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Antifreeze proteins in naturally cold acclimated leaves of *Drimys angustifolia, Senecio icoglossus*, and *Eucalyptus* ssp.

Avaliação de proteínas anticongelantes em folhas de Drimys angustifolia, Senecio icoglossus e Eucalyptus ssp. naturalmente aclimatadas ao frio

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Summary

Antifreeze proteins (AFPs) present in plants may inhibit ice recrystallization even at low concentrations, and show potential application to many frozen foods. This study evaluated the presence of antifreeze proteins in naturally cold acclimated and non-acclimated leaves of *Drimys angustifolia*, *Senecio icoglossus* and *Eucalyptus* ssp. No proteins were detected in apoplastic extracts of *Eucalyptus* ssp. Extracts of cold acclimated and non-acclimated *S. icoglossus* showed protein concentrations of 42.89 and 17.76 μg mL^{-1}, both with bands between 25 and 37 kDa in the SDS-PAGE. However, they did not inhibit recrystallization. The extract of cold acclimated *D. angustifolia* contained a protein concentration of 95.17 μg mL^{-1}, almost five times higher than the extract of non-acclimated *D. angustifolia*. In the extract of cold acclimated *D. angustifolia*, there was presence of ice recrystallization inhibitors. This extract showed a protein band just below 37 kDa and another more intense band between 20 and 25 kDa. It is the first time that the presence of antifreeze proteins in this species is being described.

Keywords: *Antifreeze protein; Drimys angustifolia; Senecio icoglossus; Eucalyptus; Natural cold acclimation; Recrystallization inhibition.*

Resumo

Proteínas anticongelantes (PACs) de plantas podem inibir a recristalização do gelo mesmo em baixas concentrações, tendo potencial para aplicação em diversos alimentos congelados. Este trabalho avaliou a presença de proteínas anticongelantes em folhas naturalmente aclimatadas ao frio e folhas não aclimatadas de *Drimys angustifolia*, *Senecio icoglossus* e *Eucalyptus* ssp. Não foram detectadas proteínas nos extratos de *Eucalyptus* ssp. Os extratos de folhas aclimatadas e não aclimatadas de *S. icoglossus* apresentaram concentrações de proteína de 42,89 e 17,76 μg mL^{-1}, respectivamente, verificando-se, em ambos os extratos, bandas entre 25 e 37 kDa no SDS-PAGE. Contudo, esses extratos não apresentaram atividade de inibição da recristalização. O extrato de folhas de *D. angustifolia* aclimatadas ao frio apresentou concentração de proteína de 95,17 μg mL^{-1}, cinco vezes maior do que extrato não aclimatado dessa espécie. Observou-se que o extrato aclimatado de *D. angustifolia* apresentou atividade de inibição da recristalização. Esse extrato apresentou uma banda logo abaixo de 37 kDa e outra mais intensa, entre 20 e 25 kDa no SDS-PAGE. É a primeira vez que a presença de proteínas anticongelantes, nessa espécie, é descrita.

Palavras-chave: *Proteína anticongelante; Drimys angustifolia; Senecio icoglossus; Eucalyptus; Aclimatação natural; Inibição da recristalização.*

1 Introduction

First described in the early 70s in cold-water fish, antifreeze proteins (AFPs) comprise the survival strategy of a large group of organisms whose habitat is located in cold places, where temperatures reach below the freezing point of their fluids. During the last two decades, different AFPs have been more thoroughly described in animals, insects, fungi, bacteria and plants and always in species and varieties that were exposed to low temperature (CAI et al., 2011).

This group of proteins has various shapes and sizes and both can modify the water freezing process. This modification may occur essentially in three ways. The first is by interfering with the nucleation process, which is the initial stage of ice formation, by binding to nucleating substances. The second is by reducing the freezing temperature of the body fluid in a non-colligative manner without causing any effect on the melting point. This property is known as thermal hysteresis (HASSAS-ROUDSARI; GOFF, 2012). The third is by inhibiting ice recrystallization, which is a capacity of another group of AFPs.

Recrystallization is the process that occurs after the formation of ice crystals when the temperature changes within a range just above the freezing point. Ice recrystallization is the growth of large ice crystals at the expense of smaller ones. Although the chemical basis for the mechanism of action of AFPs in the inhibition of recrystallization has not yet been fully elucidated, it is certain that its effects occur through adsorption of these proteins to different parts of the surface ice crystal through one or more forms of interaction, such as hydrogen bonds or Van der Waals interactions (CRUZ et al., 2009; HASSAS-ROUDSARI; GOFF, 2012).

