INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
23–0082/2023/30–2–107–112
DOI: 10.17957/IJAB/15.2064
http://www.fspublishers.org

Full Length Article

Comparative Analysis of Leaf Anatomy of Forage Grasses Submitted to Freezing Temperature Stress

João Manetti Filho[1,2], Izabeli Bertini Lara Gonçalves[2], Carolina Maria Gaspar de Oliveira[2], Ricardo de Almeida Gonçalves[1], Fernando Braz Tangerino Hernandez[1], Paulo Henrique Caramori[2] and Aline Redondo Martins[1*]
[1]*Universidade Estadual Paulista, UNESP, Campus Ilha Solteira, Ilha Solteira, São Paulo, Brazil*
[2]*Instituto de Desenvolvimento Rural do Paraná – Iapar-Emater, Londrina, Paraná, Brazil*
[*]For correspondence: aline.martins@unesp.br
Received 13 March 2023; Accepted 24 April 2023; Published 10 August 2023

Abstract

Frost causes frequent damage to pasture and limits continuous foraging under field conditions in many regions. Histological sections of plant tissues after a cold can provide important information to confirm the temperatures at which tissue death begins. This study was aimed to analyze anatomical alterations that may occur in forage leaves submitted to freezing temperatures in a controlled environment. The studied species were sorghum (*Sorghum bicolor* (L.) Moench.), Marandu (*Brachiaria brizantha* cv. Marandu), Millet (*Pennisetum glaucum* (L.) R. Br.). The plants were grown in a greenhouse for two months and then exposed in a growth chamber to temperatures of: 0.2 (control), -2.7 and -4.1ºC. After the cold exposure, leaf tissue samples were sampled to measure the thickness of the cells in the adaxial and abaxial epidermis, bulliform cells, radiated parenchyma, and total leaf thickness. The results indicated a positive relation between freezing-temperature and a decreased thickness of the analyzed tissues. In crux, thickness of lower leaf epidermis under freezing temperatures was distinctly different among the investigated forage species, and was taken as a criterion to identify cold tolerance in these species.

Keywords: Epidermis thickness; Leaf tissues; Cold damage; Correlation; Forage grasses

Introduction

One of the pillars of the Brazilian economy is the production of beef cattle, being the second most-produced commodity in the country in the years 2020 and 2021 (CNA 2021). This is a reflection of the fertile large area and favorable climate for the production of forage used for pasture. A good performance of the pasture is due to genetic quality of the forage (Van Soest 1994) and soil characteristics, water availability, luminosity, slope of the environment and temperature (Primavesi *et al.* 2001; Santos *et al.* 2003).

Temperature determines the different plant species distribution around the Earth (Alcântara *et al.* 1993). Therefore, climate and environment adapted plants can increase forage productivity (Roitsch *et al.* 2022). Species that are not adapted to freezing conditions do not have acclimation strategies, which makes them very vulnerable. On the other hand, species native to temperate zone regions have adaptation and physiological mechanisms that permit them to survive cold.

The survival of plants at low temperatures depends mainly on maintaining the integrity and fluidity of cell membranes (Levitt 1980; Steponkus 1984). With freezing, water moves from the interior of the cell to the intercellular spaces, causing cell dehydration (Steponkus 1984; Thomashow 1999). Low temperatures also form reactive oxygen species (ROS) inside cells, which causes instability and ruptures in cell membranes (Augustyniak *et al.* 2020). Thus, physiological and anatomical changes in the plant body allow it to survive in environments with temperatures close to freezing water.

Low temperatures accumulate ROS in the cells of the roots of *Cucumis sativus*, preventing the absorption and transport of water and causing visible drought stress in the leaves of the species (Lee *et al.* 2004). At low temperatures, there is also a decrease in stomatal conductance in the leaves of *Zea mays*, which decreases water transport inside the plant body (Melkonian *et al.* 2004). Thus, the main symptoms we see in plants that are not resistant to cold is the dehydration of leaf cells, caused by the cell membrane destruction and leakage of the internal content or by the reduction of the water supply from the roots to the leaves.

