK et al., 2007). When exposed to low temperatures, plants tend to use enzymatic antioxidant systems to deal with the harmful effects (ALMESELMANI et al., 2006). Studies have explored the relationship between stress tolerance under low temperatures and the enzymatic activities of antioxidant systems in plant tissues, showing that reinforcement of this antioxidant defense confers greater tolerance against oxidative stress (HUANG; GUO, 2005).

Therefore, several tests can be used in the study of tolerance to low temperatures in plant species, which mainly evaluate the different physiological responses of plants. Among these tests, the electrical conductivity test stands out as a simple, fast, efficient, and easily reproducible measurement (BARRANCO et al., 2005).

Studies that have used the electrical conductivity test to evaluate the tolerance of plants at negative temperatures have described the methodologies specific to each species. Thus, this test has been used by some authors with success, such as Silva et al. (2009), who evaluated the tolerance to cold and freezing in three hybrids of Eucalyptus by measuring the electrical conductivity to evaluate leaf damage. Manetti Filho et al. (2018) also used this test to measure the cold tolerance of several forage species. Moshtaghi et al. (2009) were successful in using the test to measure the tolerance to low temperatures in olive leaves, by evaluating the foliar density and quantity of electrolytes released, however, they concluded that more studies are needed on the conductivity test.

Thus, the objective of this work was to evaluate the variations in the methodologies of the electric conductivity test in leaf discs as an indicator of low temperature-induced damage, to estimate the cold tolerance of bean plants of IPR variety Uirapuru.

Material and Methods

The black bean cultivar IPR Uirapuru was seeded in pots and cultured in a greenhouse for a period of approximately 40 days, up to the vegetative stage V4. Ten plant pots at a time were subjected to low temperature treatments of 4 °C, 2 °C, 0 °C, -1 °C, -2 °C, -3 °C, and -4 °C, in a plant growth chamber (SS Commercial Reference) within the Laboratory of Simulation of Environments, Agrometeorology and Plant Physiology, the Agronomic Institute of Paraná - IAPAR, Londrina, PR.

The plants were conditioned in the test area inside the chamber for about 12 h followed by a 24 h acclimation period, at a minimum temperature of 5 °C and 60% relative humidity. The brightness inside the chamber had been programmed to simulate a photoperiod of approximately 12 h. After

acclimation, the temperature was reduced linearly, reaching the minimum values at approximately 6h, where it was maintained for 1 h and then increased again until reaching at 13°C after 6 h. The chamber was programmed to reproduce approximately the natural thermal conditions, based on real frosty weather.

In order to evaluate the response of plants to the various cold stress levels, the following evaluations were performed: photosystem II (Fv/Fm) efficiency, leaf anatomy, determination of total protein content, and activity of the enzymes catalase (CAT) and ascorbate peroxidase (APX).

The efficiency of photosystem II (Fv/Fm) was evaluated using an OS5p fluorometer. The initial fluorescence (F_0) was obtained at low light saturation (<0.1 μmol m^{-2} s^{-1}) and the maximum fluorescence was determined using a saturating photon pulse of 4000 μmol m^{-2} s^{-1} for 0.3 s, adjusted for a frequency of 600 Hz. The evaluations were carried out in eight homogeneous plants before and after the plants were subjected to the low temperatures, using the same leaf from the third pair of developed leaves.

For the biochemical and anatomical analyses, four leaf samples were collected after exposure to low temperatures, one for each of the two vessels, yielding a total of four replicates. The leaf samples used in the biochemical analyses were enclosed in a paper bag and stored in a freezer at -80 °C until analysis. To obtain the crude extracts of the samples, 0.250g of leaf tissue was weighed, and macerated in 5 mL50 mM potassium phosphate buffer (pH 7.0) and 4% PVP (w/v), previously cooled to 4 °C. After centrifugation for 10 min at 4 °C at 7500 rpm, the supernatant was transferred to 2 mL Eppendorf tubes and stored in a freezer at -14 °C until analysis.

The crude extracts were subjected to quantification of total proteins, performed using the method of Bradford (1976), which is based on the color change in Coomassie Brilliant Blue G-250 reagent when bound to the protein. For this purpose, the calibration curve of the reagent was used, using bovine serum albumin (BSA 0-15 μg μL^{-1}) as a standard. The total protein concentration was calculated by comparing the sample readings with those obtained from the standard curve and expressed as mg of fresh matter $protein^{-1}$ (MF).

The catalase activity was determined based on the consumption of H_2O_2, monitored by spectrophotometry at 240 nm, considering the molar extinction coefficient of H_2O_2 of 36.0 $M^{-1}cm^{-1}$ (PEIXOTO et al., 1999). The reaction solution consisted of 50 mm potassium phosphate buffer (pH 7.0) and 15 mm H_2O_2. The assay begins with the addition of 200 μL of the diluted enzyme extract in a 3mL quartz cuvette. Reading is taken at 240 nm immediately after addition of the extract, and at every 30 s for a total reaction time of 4 min. The difference in absorbance (ΔA240) was multiplied by the molar extinction coefficient of H_2O_2 and the activity of the enzyme was expressed in millimoles of H_2O_2 consumed per minute per milligram of protein (mmol H_2O_2 min^{-1}mg $protein^{-1}$).