The larger the ice crystals formed during recrystallization, the greater the possibility of there being physical damage to cells and tissues. Therefore, the recrystallization process is always a concern in sectors that use freezing as a conservation method, such as the food technology industry. Freezing is a method of conservation widely used by the food industry, even though it can cause a reduction in the sensory and nutritional quality of food products. Foods that are formed by cells, such as meat and vegetables, for example, can lose some of their nutrients and have low water retention after thawing. Products that are consumed while still frozen, such as ice cream, may have their sensory quality reduced due to the formation of larger ice crystals (WHATEN; JIA, 2005; PROVESI; AMANTE, 2015).

Although still few in number, studies conducted on the application of AFPs in the preservation of sensory and nutritional quality during processing and storage of foods such as ice cream (FEENEY; YEH, 1998), meat and its proteins (PAYNE et al., 1994; BOONSUPTHIP; LEE, 2003; YEH et al., 2009), breads and pasta (ZHANG et al., 2007, 2008; DING et al., 2014, 2015), starch gels (LI et al., 2010) and fruits and vegetables (CRUZ et al., 2009; VELICKOVA et al., 2013) have shown promising results. Some AFPs may exhibit an effective activity of recrystallization inhibition even at low concentrations as 100 μg L^{-1}. Although they usually perform low or insignificant thermal hysteresis activity, AFPs from plants are much better at inhibiting recrystallization than AFPs from insects or fishes, besides being effective from concentrations as low as 25 μg L^{-1} (ZHANG et al., 2004; HASSAS-ROUDSARI; GOFF, 2012), which is an effective concentration that is 300-500 times lower than other antifreeze substances. It is insignificant to cause sensory changes while sufficient to provide cryoprotection (BILDANOVA et al., 2013).

Since Griffith et al. (1992) described, in a pioneering way, the presence of AFPs in winter rye leaves (*Secale cereale*), which were later studied by Hon et al. (1994), Antikainen and Griffith (1997) and Lim et al. (2013), the presence of AFPs with different characteristics have been detected in wheat (*Triticum aestivum*) (ANTIKAINEN; GRIFFITH, 1997; ZHANG et al., 2007), carrots (*Daucus carota*) (SMALLWOOD et al., 1999; WANG et al., 2002; ZHANG et al., 2004; GOMEZ GALINDO et al., 2005; DING et al., 2014), ryegrass (*Lolium perenne*) (SIDEBOTTOM et al., 2000; PUDNEY et al., 2003; MIDDLETON et al., 2012), *Solanum tuberosum* (URRUTIA et al., 1992), *Solanum dulcamara* (URRUTIA et al., 1992; HUANG; DUMAN, 2002), *Forsythia suspensa* (SIMPSON et al., 2005), *Picea abis* and *Picea pungens* (JARZABEK et al., 2009), among many others.

In order for the expression of AFPs to occur, plants need to undergo a process of cold acclimation. The cold acclimation of a plant is a multigenic process, which is associated with numerous biochemical and physiological changes that culminate in increased expression of AFPs. It is a process whose details are not yet fully understood; however, it is likely that it involves hundreds of genes, calcium (Ca^{2+}) and substances such as ethylene and abscisic acid (ATICI; NALBANTOGLU, 2003; DUMAN; WISNIEWSKI, 2014). Anatomically, antifreeze proteins are located mainly in the intercellular space known as apoplast (JARZABEK et al 2009). Besides the cold, other environmental factors may also be involved in acclimation process, such as lightings, water, nutritional status, presence of diseases and others (GOMEZ-GALINDO et al., 2005).

Several plant species, especially in temperate zones, routinely have cycles of acclimation and deacclimation. For some species, one or two days are enough to induce cold acclimation while for other species higher concentrations of AFPs are only observed after some weeks. The increase to milder temperatures leads to rapid loss of this ability (QURESHI et al., 2007; BILDANOVA et al., 2013). In cold regions, where minimum temperatures are below 0 °C, this process occurs during the fall or in the first weeks of winter. With the onset of spring, there occurs the process of deacclimation and the tolerance of

plants to cold drops (JARZABEK et al., 2009). There are few studies involving AFPs in naturally acclimated plants (GOMEZ-GALINDO et al., 2005; SIMPSON et al., 2005; JARZABEK et al., 2009; CAI et al., 2011).

