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Biochemical responses to chilling injury in sweet potato after cold storage

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ABSTRACT. This study examined biochemical changes associated with chilling injury (CI) in sweet potato roots stored at low temperatures and reconditioned at ambient temperature. Sweet potato cultivars BRS Amélia and BRS Rubissol were stored at 6 or 13°C for 4 days at ambient temperature ("ambient reconditioning": 21 ± 2°C). CI on the outer surface of the roots occurred earlier in BRS Amélia than in BRS Rubissol. The CI index on the inner surface of the BRS Amélia was higher when it was stored at 6°C for 40 + 4 days. BRS Amélia showed higher proline content and electrolyte leakage when stored at 6°C. Ascorbate peroxidase was induced by storage at 6°C for 30 + 4 days in BRS Amélia and from 40 + 4 days in BRS Rubissol. The malondialdehyde and phenolic compounds of BRS Rubissol increased during storage at 6°C. CI in both cultivars was associated with increased peroxidase and polyphenol oxidase activities. Storage of sweet potato cultivars at 6°C for 50 + 4 days caused severe damage to the roots. Cultivars BRS Amélia and BRS Rubissol showed similar symptoms of CI and can be characterized as being sensitive to low temperatures.

Keywords: *Ipomoea batatas* L.; reconditioning; lipid peroxidation; oxidative enzymes.

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Introduction

Refrigeration is recognized as the simplest post-harvest technology to reduce the metabolism of vegetables, allowing a decrease in water loss, respiration, and development of post-harvest diseases (Menolli, Finger, Puiatti, Barbosa, & Barros, 2008; Vithu, Dash & Rayaguru, 2019). However, when exposed to low temperatures, some tropical and subtropical species are affected by a physiological disorder known as chilling injury (CI) (Patel, Tandel, Patel, & Patel, 2016).

The changes resulting from chilling injury are more noticeable on the external surface of the product (Zou, Zhang, Rao, Zhu, & Ye, 2014), which culminates in rejection on the part of consumers and losses to the vegetable-supply chain. Moreover, the damage may also be apparent only upon removing the vegetable from the refrigerated environment or only detected inside the product (Heyes, 2018).

In this respect, sweet potato (*Ipomoea potatoes* L.) roots are characterized as sensitive to storage at low temperatures (Xie et al., 2017), with chilling injuries appearing at temperatures below 13°C (Picha, 1987; Jackman, Yada, Marangoni, Parkin, & Stanley, 1988). The main morphological characteristics observed after storage at temperatures, around 4°C, are superficial lesions, browning of internal tissues, and susceptibility to pathogens such as soft rot and general softening (Xie et al., 2019).

Visible symptoms of chilling injury on tissues indicate intense and irreversible damage. In addition to morphological responses, physical changes take place in the lipid phase of cell membranes, and proteins undergo specific alterations. As a consequence, secondary responses occur, including increased ethylene production, respiration, and solute leakage, loss of cell compartmentalization, and changes in enzyme activity (Menolli et al., 2008).

It should also be emphasized that the ideal storage temperature varies across sweet potato cultivars, as well as their responses during chilling storage (Xie et al., 2019). This statement is corroborated by the results reported by Ji et al. (2017) and Li, Yang, and Lu (2018), who described the appearance of injury at 7 days of storage at 4°C in *Ipomoea batatas* L. 'Xinxiang', but morphological responses were observed in the *Ipomoea batatas* L. 'Yulmi' after 6 weeks of storage.

Although information is available on the behavior of sweet potato roots stored at ideal temperatures (13°C) and at low temperatures that induce chilling injury, little is known about the biochemical responses of cultivars widely grown in Brazil, where cultivars BRS Amélia and BRS Rubissol stand out. Since symptoms intensify when the product is reconditioned at ambient temperature (Luengwilai & Beckles, 2010), the present study was conducted to investigate the biochemical responses of roots of sweet potato cultivars BRS Amélia and BRS Rubissol to low-temperature storage.