The enzyme ascorbate peroxidase (APX) catalyzes the reduction of H_2O_2 to H_2O through the oxidation of ascorbate. The reaction solution consisted of 50 mm potassium phosphate buffer (pH 7), 1 mm H_2O_2, 0.5 mm ascorbate, and enzymatic extract. The assay started with the addition of 100 μL of the diluted enzyme extract in a 3mL quartz cuvette. Readings were taken at 290 nm immediately after the addition of extract, and at every 15 s for a total reaction time of 2 min. The activity of the enzyme was calculated using the molar extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ (PEIXOTO et al., 1999).

The values were expressed in units of activity per minute per milligram of fresh matter (UAmin^{-1}mg MF^{-1}), which represents the amount of enzyme that catalyzed the oxidation of 1 μmol^{-1} of ascorbate. For the evaluation of leaf anatomy, permanent slides, sectioned on a sliding microtome (Leica-SM2010R), were prepared. For that, samples of leaf segments of limb us were used. The materials were fixed in FAA (formaldehyde, acetic acid, ethyl

alcohol 70%, 1:1:18), washed in 70% alcohol, dehydrated, and embedded in paraffin (JOHANSEN, 1940). The staining was performed with basic astra blue-basic fuchsin (1:1) (O'BRIEN et al., 1964). The assembly was performed with glycerin, and sealed with colorless enamel, according to the usual methodology used in vegetal anatomy. The slides were observed under a light microscope (Olympus - CX21), and the anatomical records were stored by a computerized image capture system coupled to a microscope - MOTIC System (Zeiss - Axioskop). For each treatment, 15 sections were used to obtain an average.

In order to conduct the electrical conductivity test, leaf discs of area 1.2 cm^2 were taken from the bean plants subjected to temperatures of 4 °C, 2 °C, 1 °C, 0 °C, -1 °C, and -2 °C, and ambient conditions (control). Four leaf samples were collected after exposure to low temperatures, one for each of the two vessels, yielding a total of four replicates. For the negative temperatures, -3 °C and -4 °C, it was not possible to perform this test, as the leaves were wilted and dead, making the extraction of leaf discs impossible. In disposable plastic cups containing 30 mL distilled water, 2, 4, 6, or 8 leaf discs were immersed for 24 hours in germination chambers at soaking temperatures of 25°C, 30°C, and 35°C, with four replicates each. The reading was performed using a digital conductivity meter, and the data were expressed in $\mu mhoscm^{-1}$.

The experimental design was completely randomized and the data were subjected to analysis of variance. For the electrical conductivity, each imbibition temperature was evaluated separately, in a factorial scheme, by studying the effect of the number of discs × cold stress temperatures (4 × 7). Pearson's correlation coefficients (r) were calculated for all combinations of electrical conductivity and the usual tests for cold stress evaluation, while the significance of the r values was determined by *t*-test at 5% probability.

Results and Discussion

The photosynthetic efficiency, evaluated using the variable fluorescence ratio at maximum (Fv/Fm), was affected by temperature, as can be observed in Figure 1. The Fv/Fm ratio was lower after exposure of the plants to cold, reaching values below 0.4. This indicated that there was damage to the photosynthetic apparatus, as according to Bolhàr-Nordenkampf and Öquist (1993) the Fv/Fm ratio of a plant with its intact photosynthetic apparatus varies between 0.75 and 0.85, and a decrease in this ratio reflects the presence of photoinhibitory damage in the PSII reaction centers (BJÖRKMAN; DEMMIG, 1987).

Similar results were obtained by Yusuf et al. (2010) in leguminous crops, Rapacz et al. (2007) in wheat plants, and Percival et al. (2003) with Acer genotypes, thus, indicating that chlorophyll fluorescence response to low temperature stress was manifested by Fv/Fm reduction.

As for the leaf anatomy data, in treatments with temperatures below 0 °C, contraction, breakage, and collapse of the tissues were observed, and therefore, that the thickness of the limb (diameter) was lower in treatments with negative temperatures (Table 1 and Figure 2).

Figure 1. Variable and maximum fluorescence (Fv/Fm) measurements performed on the bean leaves before and after the plants were subjected to low temperatures.