Today, a major obstacle to the commercial use of AFPs still is the cost of extraction. The potential market for AFPs creates the need to find alternative sources of fish, insect and, especially, of cold climate plants, whether the AFPs are to be used in an isolated form or as an extract. In temperate regions of several countries, including Brazil, there are plant species that resist very well to cold and frost; however, the presence of AFPs in these plant species has never been investigated. Within this context, it is important that this assessment be conducted with a naturally occurring acclimation process. Most studies involve artificial acclimation of the plants in chambers with controlled conditions. While providing more controlled and reproducible results for other researches, this option probably would raise the cost of cultivation, thus making it too expensive.

There are several species of the *Drimys* genus. Three species grow wild in Brazil, namely, *Drimys angustifolia* Miers, *D. brasiliensis* Miers and *D. roraimensis*, the first two of which occur in southern Brazil. The *D. angustifolia* species is popularly known as "casca d'anta" or "cataia", and occurs more restrictively at high altitudes (SANTOS et al., 2013). The *Senecio icoglossus* DC. species is popularly known by several different names, the most common is "*margarida do banhado*", and it is usually found in wet or marshy areas in regions of moderate or high altitudes in the southeast and south of Brazil as well as throughout Argentina and Paraguay (TELES, 2008). *Eucalyptus* is a large genus of the Myrtaceae family, which includes 900 species and subspecies and is widely grown in many countries all over the world, including Brazil. Its leaf extract has been approved as a food additive mainly because of its antioxidant property (TYAGI; MALIK, 2011). All of these species are adapted to temperate climates.

Therefore, considering all the aforementioned, the purpose of this study was to investigate the presence of AFPs and of any recrystallization inhibition activity in plant extracts obtained from naturally cold acclimated and non-acclimated leaves of *Drimys angustifolia*, *Senecio icoglossus*, and *Eucalyptus* ssp.

2 Material and methods

2.1 Plant material

In this study, leaves of the species *Drimys angustifolia* Miers, *Senecio icoglossus* DC. and *Eucalyptus* ssp. were analyzed. The plant material was collected from plants grown under natural conditions at different rural units in the municipal district of Urupema (27°57'10" South, 49°52'23" West, 1,350 m altitude), in Santa Catarina state, Brazil.

Samples considered naturally cold acclimated and samples without acclimation period (non-acclimated) were collected from all species. The temperature range proposed by Gomez-Galindo et al. (2005) was adopted as a parameter. For the cold acclimated samples, the collection was carried out in June 2015, after a period of five consecutive days of average temperature below 6 °C and minimum temperature near or below 0 °C. Meanwhile, the collection for the non-acclimated samples occurred after a sequence of five days with average temperatures close to 10 °C and minimum temperatures equal to or greater than 6 °C. The climate data that preceded the two sample collections were provided to us as a courtesy of the EPAGRI (Santa Catarina State Agency for Agricultural Research and Rural Extension).

The samples were collected in the morning, washed in cold water and stored in a thermal box and then taken to the laboratory where the preparation of the extracts was carried out. All the reagents used in the preparation of the extracts and subsequent analysis were of analytical grade.

2.2 Extraction of apoplastic proteins

The extraction of apoplastic proteins was performed using a modification of the method proposed by Hon et al. (1994). The leaves were cut into pieces measuring 3 cm (with an approximate area of 9 cm^2) and then thoroughly rinsed with distilled water to wash off cellular proteins from the cut ends. After each rinse, the water was evaluated spectrophotometrically (Hitachi, U-1800, Japan) at 280 nm as an indicator of residues of cellular proteins. In general, four cycles of washing were sufficient to substantially reduce the residues.

The leaves were then vacuum-infiltrated with a pH 3 buffer solution that contained 20 mmol L^{-1} of L-ascorbic acid and 20 mmol L^{-1} of calcium chloride using an aspirator vacuum pump. During the process, the vacuum was ceased every 10 min for a few seconds to facilitate the infiltration of the buffer. After 40 minutes, the vacuum was ceased and the leaves were carefully blotted dry between two layers of paper towels.

The dried leaves were placed in 50 mL centrifuge bottles and the centrifugation was performed at ~3800 x *g* for 30 min. The apoplastic extract was then collected from the bottom of the tubes using an automatic pipette.