Plants originating from environments with low temperatures have distinct survival mechanisms for this condition. These plants express many genes that change the physiology and biomechanics of cells, including changes in the structure and composition of cell membranes and

To cite this paper: Filho JM, IBL Gonçalves, CMG Oliveira, RA Gonçalves, FBT Hernandez[1], PH Caramori, AR Martins (2023). Comparative analysis of leaf anatomy of forage grasses submitted to freezing temperature stress. *Intl J Agric Biol* 30:107–112

accumulation of macromolecules that prevent ice formation (Thomashow 1999; Miura and Furumoto 2013; Rihan *et al.* 2017). These plants may exhibit anatomical and physiological differences to increase survival rates. Species found in cold environments have smaller diameter conducting vessels and greater parenchymal tissue (Dolezal *et al.* 2019). Leaves of *Solanum* sp. resistant to freezing had a thicker leaf containing smaller cells and a greater amount of leaf parenchyma (Dickison 2000). This production of parenchymal tissue is related to the tendency to store macromolecules with the potential to prevent or mitigate cellular damage caused by freezing.

Cold tolerance is directly linked to the accumulation of sugars and starch in the leaves, stating that the greater the amount of these molecules, the greater the tolerance to freezing (Sasaki *et al.* 1996). A higher concentration of non-structural carbohydrates in the leaves of *Arabdopsis thaliana* increased the survival rate of the species in environments with temperatures below 0ºC, showing a lower rate of cell lysis and less loss of osmotic rate in leaf cells (Uemura and Steponkus 2003). Plants with less available water had more soluble sugars, show increased low-temperature tolerance (Xu *et al.* 2020). So the accumulation of macromolecules inside the leaf cells seems to increase the cold tolerance, preserving the leaf cells and preventing the loss of photosynthetic capacity.

Evaluating foliar structures submitted to low temperatures can contribute to species selection processes and enabling better tolerance to extreme thermal environments. Histology makes it possible to visualize the damage caused by cold in the morphological and anatomical structures in plants. This knowledge is fundamental to determine which structures were affected and the cell death mechanisms in order to find out improvement strategies aiming to increase cold tolerance in tolerant species. Hence, this study was aimed to analyze the anatomical changes in leaf cells on forages that may occur when submitted to freezing temperatures in a controlled environment.

Materials and Methods

Experimental design

A completely randomized design with five repetitions was used in this study. The treatments were formed by the forage species: *Sorghum bicolor* (L.) Moench, *Brachiaria brizantha* cv. Marandu, *Pennisetum glaucum* (L.) R. Br. All the species were sown in pots of 1 dm^3 volume with a two-part soil substrate; one-part cattle shed tanned manure and 1 kg m^{-3} of NPK 4-30-10.

The plants were grown in a greenhouse for a period of two months. After that, they were transferred to a plant growing chamber with controlled temperature and light conditions, where they were cooled progressively until they were subjected to the temperatures of 0.2 (control), -2.7 and -4.1ºC, kept for one hour at each temperature and then returned gradually to ambient temperature. This one-hour period is a simulation of frost events, which are frequent in the study region.

Plant material and anatomical analysis

After treatments were applied, five leaves of each plant were sampled with approximately 10 mm. The plant material was fixed in a solution of formaldehyde, acetic acid and alcohol 50% (Johansen 1940). The leaves were dehydrated in ethyl alcohol grades, included in hydroxyl-ethyl methacrylate (Leica Historesin) and the blocks obtained were sectioned with 8 to 10 µm thick using a rotary microtome. The material was stained with 0.05% toluidine blue O (TBO) solution prepared in phosphate buffer and citric acid, pH 4.5 (Sakai 1973) and the slides were mounted in synthetic resin "Entellan®".

Ten measurements were made of each leaf structure to find out if there was any influence of temperature on leaf structures. The biometrics was performed for the thickness of the adaxial and abaxial epidermis, radiated parenchyma, bulliform cells and the leaf total thickness using the software Image J (Schneider *et al.* 2012).