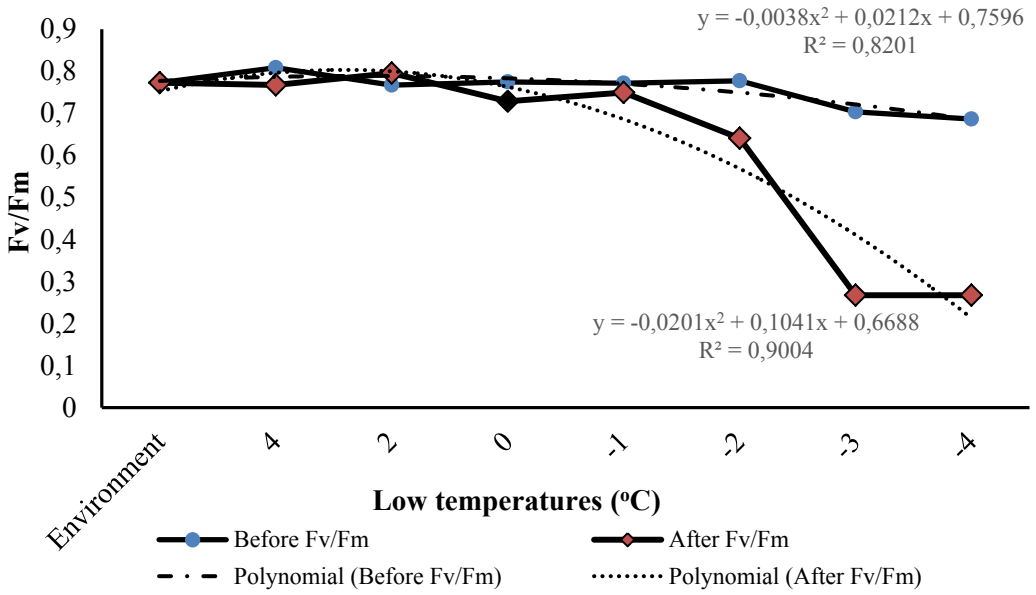


Table 1. General results of leaf anatomy analyses of bean plants subjected to cold stress under different temperatures.

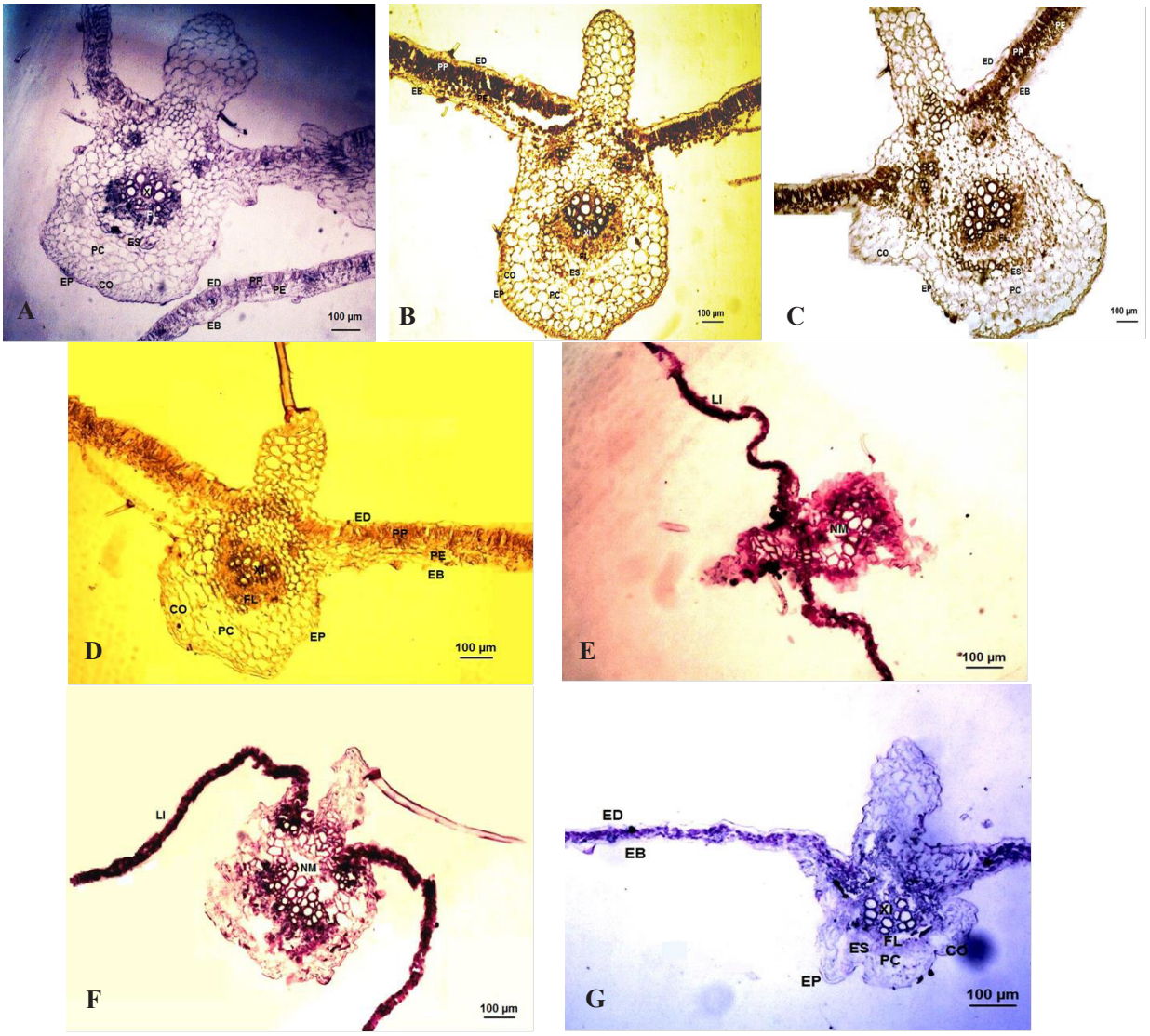
TPT (C°)	4	2	0	-1	-2	-3	-4
Total	117.28b*	136.85a	123.85b	122.03b	37.14c	47.46cd	51.05d
Ep. abaxial +Cut.	13.08cd	14.09abc	15.89a	13.67bc	0.00e	0.00e	11.17d
Ep. abaxial +Cut.	11.95b	14.54a	14.58a	11.40b	0.00c	0.00c	10.66b
Par. palisade	42.11c	53.60a	45.93bc	49.15b	0.00d	0.00d	0.00d
Par. spongy	50.13ab	54.62a	47.45b	47.81b	0.00c	0.00c	0.00c
Diameter (µm)	616.99b	539.16c	804.31a	497.99c	393.84d	439.28d	407.51d
epidermis + Cut.	13.49b	15.31b	17.95b	10.87c	0.00d	0.00d	9.96c
colenchyma	46.88b	37.29c	56.73a	36.79c	0.00e	0.00e	28.17d
Parenc. cortical	152.38a	159.13a	160.86a	158.83a	0.00c	0.00c	116.62b
Sclerenchyma	63.96b	53.25b	97.31a	0.00d	0.00d	0.00d	19.21c
phloem	66.70b	58.61b	87.27a	62.11b	0.00d	0.00d	48.43c
xylem	163.06b	140.41cd	227.95a	128.38d	0.00e	0.00e	148.79bc
Diam. Elem. Vase	29.95b	26.20c	34.01a	21.02d	0.00e	0.00e	28.23bc