Material and methods

Plant material and treatments

Two sweet potato cultivars, BRS Amélia and BRS Rubissol, were evaluated from the *Embrapa Clima Temperado* Agricultural Research Corporation. The genotypes were grown from January to June 2018 in an experimental area at the Federal University of Viçosa, Viçosa, Minas Gerais State, Brazil (20°45′49″ S and 42°49′28″ W, 650 m above sea level), following the recommended crop management practices (Montes, 2013). The roots were harvested and manually selected 160 days after transplanting. These were cured at 30°C and 90% relative humidity (RH) for 7 days to heal the peridermis and then stored at 6 or 13°C for 50 days at 90% RH. The roots were removed from storage every 10 days and kept for 4 days at ambient temperature (21 ± 2°C and 81% RH) for further evaluation.

Evaluation of chilling injury index

Sweet potato roots with dark spots and depressions on the skin surface were characterized as being affected by chilling injury on the outer surface. Physiological disorders on the inner surface were identified by browning of the vascular rings and irregularity of the flesh surface. For this evaluation, cross-sections were made in the respective roots of each cultivar at each predefined storage time. The injury index was based on a subjective scale where 1 = no damage, 2 = up to 25% of the surface damaged, 3 = 26–50% of the surface damaged, 4 = 51–75% of the surface damaged, and 5 = more than 75% of the surface damaged. The chilling injury index was calculated according to the formula proposed by Pesis, Marinansky, Zauberman, and Fuchs (1994):

$$\text{Chilling injury index} = \sum(\text{degree of injury}).\frac{\text{number of roots showing this degree}}{\text{total number of roots}}$$

Analysis of total soluble phenolic compounds

Total phenolic compounds were determined by the method described by Fu et al. (2010) with modifications, using 80% ethanol as the extractor. For quantification, a 0.2-mL aliquot of extract, 1.0 mL of the Folin-Ciocalteu reagent (1:10), and 0.8 mL of a 7.5 % sodium carbonate (Na_2CO_3) solution were added to the test tube. The tube was then left to sit at 25°C for 30 min. in a dark environment. After this process, the absorbance of the samples was measured in a spectrophotometer at 760 nm using gallic acid as the standard for building the curve. Results were expressed in milligrams of gallic acid equivalents (GAE) per 100 g of fresh weight.

Determination of proline content

Proline was determined according to Bates, Waldren, and Teare (1973), with modifications. Approximately 0.1 g of flesh was crushed in 2 mL of a 3% (w/v) sulfosalicylic acid solution and centrifuged at 2,000× *g* for 10 min. In a test tube, 1 mL of the supernatant (extract), 1 mL acid ninhydrin, and 1 mL glacial acetic acid were homogenized and incubated in boiling water (100°C) for 1h. Subsequently, the reaction was stopped on ice and absorbance was measured at 520 nm. Results are expressed in mmol of proline g^{-1} fresh weight.

Malondialdehyde (MDA) content

Lipid peroxidation was measured based on malondialdehyde (MDA) quantification, following the methodology proposed by Heath and Packer (1968). The MDA content was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}, with values expressed in nmol g^{-1} of fresh weight.

Measurement of electrolyte leakage

Electrolyte leakage was measured according to the method of Lima, Damatta, Pinheiro, Totola, and Loureiro (2002), with modifications. Disks (10 mm in diameter) of sweet potato flesh from each treatment were washed in deionized water to remove the ruptured cells during removal and then immersed in 20 mL of

deionized water. The initial electrical conductivity (L1) of the suspension liquid was measured using a conductivity meter (DM-31, Digimed) after incubation for 6 h at room temperature. The percentage of total conductivity (L2) was obtained after placing the flasks containing the disks in an oven at 90°C for 2h. Membrane permeability was calculated as EL% = (L1/L2) × 100.

Enzyme analysis

The samples for the analysis of peroxidase (POD) and ascorbate peroxidase (APX) consisted of 0.3 g). The material was macerated and homogenized in 2 mL of extraction buffer composed of 1% (m/v) polyvinylpyrrolidone (PVP), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 mM potassium phosphate (pH 7.0). The homogenate was centrifuged for 15 min. at 14,000 × *g* at 4°C, and the supernatant was used to determine the activity of the respective enzymes.