Data analysis

The obtained data were evaluated using R software version 4.1.3 (R Core Team 2022) with R Studio interface. For non-parametric data, we used Kruskal-Wallis test and Wilcox test for peer-to-peer comparison. For parametric data with normal distribution, we analyzed ANOVA and peer-to-peer comparison using Tukey test. Pearson's correlation was also drawn to evaluate the anatomic leaves modifications in response to treatments. All the differences were considered significant at $p < 0.05$.

Results

Data revealed that the thickness of the analyzed tissues decreased significantly at the lower temperatures, and sometimes tissues were damaged by intense stress (Table 1; Fig. 1). The data did not present normal distribution, being analyzed with Kruskal Wallis, where it was evidenced that the epidermis (adaxial and abaxial) differed between treatments ($P < 0.01$), as well as the thickness of the leaves ($P < 0.01$). The size of bulliform cells also differed significantly between treatments ($P < 0.01$) as well as the radial mesophyll of forage species ($P < 0.01$). Wilcox test verified the significant differences among treatments for each studied anatomical variable (Table 1).

Pearson's correlation verified that the negative temperatures of the treatments positively influenced the anatomical variables. Therefore, the more negative the temperature, smaller were the leaf cells (Table 2). In *P. glaucum*, *S. bicolor*, and *B. brizantha*, a decrease in the total leaf thickness was noticed. However, in certain treatments

Table 1: Average thickness of the adaxial and abaxial epidermis, bulliform cells, radiated parenchyma, and leaf total thickness of all species subjected to different freezing temperatures for 1 h

Temperature (°C)	Adaxial Epidermis (µm)	Abaxial Epidermis (µm)	Bulliform cells (µm)	Radiated parenchyma (µm)	Total thickness (µm)
0.2	11.46 ± 3.79a	11.04 ± 3.67a	35.05 ± 18.5a	28.35 ± 30.16a	111.65 ± 35.47a
-2.7	11.43 ± 3.68ab	11.04 ± 3.73ab	30.48 ± 17.73b	18.15 ± 9.33b	103.53 ± 33.59b
-4.1	11.61 ± 3.57ac	11.34 ± 3.53bc	31.76 ± 17.37c	24.14 ± 22.68a	107.84 ± 35.31c

The values correspond to average ± standard deviation
Averages followed by the same letter in the column do not differ statistically from each other ($p<0.05$)

Table 2: Correlation between anatomical variables and treatments with different temperatures. The numbers correspond to the Pearson's correlation r value. *** = $p<0.01$

	Adaxial Epidermis	Abaxial Epidermis	Bulliform Cells	Radiated Parenchyma	Total Thickness
Temperature (°C)	0.21***	0.13***	0.28***	0.21***	0.30***