*Means followed by the same letter in a row do not differ statistically from each other, based on the Tukey averages test at 5% probability.

In addition, due to this collapse, it was not possible to differentiate the palisade parenchyma from the spongy parenchyma in treatments -2 °C, -3 °C, and -4 °C. At 0 °C and -1 °C, the spongy parenchyma was less thicker than that at 4 °C and 2°C. The adaxial and abaxial epidermal faces were also thicker or collapsed in treatments with temperatures below 0 °C. At -1 °C and -4°C, they were thinner than those at positive temperatures; and at -2 °C and -3 °C, they collapsed, making

it impossible to measure them separately. Thus, in Table 1, some measurements show a value of 0.0, indicating that the tissue was absent, either collapsed or degraded. It is inferred that this result was caused by breakage of the cell walls, structural weakening, and tissue collapse caused by freezing (McCONNELL; SHEEHAN, 1978).

Figure 2. Medium vein and leaf blade limb subjected to low temperatures: (A) 4°C; (B) 2°C; (C) 0°C; (D) -1°C; (E) -2°C; (F) -3°C (G) -4°C.



CO - colenchyma; EB - abaxial epidermis; ED - adaxial epidermis; EP-epidermis; ES - Sclerenchyma; PC - cortical parenchyma; PE - spongy parenchyma; PP - palisade parenchyma; FL - phloem; XI - xylem, LI - limbo; NM - median vein.

At negative temperatures, the total diameter of the median rib was smaller, and, individually, the tissues showed lower thickness when subjected to negative temperatures. However, cell damage to mesophyll cells may be accompanied by other changes, such as reduction of intercellular space volume and increase in leaf thickness (BANDURSKI; GREINER, 1953).

Chinnusamy et al. (2007) also observed leaf damage after cold and stated that low temperature stress impedes the expression of the physiological potential of plants due to the inhibition of metabolic reactions and, indirectly, by restricting water absorption and cell dehydration, induced by freezing.

In the biochemical analyses, it was noted that with a decrease in temperature, there was an increase in the protein content, especially at the temperature of -3 **°C** (Table 2).This is suggested as a mechanism of tolerance to the low temperatures, because, by increasing the protein content of the cells, the osmotic potential of the cytoplasm increases, decreasing the freezing temperature of the cell (TAIZ; ZEIGER, 2017). Several other authors have also observed changes in proteins as an inherent characteristic of cold tolerance (TSENG; LI, 1991).

Table 2. Analysis of the total protein content (mg g^{-1} protein of FM) and enzymatic activity of ascorbate peroxidase (APX) (UAmin^{-1}mg FM^{-1}) and catalase (CAT) (mmol H_2O_2 min mg^{-1} protein^{-1}) in black bean leaves subjected to cold stress at temperatures of 4 °C, 2 °C, 0 °C, -1 °C, -2 °C, -3 °C, and -4 °C.

Temperatures	**Protein**		**APX**		**CAT**	
4 °C	3.9979	b*	0.0008	ab	0.4160	ac
2 °C	4.5661	ab	0.0004	b	0.4474	a
0 °C	6.6642	ab	0.0023	a	0.2642	bc
-1 °C	5.3851	ab	0.0006	ab	0.3989	b
-2 °C	3.8912	b	0.0019	ab	0.2590	abc
-3 °C	8.3515	a	0.0012	ab	0.3305	bc
-4 °C	6.6923	ab	0.0015	ab	0.2281	ac

*Means followed by the same letter in a row do not differ statistically from each other, based on the Tukey averages test at 10% probability.