Peroxidase (POD)

POD activity was quantified as described by Kar and Mishra (1976), where the reaction medium consisted of 20 mM guaiacol, 25 mM potassium phosphate buffer (pH 6.5), and 20 mM H_2O_2. The activity was determined based on the rate of tetraguaiacol production at 470 nm and expressed in ηmol $min.^{-1}$ mg^{-1} of protein, using a molar extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Ascorbate peroxidase (APX)

APX activity was based on the method described by Nakano and Asada (1981), with modifications. The extract was composed of 0.3 mM of H_2O_2, 50 mM potassium phosphate buffer (pH 7.8), and 0.25 mM ascorbic acid. Enzyme activity was measured by reducing the absorbance for 1 min. at 290 nm and expressed in ηmol $min.^{-1}$ mg^{-1} protein, using a molar extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Polyphenol oxidase (PPO)

PPO activity was measured using the method of Benjamin and Montgomery (1973), with modifications. Approximately 0.3 g of flesh was macerated and homogenized in an extraction solution containing 1 mM PMSF and 0.1 M potassium phosphate buffer (pH 6.5). Subsequently, the material was centrifuged for 15 min. at 14,000 × *g* at 4°C. The reaction medium consisted of 120 mM pyrocatechol, enzymatic extract, and 0.1 M potassium phosphate buffer (pH 5.0). Readings were taken using a spectrophotometer at a wavelength of 420 nm for 3 min. The activity was expressed in ηmol min^{-1} mg^{-1} protein, using a molar extinction coefficient of 3450 M^{-1} cm^{-1}.

Statistical analysis

The experiment was laid out in a completely randomized split-plot design with four replicates in a 2 × 2 factorial arrangement consisting of 2 cultivars (BRS Amélia and BRS Rubissol) and 2 temperatures (6 and 13°C) in the plot, as well as 6 storage times (0, 10 + 4; 20 + 4; 30 + 4; 40 + 4; and 50 + 4 days) in the subplot. The data were subjected to analysis of variance (ANOVA), and the means of the temperature factor were compared using the F test ($p \leqslant 0.05$) and Sisvar 5.6 statistical software (Ferreira, 2014).

Results and discussion

Chilling injury index

Cold storage at 6°C for 4 days at ambient temperature induced morphological changes on the outer and inner surfaces of the roots of cultivars BRS Amélia and BRS Rubissol (Figure 1A and D). After 50 days of storage and 4 days at ambient temperature (50 + 4 days), the roots showed slight discoloration, brown lesions, and depressions on the outside, as well as browning on the inside with a uniform pattern in BRS Amélia and an uneven pattern in BRS Rubissol. This browning is associated with chilling injury, which has already been reported in arracacha (*Arracacia xanthorrhiza*) and arrowleaf elephant ear (*Xanthosoma sagittifolium*) (Menolli et al., 2008; Souza & Finger, 2014). Storage at 13°C did not interfere with morphological quality, with no significant changes observed between days 0 and 50 + 4.

The CI was higher when the vegetables were stored at 6°C than at 13°C, regardless of the cultivar (Figure 1B, C, E, and F). For BRS Amélia, the injury index on the outer surface of the roots increased from 2.25 at 30 + 4 days at 6°C to 4.0, at 50 + 4 days (Figure 1B). In BRS Rubissol, injury at 6°C started to appear later, at 40 + 4 days, with an index of 1.75, which reached 3.0% at 50 + 4 days (Figure 1C). The earliness and greater extent

of damage caused by chilling injuries in BRS Amélia, in relation to BRS Rubissol.is shown in Figure 1A. On the inner surface, however, storage at 6°C induced greater chilling injury from 40 + 4 days in both cultivars (Figure 1E and F). At 50 + 4 days, storage at 6°C induced a higher percentage of injury than storage at 13°C, in both cultivars: 266.7 and 300% on the outer surface and 300 and 333.3% on the inner surface, respectively.

Chilling injury develops due to the sensitivity of some tropical and subtropical species, such as sweet potatoes, to temperatures below 10°C (Ji et al., 2017). Li et al. (2018) observed chilling injury at 7 days of storage at 4°C in sweet potato Xinxiang, but no injury occurred at 13°C. Storage at 4°C promoted internal browning of the tissue and dark and deep injuries on the outer surface of the Yulmi, whereas the roots stored at 13°C did not show this decline in morphological quality (Ji et al., 2017). In sweet potato roots, the stress caused by cold generates an imbalance in cellular homeostasis that leads to membrane disorganization, which is driven by oxidative processes that, in turn, increase the levels of reactive oxygen species (Sevillano, Sanchez-Ballesta, Romojaro, & Flores, 2009).