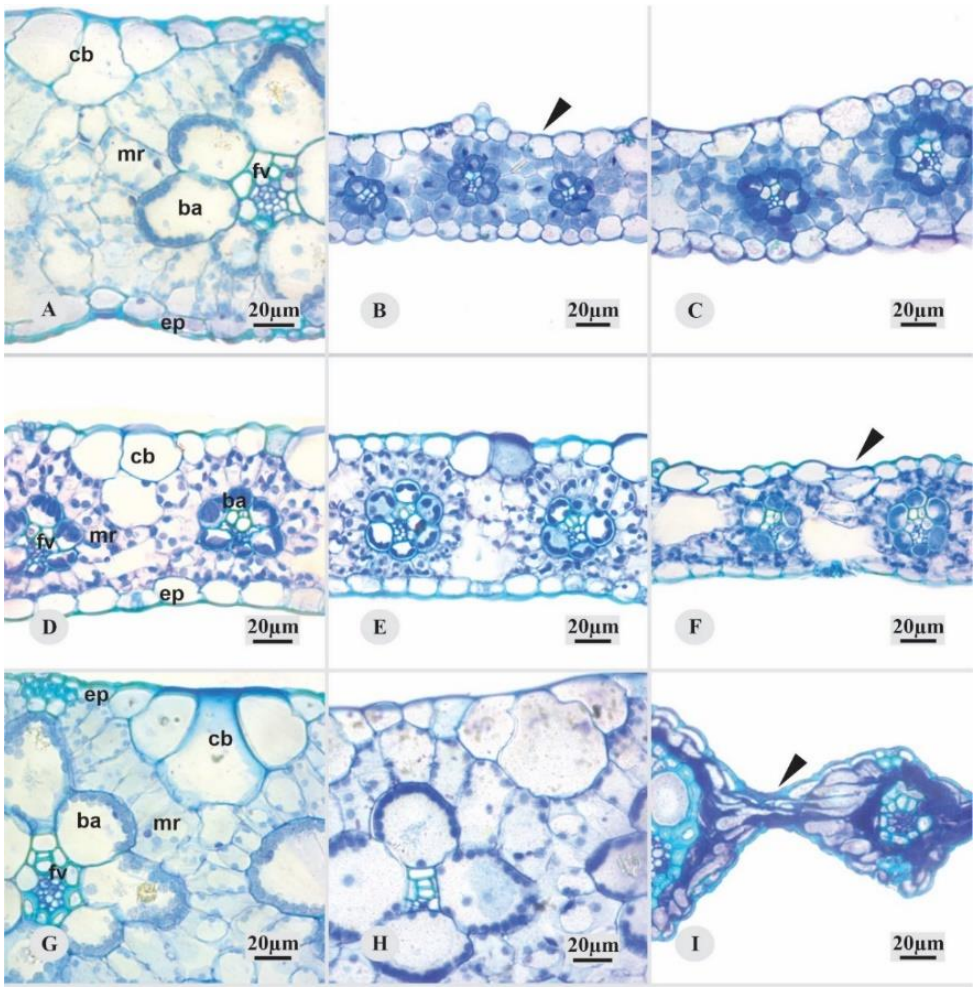

Fig. 1: Cross sections of *P. glaucum* leaves (A–C), *S. bicolor* leaves (D–F), *B. brizantha* leaves (G–I) subjected to certain treatments of freezing temperature in a plant growth chamber with controlled temperature and light conditions: A, D, G = 0.2°C; B, E, H = -2.7°C; C, F, I = -4.1°C. ep = epidermis; ba = bundle sheath; cb = bulliform cell; fv = vascular bundle; mr = radiated mesophyll; arrow head = evident damages

and species, there was a significant increase in thickness of some structures. In *P. glaucum* subjected to the treatment of -4.1°C there was an increase in the adaxial and abaxial epidermis, also in the radiated parenchyma (Table 3; Fig. 1A–C).

For *S. bicolor*, at control (0.2°C) temperature there was a decrease in bulliform cells and radiated parenchyma in relation to treatment -2.7°C (Table 4; Fig. 1D–F). In treatment -2.7°C it was possible to observe that the tissue degradation was caused due to freezing temperature (Fig. 1E). In *B. brizantha* leaf there was a decrease in all the structures analyzed in treatment -2.7°C, but they increased

Table 3: Average thickness of adaxial and abaxial epidermis, bulliform cells, radiated parenchyma and leaf total thickness of, *P. glaucum* subjected to 0.2; -2.7 and -4.1°C for 1 h

Temperature (°C)	Adaxial Epidermis (µm)	Abaxial Epidermis (µm)	Bulliform cells (µm)	Radiated parenchyma (µm)	Total thickness (µm)
0.2	12.52 ±2.44a	10.27± 2.19a	46.64± 12.12a	22.72±7.94a	142.82± 24.32a
-2.7	11.71±2.42a	11.96 ± 2.47b	21.73 ± 5.03b	19.55±4.28a	78.87±10.10b
-4.1	15.71 ±2.82b	15.87 ± 2.89c	26.95± 4.74c	27.54 ±4.74b	100.50± 12.09c