The concentrations of apoplastic proteins in the extracts of the acclimated and of the non-acclimated leaves were determined according to the methodology proposed by Bradford (1976), i.e., using a UV-Vis spectrophotometer (Hitachi, U-1800, Japan) (695 nm) and BSA (bovine serum albumin) as a standard.

2.3 Antifreeze activity assay

The antifreeze activity was determined by measuring ice recrystallization inhibition, using the sucrose sandwich splat assay proposed by Smallwood et al. (1999) with modifications. In the methodology used, about 5 µL of the solution under investigation in 30% (w/w) sucrose was compressed between two microscope slides. The 'sandwich' was flash frozen at –40 °C in an ultra freezer (Klimaquip, UK-05, Brazil). Then the temperature was increased to –10 °C and the samples were incubated for 60 to 120 min. After incubation, the growth of ice crystal was observed in an optical microscope (Kozo, XJS900, China), with 20x objective, coupled to a digital photography camera and using image processing software (ToupTek, ToupView, China).

A visual assessment to detect any degree of recrystallization was made by comparing the size of the ice crystals of the test sample with those of a control sample (without plant extract) (WANG et al., 2002).

2.4 Protein electrophoresis

The apoplastic protein extracts were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) carried out in 4% (w/v) polyacrylamide-stacking gel and a 12% (w/v) polyacrylamide-resolving gel, in accordance with standard protocols using a Bio-Rad Mini-PROTEAN system (Mini-Protean II; Bio-Rad Laboratories, Richmond, CA, USA), and 250 kD-10 kD Precision Plus Protein Standards (Bio-Rad Laboratories, Richmond, CA) as molar mass markers. 200 µL of the extracts were centrifuged for 20 min at 20000 x *g* at 4 °C, the supernatant was discarded, then the resulting pellets were suspended in 300 µL of rehydration solution (7 mol/L Urea, 2 mol L^{-1} Thiourea, 0.3 g L^{-1} CHAPS, 0.15 g L^{-1} DDT) and heated at 96 °C for 5 min. 15 µL of the solution cooled to room temperature (20 °C) was loaded into the gel at 15 mA/gel free voltage for 15 min, followed by 30 mA/gel free voltage for 1 h. The 5x SDS-running buffer (distilled water, 0.12 mol L^{-1} Tris pH 8.3, 7.2% glycine, 0.5% SDS) was diluted to 1x with distilled water. The gels were stained for 1 h with 0.1% (w/v) Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (25:10:65, v/v).

2.5 Statistical analysis

All the analyses were performed in triplicate. The results of the protein concentration in the extracts were expressed as the mean ± standard deviation. They were submitted to analysis of variance (ANOVA) and mean separations using Tukey's multiple-range test ($P \leq 0.05$) for comparison between the acclimated and the non-acclimated extracts of each of the plant species. In all the statistical analyses, ANOVA assumptions, such as independence and normal distribution of the residues and homogeneity of variances, were observed.

3 Results and discussion

Figure 1 shows the climate data on the days previous to the material collection. The collections of the acclimated samples were carried out on the days following a minimum period of 5 days with an average temperature below 6 °C and minimum temperatures near or below 0 °C (Figure 1a). The non-acclimated samples were collected after a minimum of 5 days with average and minimum temperatures above 6 °C (Figure 1b).

There are basically two ways to extract antifreeze proteins from plants. In the conventional AFPs extraction