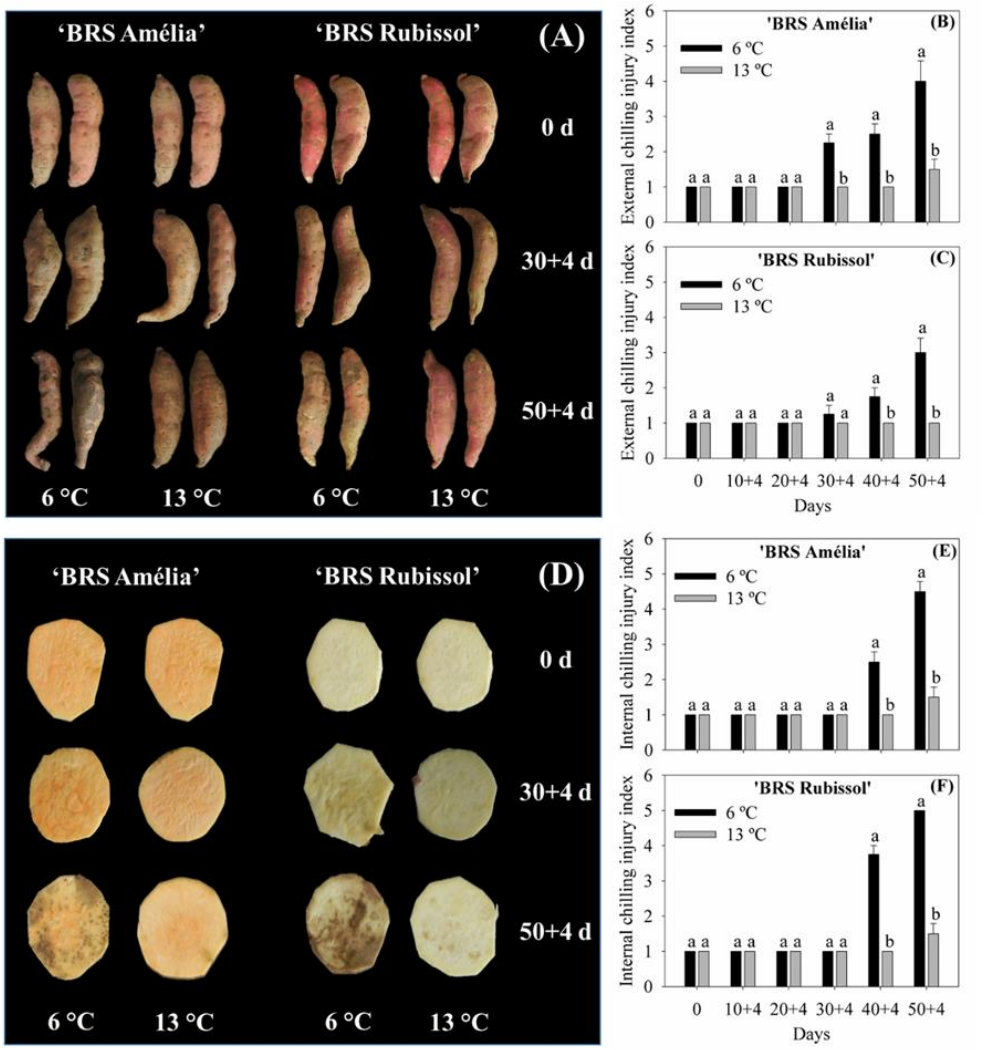

Figure 1. Chilling injury on the outer (A) and inner surfaces (B) of sweet potato roots stored at 6 and 13°C for 50 days, plus 4 days of ambient reconditioning. Chilling injury index of the outer and inner surfaces of cultivars BRS Amélia (B and E) and BRS Rubissol (C and F). Data represent the mean ± standard error (n = 4). Common and uncommon letters at the same storage time indicate non-significant ($p > 0.05$) and significant ($p \leq 0.05$) differences by the F test.