Table 4: Average thickness of adaxial and abaxial epidermis, bulliform cells, radiated parenchyma and leaf total thickness of *S. bicolor* subjected to 0.2; -2.7 and -4.1°C for 1 h

Temperature (°C)	Adaxial Epidermis (µm)	Abaxial Epidermis (µm)	Bulliform cells (µm)	Radiated parenchyma (µm)	Total thickness (µm)
0.2	13.82±2.11a	14.45± 2.13a	27.57±4.05a	21.38±3.79a	93.47±7.11a
-2.7	10.35 ±3.17b	12.29±2.65 b	20.67±10.83b	15.93±7.91b	79.75± 17.03b
-4.1	10.67 ±2.69b	12.54±2.63 b	25.94±4.72ab	19.58± 3.80ab	84.54±17.43b

The values correspond to average ± standard deviation
Averages followed by the same letter in the column do not differ statistically from each other ($p<0.05$)

Table 5: Average thickness of adaxial and abaxial epidermis, bulliform cells, radiated parenchyma and leaf total thickness of *B. brizantha* subjected to 0.2; -2.7 and -4.1ºC for 1 h

Temperature (°C)	Adaxial Epidermis (µm)	Abaxial Epidermis (µm)	Bulliform cells (µm)	Radiated parenchyma (µm)	Total thickness (µm)
0.2	12.59± 3.03a	11.28 ±2.33a	53.67± 11.67a	25.74± 7.99a	147.05±25.09a
-2.7	10.84± 3.52b	10.50±3.68ac	38.24 ±29.89ac	17.74± 13.91a	123.23 ±45.33a
-4.1	13.67 ± 2.96a	12.43 ±2.49ad	50.28 ±7.38ac	23.93± 7.38a	144.22 ±14.75a

The values correspond to average ± standard deviation
Averages followed by the same letter in the column do not differ statistically from each other ($p<0.05$)

again at the temperature -4.1°C (Table 5; Fig. 1G–I). In some samples at -2.7 and -4.6°C it was not possible to assess the thickness of structures, because of the severe damage caused to the leaves by the treatments (Fig. 1i).

It is possible that differential variation (decrease/increase) in thickness of the analyzed structures in the species *P. glaucum*, *S. bicolor* and *B. brizantha* were due to stress caused by critical temperatures. In addition, the deteriorated structure at -2.7°C and -4.6°C in *B. brizantha* presented a susceptibility to the intense cold.

Discussion

The analysis demonstrated that the critical temperatures caused damages to foliar anatomical structures, with differentiated responses according to the tolerance of the assessed species. Under temperate conditions, to remain alive during the winter season, the plants must prevent their tissues from freezing, specially the vascular system (Cavender-Bares 2005). In plant tissues, freezing may cause the formation of intracellular ice, that might result in cells death, or the formation of extracellular ice might protect the cells against damages. Within the vessel elements, freezing temperatures could cause cavitation resulting in loss of xylem function, depending on the size of vessel elements as well as the freezing temperature. Phloem transport is also limited by low temperatures, as the phloem sap becomes more viscous (Cavender-Bares 2005). *Arabidopsis* has been used as a model plant to investigate the tolerance to controlled freezing (Livingston III *et al.* 2007). Such species presented analogous damage to the root vascular tissue and foliar axis of winter cereals, but no damage to meristematic regions after being cultivated under freezing conditions for seven days. Pearce and Mcdonald (1977) evaluated damages to the ultrastructure of mesophyll tissue in *Festuca arundinacea* Scherb, subjected to freezing, followed by defrost. They concluded that the cytoplasm was contracted, the organelles were swollen or partially destroyed, tonoplast and nuclear membranes broken or missing and the vacuoles sometimes destroyed.

Some of the responses to cold stress were also found in plants under water stress, in which water loss from cells occurred. The concentrations of abscisic acid (ABA), sugars and compatible solute levels could be increased by low temperature stress, correlating the ABA to freezing tolerance in several species (Maldonado *et al.* 1997). According to Sparks *et al.* (2000) the ABA had a direct role in the cell response to dehydration during freezing. More likely, it was involved in the control of gene expression during the cold acclimatization. The unfrozen water was found especially in the apoplast.