In terms of catalase activity, there was a significant statistical difference, indicating its performance in the elimination of oxidative stress caused by the low temperatures, as seen in table 2. At the temperature of 2 **°C**, there was a higher activity of the enzyme relative to the other temperatures, as also observed by Nir et al. (1986) and Dat et al. (2000). Catalase is involved in the elimination of H_2O_2 and its accumulation is continuous, however, its activity may get significantly reduced when subjected to stress at low temperatures, as observed, where catalase showed lower activity at -4 °C, signaling the death of tissues.

As for ascorbate peroxidase, the data obtained presented a statistically significant difference, and the temperatures of 0 **°C**, -2 **°C**, -3 **°C**, and -4 **°C**had a higher activity of the enzyme relative to the others, demonstrating the plant response to a stress situation. Siegel et al. (1993) also observed that oxidative enzymes, involved in various physiological processes, often have their response enhanced by stress at low temperatures. Thus, APX activity is related to signaling processes through a stress condition (MITTLER, 2002).

These results showed that enzymatic systems are dynamic, and depending on the situation, one or the other enzyme is activated in an attempt to eliminate oxidative systems(in this case, hydrogen peroxide). Thus at -1 **°C**, a low ascorbate peroxidase performance is observed, along with the highest value of catalase activity, demonstrating that there was low temperature stress in the bean plants at all the treatment temperatures.

The results of the electrical conductivity test showed a significant interaction between cold stress temperatures and number of discs, at all imbibition temperatures (Table 3). In general, it was verified for all treatments that with an increase of the number of discs, the conductivity value was also higher, following the increase in the concentration of solutes in the imbibition solution.

In the context of low temperatures, most combinations of number discs and soak temperatures were able to detect cold stress, with an exception of 2 and 4 discs at 25 °C. This result is unexpected, as according to Guye et al. (1987), the loss of membrane integrity resulting from prolonged exposure to low temperature is the main response of plants sensitive to cold and may cause photodamage. This loss of membrane integrity due to cold results in the release of electrolytes, which can be estimated by the electrical conductivity test. Manetti Filho et al. (2018) also verified that the electrical conductivity test is an efficient way to verify cold stress in forage species. Thus, greater the stress due to cold, higher should be the value of electrical conductivity.

Table 3. Electric conductivity (μmhoscm^{-1}) performed with different numbers of leaf discs (NDF) and under different soaking temperatures (TE), on cold-stressed bean leaves at temperatures of 4 °C, 2 °C, 0 °C, -1 °C, and -2 °C, and ambient temperature at 20.5 °C.

TE	NDF	Environment	Minimum Temperatures				
		20.5 °C	**4 °C**	**2 °C**	**0 °C**	**-1°C**	**-2°C**
25°C	2	11.08 Ca*	14.15 Da	20.36 Ca	16.38 Ca	14.41 Ca	17.93 Ca
	4	23.62 BCa	31.19 Ca	26.12 Ca	26.31 Ca	32.49 Ba	30.69 Ca
	6	29.52 Bc	46.12 Bb	40.76 Bbc	44.33 Bb	41.79 ABbc	70.53 Ba
	8	77.47 Ab	86.82 Aab	55.77 Ac	96.77 Aa	51.36 Ac	98.47 Aa
CV%	17.01						
30°C	2	15.11Cab	9.16 Db	16.09 Dab	12.06 Cab	16.67 Dab	16.92 Da
	4	21.78 Cb	22.18 Cb	23.15 Cab	30.04 Ba	29.22 Cab	24.54 Cab
	6	65.21Ba	29.65 Be	34.03 Bde	40.46 Acd	50.54 Bb	41.77 Bc
	8	103.26 Aa	38.53 Ad	49.83 Ac	45.35 Acd	65.88 Ab	59.79 Ab
CV%	10.30						
35°C	2	23.36 Ca	15.74 Dab	16.41Dab	13.66 Db	21.53 Dab	18.61 Cab
	4	29.93 Cab	24.57 Cb	25.09 Cb	23.77 Cb	37.19 Ca	29.85 Bab
	6	57.66 Bab	50.98 Bbc	35.74 Bd	35.85 Bd	48.35 Bc	61.46 Aa
	8	105.56 Ab	70.04 Ac	49.96 Ad	65.33 Ac	117.73 Aa	64.17 Ac
CV%	9.80						

*Averages followed by the same lowercase letter in the row, and the same uppercase letter in the column do not differ statistically from each other by the Tukey averages test at 5% probability. CV= Coefficient of Variation.

Such a continuous increase in the electrical conductivity with a decrease in the stress temperature was observed only for the combinations treatments 6 discs in the temperature of 25 **°C** and 4 discs in the temperature of 30 **°C**.