Total phenolic compound content

The phenolic compound content increased with storage time in the studied cultivars and at different temperatures (Figure 2A and B). Cultivar BRS Amélia stored at 6°C showed a significant increase in phenolic content, which reached 51.68 mg GAE 100 g^{-1} at the last evaluation time, a 1.57 times higher content than that measured in the roots stored at 13°C (Figure 2A). In contrast, the phenolic content of BRS Rubissol increased gradually, with values 2.35 times higher than those obtained in storage for 50 + 4 days at 13°C (Figure 2B).

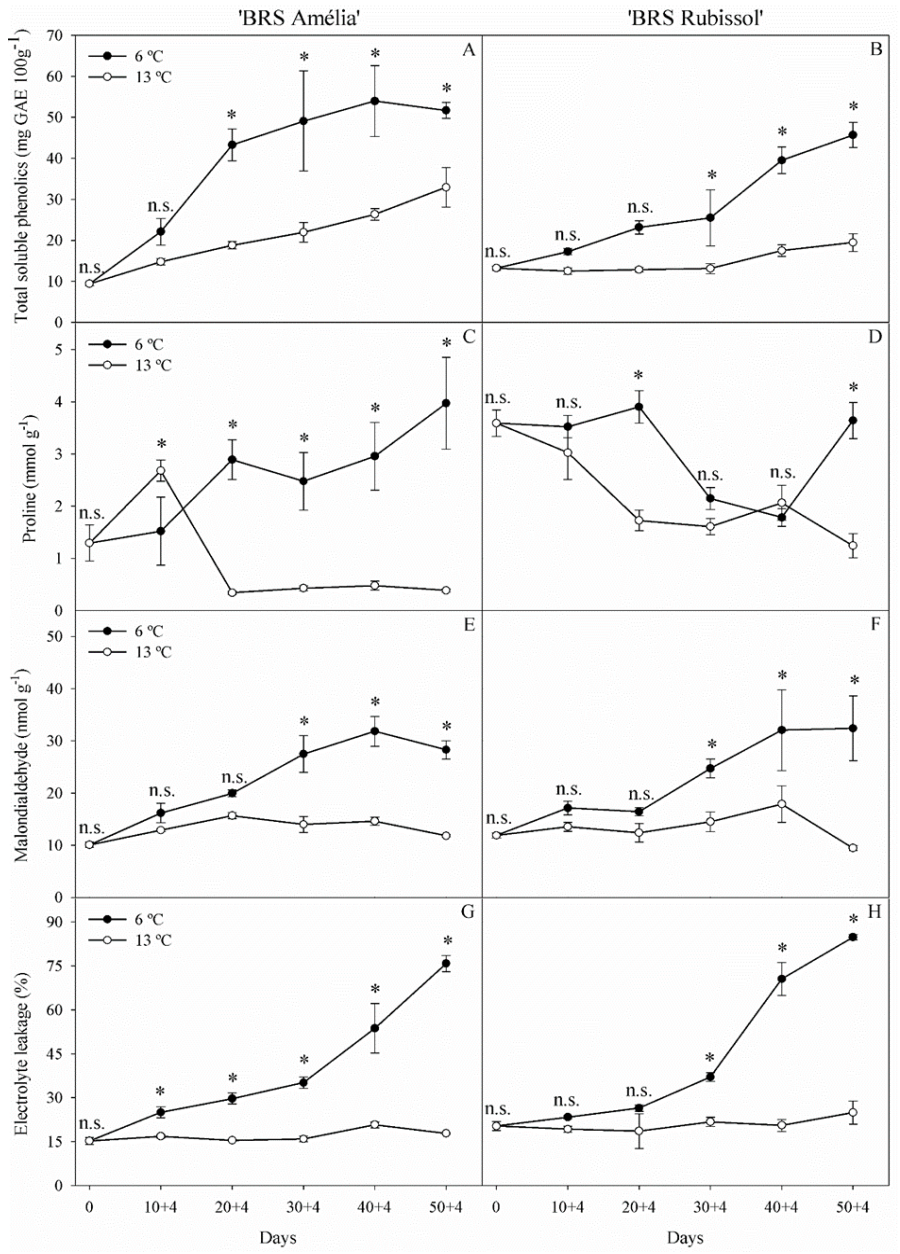

Figure 2. Phenolic compounds content (A and B), proline content (C and D), MDA content (E and F), and electrolyte leakage (G and H) in roots of sweet potato cultivars BRS Amélia and BRS Rubissol during storage at 6 and 13°C plus four days of ambient reconditioning. Data represent the mean ± standard error (n = 4). $^{n.s.}$ and * indicate non-significant $p > 0.05$) and significant ($p \leqslant 0.05$) differences, respectively, by the F test.