According to the obtained data in the present study, it could be observed that the thickness of the analyzed tissues tended to decrease due to the damage caused by freezing temperature intense stress. It could be due to the dehydration caused by the freezing of intercellular spaces followed by dehydration and death of tissues. Åström *et al.* (2015) observed that the winter leaves of *Fragaria vesca* were smaller and had abundant hairs, their stomatal density was higher and their mesophyll was denser with more palisade parenchyma cells. Many of these characteristics could be considered by the authors as adaptations for light intensity, freezing temperatures and frost desiccation stress in winter, in which photosynthetic capacity does not change depending on the season of the year. Rhizomatous grasses such as *Arundo donax* L. (type C3 metabolism) and

Panicum virgatum L. (type C4 metabolism) presented several changes in photosynthesis parameters when subjected to a combination of low temperature and darkness (Sánchez *et al.* 2016). According to Sánchez *et al.* (2016), *P. virgatum* seemed to be more tolerant to cold, water stress, salinity and continuous darkness.

Conclusion

The leaves of grass species responded to freezing temperatures differently; *B. brizantha* showing intense sensitivity. The lower epidermis under freezing temperatures was significantly different among the investigated forage species, which was taken as a criterion of freezing temperature tolerance. Cold stress damaged cells and disrupted membranes due to ice forming inside the leaves, decreasing the thickness of leaves.

Acknowledgements

Instituto de Desenvolvimento Rural do Paraná – IAPAR-EMATER" contributed to the publication of this article.

Author Contributions

JMF, FBTH, PHC and ARM designed the experiments; JMF, IBLG and CMGO collected and analyzed the data; JMF, IBLG, CMGO and RAG interpreted the results and revised the manuscript. All authors did the final revision of the manuscript.

Conflicts of Interest

All authors declare no conflict of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author

Ethics Approval

Not applicable to this paper

References

Alcântara PB, MJ Pedro Junior, PL Donzelli (1993). *Zoneamento Edafoclimático de Plantas Forrageiras*. Simpósio Sobre Ecossistemas de Pastagens, 16. Jaboticabal, Brazil

Åström H, E Metsovuori, T Saarinen, R Lundell, H Hänninen (2015). Morphological characteristics and photosynthetic capacity of *Fragaria vesca* L. winter and summer leaves. *Flora* 215:33–39

Augustyniak A, I Pawłowicz, K Lechowicz, K Izbiańska-Jankowska, M Arasimowicz-Jelonek, M Rapacz, D Perlikowski, A Kosmala (2020). Freezing tolerance of *Lolium multiflorum*/*Festuca arundinacea* introgression forms is associated with the high activity of antioxidant system and adjustment of photosynthetic activity under cold acclimation. *Intl J Mol Sci* 21:5899

Cavender-Bares J (2005). Impacts of freezing on long distance transport in woody plants. In: *Vascular Transport in Plants*, pp:401–424. Holbrook NM, MA Zwieniecki (Eds.). Elsevier BV, Amsterdam, The Netherlands

CNA (2021). *Panorama do Agro*. Available at: https://cnabrasil.org.br/cna/panorama-do-agro#:~:text=O%20segundo%20lugar%20no%20ranking,%24%2067%2C2%20bilh%C3%B5es (Accessed: 29 September 2022)

Dickison WC (2000). *Integrative Plant Anatomy*, 1st edn. Academic Press, New York, USA

Dolezal J, A Klimes, M Dvorsky, P Riha, J Klimesova, F Schweingruber (2019). Disentangling evolutionary, environmental and morphological drivers of plant anatomical adaptations to drought and cold in Himalayan graminoids. *Oikos* 128:1576–1587

Johansen DA (1940). *Plant Microtechnique*. McGraw-Hill Book Company, New York, USA