In the correlation of the conductivity with the results of the tests that evaluated the cold stress response (Table 4), only the 6-disc treatment at the 25 **°C** imbibition temperature correlated significantly and negatively with the fluorescence (Fv/Fm), indicating that higher the conductivity, the lower is the fluorescence, and greater is the

stress to the plants. Thus, this combination of 6 discs at 25 **°C** can be used to evaluate the damage caused to the membranes by cold stress, as they presented similar pattern to the fluorescence test, which according to Stirbet and Govindjee (2011) has been largely applied to evaluate the damage in photosynthetic metabolism under stress conditions, as photosystem II (FSII) is one of the main targets of low temperature stress (BERTAMINI et al., 2007). The electrical conductivity is not a direct measure of the damage caused to the cell, but it is valid way to express the integrity of the foliar cell membrane (BARTOLOZZI; FONTANAZZA, 1999), so it must be used in conjunction with other tests.

Table 4. Pearson correlation coefficient (r) between the means of the electrical conductivity tests performed with different numbers of leaf discs (NDF) and under different soaking temperatures (°C), with the results of the variable and maximum fluorescence analyses performed before (Fv/Fm Before) and after (Fv/Fm After) cold stress, total protein content (Protein), catalase enzyme activity (CAT), and ascorbate peroxidase (APX) in bean plants subjected to low temperature stress.

	Immersion Temperature (°C)											
	25				30				35			
NDF	2	4	6	8	2	4	6	8	2	4	6	8
Fv/Fm Before	-0.29^{ns}	0.43^{ns}	0.16^{ns}	0.40^{ns}	-0.82*	-0.34^{ns}	-0.49^{ns}	-0.44^{ns}	-0.32^{ns}	-0.34^{ns}	0.25^{ns}	-0.14^{ns}
Fv/Fm After	-0.20^{ns}	-0.39^{ns}	-0.88*	-0.63^{ns}	-0.27^{ns}	-0.26^{ns}	0.02^{ns}	0.08^{ns}	0.04^{ns}	-0.18^{ns}	-0.50^{ns}	0.14^{ns}
Protein	0.58^{ns}	0.18^{ns}	0.92*	0.58^{ns}	0.28^{ns}	-0.02^{ns}	-0.31^{ns}	-0.26^{ns}	-0.28^{ns}	-0.17^{ns}	0.29^{ns}	-0.58^{ns}
CAT	-0.33^{ns}	-0.54^{ns}	-0.59^{ns}	-0.08^{ns}	0.06^{ns}	0.57^{ns}	0.57^{ns}	0.38^{ns}	0.12^{ns}	0.10^{ns}	-0.40^{ns}	0.40^{ns}
APX	0.52^{ns}	0.28^{ns}	0.35^{ns}	-0.36^{ns}	0.78^{ns}	0.63^{ns}	0.07^{ns}	-0.04^{ns}	0.11^{ns}	0.54^{ns}	-0.18^{ns}	0.08^{ns}

*Significant at 5% probability. ns not significant.

Conclusions

The methodology to be used for testing electric conductivity in bean leaves involves a combination of six leaf discs, kept at 25**°C** for 24 hours. The electrical conductivity test presents results similar to those obtained from other physiological and anatomical tests, and thus, it can be used to evaluate the damage caused by low temperatures in bean plants.

References

ALMESELMANI, M.; DESHMUKH, P. S.; SAIRAM, R. K.; KUSHWAHA, S. R.; SINGH, T. P. Protective role of antioxidant enzymes under high temperature stress. *Plant Science*, Davis, v. 171, n. 3, p. 382-388, 2006. DOI: 10.1016/j.plantsci.2006.04.009

BANDURSKI, R. S.; GREINER, C. M. The enzymatic synthesis of oxalacetate from phosphoryl-enopyruvate and carbon dioxide. *The Journal of Biological Chemistry*, Massachusetts, v. 204, n. 1, p. 781-786, 1953.

BARRANCO, D.; RUIZ, N.; GÓMEZ-DEL CAMPO, M. Frost tolerance of eight Olive cultivars. *HortScience*, Baton Rouge, v. 40, n. 3, p. 558-560, 2005.

BARTOLOZZI, F.; FONTANAZZA, G. Assessment of frost tolerance in olive (*Olea europaea* L.). *Scientia Horticulturae*, Agassiz, v. 81, n. 3, p. 309-319, 1999. DOI: 10.1016/S0304-4238(99)00019-9

BERTAMINI, M.; ZULINI, L.; MUTHUCHELIAN, K.; NEDUNCHEZHIAN, N. Low night temperature effects on photosynthetic performance on two gravepine genotypes. *Biologia Plantarum*, Prague, v. 51, n. 2, p. 381-385, 2007.

BJÖRKMAN, O.; DEMMIG, B. Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at

77 K among vascular plants of diverse origins. *Planta*, Berlim, v. 170, n. 4, p. 489-504, 1987.

BOLHÀR-NORDENKAMPF, H. R.; ÖQUIST, G. Chlorophyll fluorescence as a tool in photosynthesis research. In: HALL, D. O.; SCURLOCK, J. M. O.; BOLHÀR-NORDENKAMPF, H. R.; LEEGOOD, R. C.; LONG, S. P. (Ed.). *Photosynthesis and production in a changing environment:* a field and laboratory manual. London: Chapman & Hall, 1993. p. 193-206.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, Bethesda, v. 72, n. 1, p. 248-254, 1976. DOI: 10.1016/0003-2697(76)90527-3