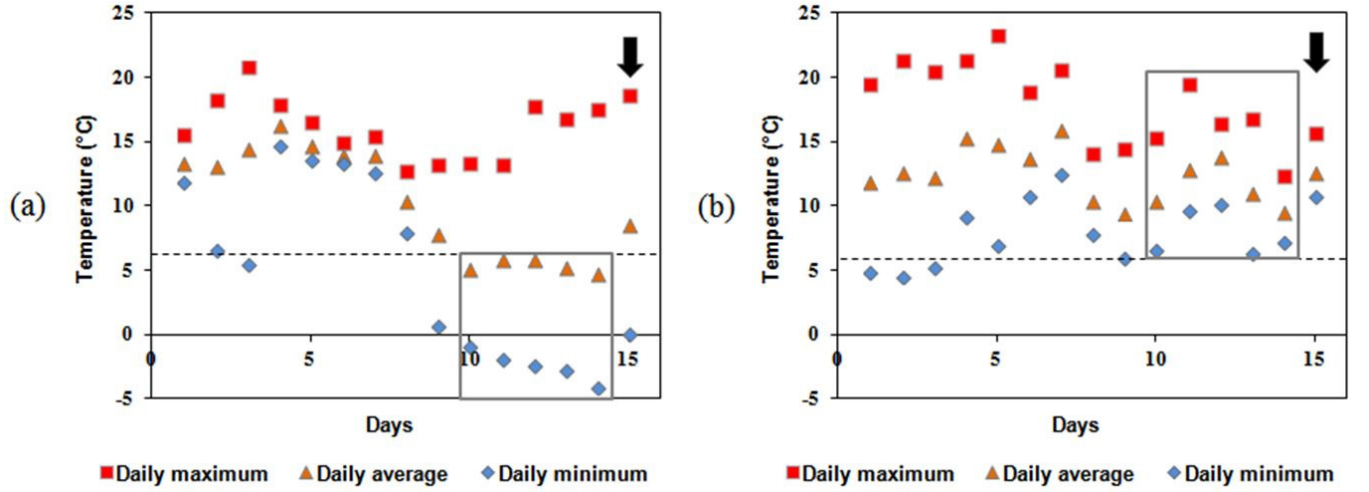

Figure 1. Daily average, maximum and minimum temperature in the municipality of Urupema, in Santa Catarina state, Brazil, in the periods preceding the collection of (a) the cold acclimated and (b) the non-acclimated samples. The samples were collected on the morning of the 15th day, indicated by the arrow.

method, grinding and stirring are adopted. This method performs the complete extraction of the AFPs; however, the analysis of these proteins would be masked by other intracellular proteins, and thus requiring many subsequent purification steps. Meanwhile, by the infiltration-centrifugation method, it is possible to collect the apoplastic fluid from plants with less plasmic contamination, especially Rubisco. Although this method has a lower protein extraction rate, it is more specific to apoplastic proteins (HON et al., 1994; DING et al., 2015). Table 1 shows the concentrations of apoplastic proteins (in the extract and leaf) for the acclimated and the non-acclimated samples with the proposed methodology of extraction.

Apoplastic proteins were not detected in the acclimated and non-acclimated *Eucalyptus* ssp. leaves. For cold acclimated *Drimys angustifolia* and *Senecio icoglossus* leaves the concentration of apoplastic proteins increased 4.9 and 2.4 fold, respectively, compared with non-acclimated samples of the same species. Similar relationships, 3.1 and 2.3 fold, were described for acclimated and non-acclimated samples of Norway spruce (*Picea abies L.* Karst) and blue spruce (*Picea pungens* Engelm), respectively (JARZABEK et al., 2009). Antikainen and Griffith (1997) evaluated twelve herbaceous plants for 7 weeks of cold acclimation and also described a considerable increase in the apoplastic protein concentration for most species.

In relation to the protein concentrations, comparisons with other studies are difficult to make due to many factors that may cause variations in the concentrations of proteins, like variation of species and different conditions of acclimation, extraction, purification method, among other factors.

Hon et al. (1994) described concentrations ranging from 320 to 660 μg mL^{-1} in extracts of the same samples of winter rye leaves (*Secale cereale* L.) depending only on the buffer used for infiltration. The concentrations of AFPs were much higher than those observed in this present study; however, it is important to note that the authors used artificial acclimation and the cultivation time was of 7 or 8 weeks. Plant seedlings usually have higher concentrations of antifreeze protein because plants at this stage are sensitive and more prone to be cold acclimated (DING et al., 2015). In another research involving mature and naturally cold acclimated plants, the concentrations observed were 4.7 ± 0.6 μg g^{-1} and 4.4 ± 0.4 μg g^{-1} for fresh needles Norway spruce (*Picea abies L.* Karst) and blue spruce (*Picea pungens* Engelm), respectively. For plants collected in the summer (non-acclimated) these values were lower, 1.5 ± 0.6 μg g^{-1} and 1.9 ± 0.1 μg g^{-1} fresh needles, respectively (JARZABEK et al., 2009).