Lee SH, AP Singh, GC Chung (2004). Rapid accumulation of hydrogen peroxide in cucumber roots due to exposure to low temperature appears to mediate decreases in water transport. *J Exp Bot* 55:1733–1741

Levitt J (1980). *Responses of Plant to Environmental Stresses. Chilling, Freezing and High Temperature Stresses*, 2nd edn. Academic Press, New York, USA

Livingston III DP, K Van, R Premakumar, SP Tallury, EM Herman (2007). Using *Arabidopsis thaliana* as a model to study subzero acclimation in small grains. *Cryobiology* 54:154–163

Maldonado CA,GE Zuñiga, LJ Corcuera, M Alberdi (1997). Effect of water stress on frost resistance of oat leaves. *Environ Exp Bot* 38:99–107

Melkonian J, L Yu, TL Setter (2004). Chilling responses of maize (*Zea mays* L.) seedlings: Root hydraulic conductance, abscisic acid, and stomatal conductance. *J Exp Bot* 55:1751–1760

Miura K, T Furumoto (2013). Cold signaling and cold response in plants. *Intl J Mol Sci* 14:5312–5337

Pearce RS, I McDonald (1977). Ultrastructural damage due to freezing followed by thawing in shoot meristem and leaf mesophyll cells of tall fescue (*Festuca arundinacea* Schreb.). *Planta* 134:159–168

Primavesi O, AR Freitas, ACPA Primavesi, AC Camargo, J Rocha Filho, LA Castro Jorge, MAV Ligo, SR Vieira (2001). *Qualidade Ambiental em Sistema Intensivo de Produção de Bovinos de Leite, na Microbacia do Ribeirão Canchom: Indicadores, Manejo e Problemas*. Embrapa Pecuária Sudeste. Boletim de Pesquisa nº 7, Brasil

R Core Team (2022). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org/

Rihan HZ, M Al-Issawi, MP Fuller (2017). Advances in physiological and molecular aspects of plant cold tolerance. *J Plant Interact* 12:143–157

Roitsch T, K Himanen, A Chawade, L Jaakola, A Nehe, E Alexandersson (2022). Functional phenomics for improved climate resilience in Nordic agriculture. *J Exp Bot* 73:5111–5127

Sakai WS (1973). Simple method for differential staining of paraffin embedded plant material using toluidine blue O. *Stain Technol* 48:247–249

Sánchez E, S Gil, J Azcón-Bieto, S Nogués (2016). The response of *Arundo donax* L. (C3) and *Panicum virgatum* (C4) to different stresses. *Biomass Bioener* 85:335–345

Santos HP, RS Fontaneli, GO Tomm, JE Denardin (2003). Atributos químicos e físicos de solo em sistemas de produção com pastagens anuais de inverno, sob plantio direto. In: *Embrapa Trigo*. Passo Fundo, Brazil

Sasaki H, K Ichimura, M Oda (1996). Changes in sugar content during cold acclimation and deacclimation of cabbage seedlings. *Ann Bot* 78:365–369

Schneider CA, WS Rasband, KW Eliceiri (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat Meth* 9:671–675

Sparks JP, GS Campbell, RA Black (2000). Liquid water content of wood tissue at temperatures below 0°C. *Can J For Res* 30:624–630

Steponkus PL (1984). Role of the plasma membrane in freezing injury and cold acclimation. *Annu Rev Plant Physiol* 35:543–584

Thomashow MF (1999). Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Physiol Plant Mol Biol* 50:571–599

Uemura M, PL Steponkus (2003). Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from *Arabidopsis thaliana* leaves. *Plant Cell Environ* 26:1083–1096

Van Soest PJ (1994). *Nutritional Ecology of the Ruminant*, 2nd edn. Cornell University Press, Ithaca, New York, USA

Xu H, Z Li, Z Tong, F He, X Li (2020). Metabolomic analyses reveal substances that contribute to the increased freezing tolerance of alfalfa (*Medicago sativa* L.) after continuous water deficit. *BMC Plant Biol* 20:15