CALATAYUD, Á.; GORBE, E.; ROCA, D.; MARTÍNEZ, P. F. Effect of two nutrient solution temperatures on nitrate uptake, nitrate reductase activity, NH_4^+ concentration and chlorophyll a fluorescence in rose plants. *Environmental and Experimental Botany*, Barcelona, v. 64, n. 1, p. 65-74, 2008. DOI: 10.1016/j.envexpbot.2008.02.003

CHINNUSAMY, V.; ZHU, J.; ZHU, J. K. Cold stress regulation of gene expression in plants. *Trends in Plant Science*, Massachusetts, v. 12, n. 10, p. 444-451, 2007. DOI: 10.1016/j.tplants.2007.07.002

DAT, J.; VANDENABEELE, S.; VRANOVÁ, E.; VAN MONTAGU, M.; INZÉ, D.; VAN BREUSEGEM, F. Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences*, Freiburg, v. 57, n. 5, p. 779-795, 2000.

ENNAHLI, S.; EARL, H. J. Physiological limitations to photosynthetic carbon assimilation in cotton under water stress. *Crop Science*, Washington, v. 45, n. 6, p. 2374-2382, 2005.

FILGUEIRA, F. A. R. *Novo manual de olericultura:* agrotecnologia moderna na produção e comercialização de hortaliças. 2. ed. Viçosa, MG: UFV, 2008. 421 p.

FLOWERS, M. D.; FISCUS, E. L.; BURKEY, K. O.; BOOKER, F. L.; DUBOIS, J. J. B. Photosynthesis, chlorophyll fluorescence, and yield of snap bean (*Phaseolus vulgaris* L.) genotypes differing in sensitivity to ozone. *Environmental and Experimental Botany*, Barcelona, v. 61, n. 2, p. 190-198, 2008.

GUYE, M. G.; VIGH, L.; WILSON, J. M. Recovery after chilling: an assessment of chill-tolerance in *Phaseolus* spp. *Journal of Experimental Botany*, Colchester, v. 38, n. 4, p. 691-701, 1987. DOI:10.1093/jxb/38.4.691

HUANG, M.; GUO, Z. Responses of antioxidative system to chilling stress in two rice cultivars differing in sensitivity. *Biologia Plantarum*, Prague, v. 49, n. 1, p. 81-84, 2005.

JOHANSEN, D. A. *Plant microtechnique*. New York: Mc Graw- Hill Book Company, 1940. 523 p.

KOTAK, S.; LARKINDALE, J.; LEE, U.; VON KOSKULL-DÖRING, P.; VIERLING, E.; SCHARF, K. D. Complexity of the heat stress response in plants. *Current Opinion in Plant Biology*, London, v. 10, n. 3, p. 310-316, 2007. DOI: 10.1016/j.pbi.2007.04.011

KRAUSE, G. H.; WEIS, E. Chlorophyll fluorescence and photosynthesis: the basics. *Annual Review of Plant Biology*, Berkeley, v. 42, n. 1, p. 313-349, 1991.

LUO, Q. Temperature thresholds and crop production: a review. *Climatic Change*, Princenton, v. 109, n. 3-4, p. 583-598, 2011. DOI 10.1007/s10584-011-0028-6

MANETTI FILHO, J.; OLIVEIRA, C. M. G.; CARAMORI, P. H.; NAGASHIMA, G. T.; HERNANDEZ, F. B. T. Cold tolerance of forage plant species. *Semina: Ciências Agrárias*, Londrina, v. 39, n. 4, p. 1469-1476, 2018. DOI: 10.5433/1679-0359.2018v39n4p1469

MASSACCI, A.; NABIEV, S. M.; PIETROSANTI, L.; NEMATOV, S. K.; CHERNIKOVA, T. N.; THOR, K.; LEIPNER, J. Response of the photosynthetic apparatus of cotton (*Gossypium hirsutum*) to the onset of drought stress under field conditions studied by gas-exchange analysis and chlorophyll fluorescence imaging. *Plant Physiology and Biochemistry*, Bari, v. 46, n. 2, p. 189-195, 2008. DOI:10.1016/j.plaphy.2007.10.006

McCONNELL, D. B.; SHEEHAN, T. J. Anatomical aspects of chilling injury to leaves of *Phalaenopsis*. *HortScience*, Baton Rouge, v. 13, n. 1, p. 705-706, 1978.

MITTLER, R. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, Massachusetts, v. 7, n. 9, p. 405-410, 2002. DOI: 10.1016/S1360-1385(02)02312-9

MOSHTAGHI, E. A.; SHAHSAVAR, A. R.; TASLIMPOUR, M. R. Ionic leakage as indicators of cold hardiness in olive (*Olea europaea* L.). *World Applied Sciences Journal*, Babol, v. 7, n. 10, p. 1308-1310, 2009.