The plant extracts were then evaluated for their antifreeze activity, more specifically their activity on the recrystallization inhibition (RI). The assessment of RI activity involves monitoring the rate of recrystallization of ice by flash-freezing a solution to obtain small ice crystals, maintaining the sample at temperatures just below 0 °C (–6 to –10 °C), and observing changes in crystal size. As recrystallization may be affected by solutes other than AFPs, recrystallization experiments are generally conducted at high solute concentrations to minimize nonspecific effects (GRIFFITH; YAISH, 2004). The modified sucrose-sandwich-splat assay proposed by Smallwood et al. (1999) allows for unequivocal identification of RI activity in plant extracts using high levels of sucrose as solute.

Figure 2 shows a sucrose-sandwich-splat microscopy for extracts of acclimated *Drimys angustifolia*, *Senecio icoglossus* and *Eucalyptus* ssp. before and after the incubation period. It is possible to clearly observe that the ice crystals in the control solution and acclimated *Eucalyptus* ssp. extract have grown significantly. The same happened with cold acclimated *Senecio icoglossus* extract, in which apoplastic proteins were detected in spite of showing no RI activity in the evaluated concentrations. Only the ice crystals of the cold acclimated *Drimys angustifolia* extract the remained small after the incubation period, indicating the presence of inhibitors of ice recrystallization in the analyzed concentrations. The microscopy of the non-acclimated extracts are not shown; however, growth of ice crystals was observed in all of them, as occurred with the control group and with the acclimated *Senecio icoglossus* and *Eucalyptus* ssp. extracts.

Other authors have also reported RI activity in plant extract in similar or even lower concentrations. RI

Table 1. Apoplastic proteins levels in obtained apoplastic extract and fresh leaves of acclimated and non acclimated *Drimys angustifolia*, *Senecio icoglossus* and *Eucalyptus* ssp.

Plant	Apoplastic Extract (μg proteins mL^{-1} extract)		Leaves (μg proteins g^{-1} fresh leaves)	
	Acclimated	Non acclimated	Acclimated	Non acclimated
Drimys angustifolia	95.17 ± 4.72[a]	23.19 ± 5.93[b]	19.03 ± 0.94[a]	3.86 ± 0.99[b]
Senecio icoglossus	42.89 ± 4.02[a]	17.76 ± 3.87[b]	5.36 ± 0.50[a]	2.22 ± 0.48[b]
Eucalyptus ssp.	nd	nd	nd	nd

The data are presented as means ± standard deviation (SD) (n = 3); The values on the same line with different superscript letters are significantly different ($P < 0.05$), according to Tukey's test; nd, not detected.