The accumulation of phenolic compounds in sweet potato roots seems to be related to a defense mechanism against pests and diseases (Harrison, Peterson, Snook, Bohac, & Jackson, 2003). In addition, the synthesis of phenolic compounds intensifies in response to abiotic stresses, such as cold stress (Padda & Picha, 2008). Wang et al. (2019) observed a higher phenolic content in sweet potato cultivars stored at 4°C than at 16°C. A similar result was also found in sweet potato roots by Ji et al. (2017), who reported an increase in phenolic synthesis under cold stress, with an increase in the activity of phenylalanine ammonia lyase.

Proline content

After 20 + 4 days of storage, the proline content, of BRS Amélia increased sharply at 6°C but remained constant at 13°C (Figure 2C). Although the roots stored at 13°C were influenced by the low temperature, the proline content initially remained constant after 20 + 4 days, with values 10.34 times lower at the last storage time than those achieved at 6°C (Figure 2C). The proline content of BRS Rubissol was reduced to 40 + 4 days. However, in the last evaluation period (50 + 4 days), the proline content was 2.93 times higher (1.24 mmol g^{-1}) than that seen in the roots stored at 13°C (Figure 2D).

These results are consistent with those described by Wang et al. (2019), who observed an increase in proline content in sweet potato roots under cold stress at 4°C. Proline functions as a regulator of cellular osmosis, maintains turgor, and protects protein integrity (Li, Zheng, Liu, & Zhu, 2014). In fruits and vegetables stored at low temperatures, proline accumulates as a response stimulus for defense against cold (Gao et al., 2016; Ge et al., 2019). We can thus infer that BRS Amélia has a more immediate adaptation mechanism than BRS Rubissol when subjected to temperatures below the critical temperature. The presented results are overwhelming, indicating that proline accumulation in BRS Amélia was higher than that observed in BRS Rubissol, with a total increase of 2.68 mmol g^{-1}.

MDA content and electrolyte leakage

The malondialdehyde (MDA) content was similar in the two studied cultivars over the storage period (Figure 2E and F). In both cultivars stored at 6°C, the MDA content did not show significant differences ($p > 0.05$) until 20 + 4 days when compared with the levels observed after storage at 13°C. After this period, storage at 6°C induced greater MDA accumulation until 50 + 4 days, when the contents were 2.39 and 3.42 times higher than those obtained during storage at 13°C in cultivars BRS Amélia (Figure 2E) and BRS Rubissol (Figure 2F), respectively.

The MDA content is considered an indicator of membrane damage during stress, as it measures the degree of lipid peroxidation (Ge et al., 2019). Therefore, it is understood that the temperature of 6°C influenced the oxidative degradation of the lipids considerably and in a very similar fashion between the cultivars. Wang et al. (2019) and Ji et al. (2017) also observed a significant accumulation of MDA in sweet potatoes stored at low temperatures.

Regardless of temperature, electrolyte leakage responded similarly during storage between the two cultivars (Figure 2G and H). The roots of cultivars BRS Amélia and BRS Rubissol stored at 6°C exhibited a gradual increase in leakage up to 30 + 4 days, followed by a marked increase from that storage time onwards. Electrolyte leakage at this temperature was 4.97 and 4.17 times higher than the initial value measured in cultivars BRS Amélia (Figure 2G) and BRS Rubissol (Figure 2H), respectively. Storage at 13°C did not influence electrolyte leakage in the studied cultivars, which remained constant since the start of storage.

Like MDA, electrolyte leakage can also be used to measure the extent of damage caused by tissue leachate to membranes (Côté, Thompson, & Willemot, 1993). Increased leakage induced by chilling injury in post-harvest products is well established in the literature. In sweet potato, Li et al. (2018) observed that storage at 4°C for 28 days considerably increased leakage, when compared with storage at 13°C. These results corroborate those described here regarding the role of lipid peroxidation as a primary event in the manifestation of chilling injury symptoms.