NAUMANN, J. C.; YOUNG, D. R.; ANDERSON, J. E. Leaf chlorophyll fluorescence, reflectance, and physiological response to fresh water and salt water flooding in the evergreen shrub, *Myrica Cerifera*. *Environment Experimental Botany*, Barcelona, v. 63, n. 1-3, p. 402-409, 2008. DOI:10.1016/j.envexpbot.2007.12.008

NIR, G.; SHULMAN, Y.; FANBERSTEIN, L.; LAVEE, S. Changes in the activity of catalase (EC. 1.11.1.6) in relation to the dormancy of grapevine (*Vitis vinifera* L.). *Plant Physiology*, Glasgow, v. 81, n. 4, p. 1140-1142, 1986. DOI: 10.1104/pp.81.4.1140

O'BRIEN, T. P.; FEDER, N.; MCCULLY, M. E. Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma*, Verlag, v. 59, n. 2, p. 368-373, 1964.

PEISKER, M.; TICHÁ, I. Effects of chilling on CO_2 Gas Exchange in two cultivars of *Phaseolus vulgaris* L. *Journal of Plant Physiology*, New York, v. 138, n. 1, p. 12-16, 1991. DOI: 10.1016/S0176-1617(11)80722-4

PEIXOTO, P. H. P.; CAMBRAIA, J.; SANT'ANNA, R.; MOSQUIM, P. R.; MOREIRA, M. A. Aluminum effects on lipid peroxidation and on the activities of enzymes of oxidative metabolism in sorghum. *Revista Brasileira de Fisiologia Vegetal*, Campinas, v. 11, n. 3, p. 137-143, 1999.

PEÑA-VALDIVIA, C. B.; LAGUNES E, L. del C.; PERALES R, H. R. Chilling effects on leaf photosynthesis and seed yields of *Phaseolus vulgaris*. *Canadian Journal of Botany*, Soskatchewan, v. 72, n. 1, p. 1403-1411, 1994. DOI: 10.1139/b94-173

PERCIVAL, G. C.; FRASER, G. A.; OXENHAM, G. Foliar salt tolerance of *Acer* genotypes using chlorophyll fluorescence. *Journal of Arboriculture*, Urbana, v. 29, n. 2, p. 61-65, 2003.

RAPACZ, M.; GA˛SIOR, D.; KOS´CIELNIAK, J.; KOSMALA, A.; ZWIERZYKOWSKI, Z.; HUMPHREYS, M. W. The role of the photosynthetic apparatus in cold acclimation of *Lolium multiflorum*: characteristics of novel genotypes low-sensitive to PSII over-reduction. *Acta Physiologiae Plantarum*, Poznań, v. 29, n. 4, p. 309-316, 2007.

SANGHERA, G. S.; WANI, S. H.; HUSSAIN, W.; SINGH, N. B. Engineering cold stress tolerance in crop plants. *Current Genomics*, Bentham, v. 12, n. 1, p. 30-43, 2011. DOI: 10.2174/138920211794520178

SIEGEL, V.; JONGENS, T. A.; JAN, L. Y.; JAN, Y. N. Pipsqueak, an early acting member of the posterior group of genes, affects vasa level and germ cell-somatic cell interaction in the developing egg chamber. *Development*, London, v. 119, n. 4, p. 1187-1202, 1993.

SILVA, A. L. L. da; OLIVEIRA, Y. de; ALCANTARA, G. B. de; SANTOS, M. dos; QUOIRIN, M. Tolerância ao resfriamento e congelamento de folhas de eucalipto. *Biociências*, Porto Alegre, v. 17, n. 1, p. 86-90, 2009.

STIRBET, A.; GOVINDJEE. On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: basics and applications of the OJIP fluorescence transient. *Journal of Photochemistry and Photobiology*, Debrecen, v. 104, n. 2, p. 236-257, 2011. DOI:10.1016/j.jphotobiol.2010.12.010

TAIZ, L.; ZEIGER, E. *Fisiologia vegetal*. 6. ed. Porto Alegre: Artmed, 2017. 858 p.

TSENG, M. J.; LI, P. H. Changes in protein synthesis and translatable messenger RNA populations associated with ABA induced cold hardiness in potato (*Solanum commersonii*). *Physiologia Plantarum*, Umeå, v. 81, n. 3, p. 349-358, 1991. DOI: 10.1111/j.1399-3054.1991.tb08743.x

YUSUF, M. A.; KUMAR, D.; RAJWANSHI, R.; STRASSER, R. J.; TSIMILLI-MICHAEL, M.; GOVINDJEE; SARIN, N. B. Overexpression of y-tocopherol methyl transferase gene in transgenic *Brassica juncea* plants alleviates abiotic stress: physiological and chlorophyll a fluorescence measurements. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, Amsterdam, v. 1797, n. 8, p. 1428-1438, 2010. DOI: 10.1016/j.bbabio.2010.02.002

ZABOT, L.; DUTRA, L. M. C.; JAUER, A.; LUCCA FILHO, O. A.; UHRY, D.; STEFANELO, C.; LOSEKAN, M. E.; FARIAS, J. R.; LUDWIG, M. P. Análise de crescimento da cultivar de feijão BR IPAGRO 44 Guapo Brilhante cultivada na safrinha, em quatro densidades de semeadura em Santa Maria - RS. *Revista de Ciências Agroveterinárias*, Lages, v. 3, n. 2, p. 105-115, 2004.