Peroxidase, polyphenol oxidase and ascorbate peroxidase

From 20 + 4 days, the roots of the sweet potato BRS Amélia stored at 6°C showed higher peroxidase (POD) activity than those stored at 13°C (Figure 3A). For BRS Rubissol roots stored at 6°C showed higher POD activity between 20 and 40 + 4 days than those stored at 13°C (Figure 3B). In BRS Amélia stored at 6°C, POD activity peaked at 30 + 4 days, exceeding the activity observed at 13°C by 5.8 times (Figure 4A). Conversely, the peak of POD activity in BRS Rubissol stored at 6°C occurred earlier (20 + 4 days), exceeding the activity seen at 13°C by 2.8 times (Figure 3B).

Peroxidase is an oxidoreductase that is normally induced by biotic and abiotic stress conditions (Singh et al., 2016). Its ability to catalyze the oxidation of phenolic compounds in the presence of hydrogen peroxide makes it an important detoxifying enzyme (Menolli et al., 2008; Raimbault et al., 2011; Menolli, Finger, Barbosa, Correia, & Vieira, 2011). However, o-quinones, the product of phenol oxidation, are highly reactive molecules that polymerize into brown pigments (Trejo-Márquez, Ramírez-Villatoro, & Camacho de la Rosa, 2010). Therefore, the induction of POD activity at 6°C may be involved in the browning of the periderm of both sweet potato cultivars.

In addition to POD, the reaction catalyzed by polyphenol oxidase (PPO) is also generally associated with the enzymatic browning characteristic of chilling injury in fruits and vegetables (Menolli et al., 2008; Trejo-Márquez et al., 2010). In BRS Amélia, the studied temperatures practically did not affect PPO activity (Figure 3C). In contrast, the PPO activity in BRS Rubissol was induced at a temperature of 6°C, exceeding by 2.4 times the activity obtained at 13°C at 50 + 4 days, when it was at its peak (Figure 3D). These results suggest

that the enzymatic mechanism involved in the browning of the periderm under cold stress differed between the studied genotypes. In BRS Amélia, the progress of external browning under ambient conditions after exposure to 6°C seems to be more related to the increase in POD than PPO. In contrast, the simultaneous increase in PPO activity and browning in the BRS Rubissol suggests that the accumulation of browned compounds results mainly from the oxidation of phenolic compounds by PPO, whereas the reaction catalyzed by POD acted more as an initial stress response (Menolli et al., 2011).

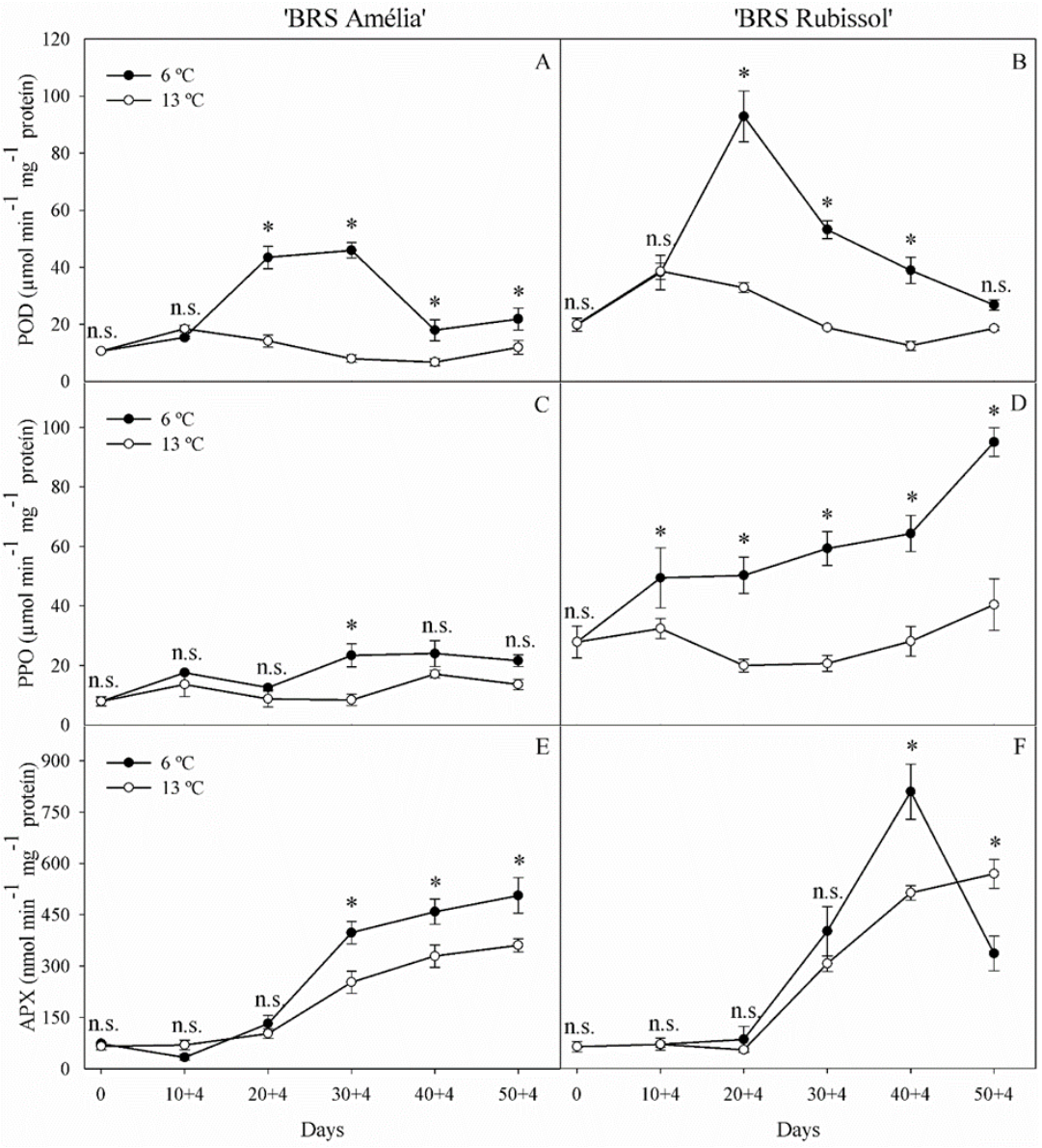

Figure 3. Peroxidase (A and B), polyphenol oxidase (C and D), and ascorbate peroxidase (E and F) in roots of sweet potato cultivars BRS Amélia and BRS Rubissol during storage at 6 and 13 °C and 4 days of ambient reconditioning. Data represent the mean ± standard error (n = 4). The symbols $^{n.s.}$ and * indicate non-significant ($p > 0.05$) and significant ($p \leq 0.05$) differences, respectively, by the F test.

Ascorbate peroxidase (APX) activity did not change until 20 + 4 days, regardless of the genotypes and temperatures tested. After 30 + 4 days, APX activity in the BRS Amélia was induced at a temperature of 6°C, reaching a value of up to 1.6 times higher than that observed at 13°C (Figure 3E). In BRS Rubissol, APX activity at 6°C was higher than that at 13°C only at 40 + 4 days (Figure 3F). Although APX is known to be an important component of the protection system against oxidative stress, in the present study, the increased APX activity in BRS Amélia stored at 6°C was not sufficient to mitigate damage caused by cold. A similar result was observed in a cold-sensitive Chinese sweet potato cultivar (Wang et al., 2019). Despite the increase in APX activity, storage at 4°C was not able to mitigate the effects of chilling injury in Yanshu 25 compared with the temperature of 13°C for 16 days of storage.

Conclusion

The sweet potato cultivars BRS Amélia and BRS Rubissol can be stored to 20 days at 6°C and 4 days at ambient temperature without compromising their quality. On the other hand, storing for 50 days at 6°C followed by 4 days at ambient temperature caused severe external and internal damage to the roots, regardless of the cultivar. Therefore, BRS Amélia and BRS Rubissol can be classified as sensitive to low temperatures, and the manifestation of chilling injury symptoms in their roots is closely related to the increase in lipid peroxidation, activity of oxidative enzymes and oxidation of phenolic compounds.

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