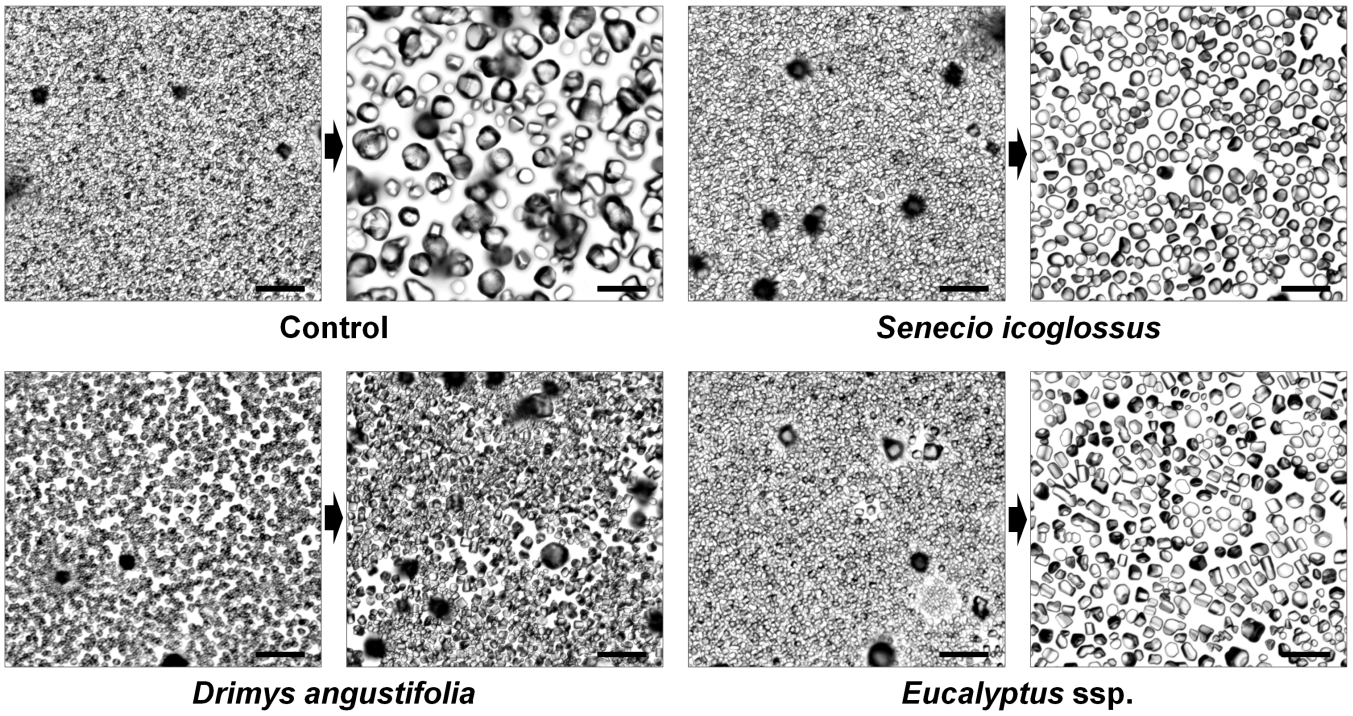

Figure 2. Sucrose-sandwich-splat assays to evaluate the inhibition of ice recrystallization in apoplastic extract from cold acclimated leaves of *Drimys angustifolia*, *Senecio icoglossus* and *Eucalyptus* ssp. The bar represents 50 µm.

activity was detectable in cold-acclimated carrot root homogenates with concentrations as low as 150 µg mL^{-1}, but for purified protein this activity could still be detected at a concentration of 1 µg mL^{-1} (SMALLWOOD et al., 1999). The apoplastic extract is an intermediate form between the plant homogenates and purified protein. For *Lollium perenne* AFPs, a high specific activity in the ice recrystallization inhibition was described with a concentration of less than 10 µg mL^{-1} (SIDEBOTTOM et al., 2000; PUDNEY et al., 2003).

The plant extracts were analyzed in relation to their electrophoretic profile (Figure 3). Despite only the cold acclimated *Drimys angustifolia* extract having been submitted to antifreeze activity, the other extracts were also analyzed for comparison and confirmation.

The *Senecio icoglossus* extract showed two bands, between 25 and 37 kDa markers, both of which are present in the acclimated and non-acclimated samples (2A and 2NA slots); however, with more intensity in the first group. In the concentrations evaluated in this study, the extract of acclimated *Senecio icoglossus* leaves showed no RI activity. The apoplastic extract of acclimated and non-acclimated *Eucalyptus* ssp. (3A and 3NA slots), as expected, showed no bands in the gel.

The SDS-PAGE analysis of the apoplastic extract of cold acclimated *Drimys angustifolia* (1A slot) showed two different bands, a less intense band just below 37 kDa and a more intense band between 20 and 25 kDa. For the non-acclimated sample of this species (1NA slot), only the second band was observed in a lower intensity.

For bark and leaves of *Forsythia suspensa*, also cold-acclimated under natural conditions, for example, the antifreeze activity was observed in a single protein with apparent molecular mass of 20 kDa (SIMPSON et al., 2005). Kontogiorgos et al. (2006) reported that apoplastic extract of the cold-acclimated winter wheat grass contained only heat-stable RI protein with a molecular weight below 40 kDa.

Needles of *Picea abies* and *Picea pungens* showed 5-9 peptides ranging from 7 to 80 kDa. A 16 kDa polypeptide played an important role in the the cryoprotective activity (JARZABEK et al., 2009). A fraction containing protein of 27 kDa with antifreeze activity was analyzed and it showed a significant difference in the concentration in the plant material collected in the winter compared with the samples collected in the summer.

The extract of acclimated barley seeds (*Hordeum vulgare* L.) obtained by the infiltration-centrifugation method was abundant in proteins between 20.1 and 31 kDa and proteins around 14.4 kDa (DING et al., 2015). Apoplastic extract from acclimated winter rye (*Secale cereale* L.) leaves contains five polypeptides, with molecular weights of 19, 26, 32, 34 and 36 kDa and with a high degree of antifreeze activity (HON et al., 1994). The main AFP extracted from carrot (*Daucus carota*) showed values close to 36 kDa (SMALLWOOD et al.,

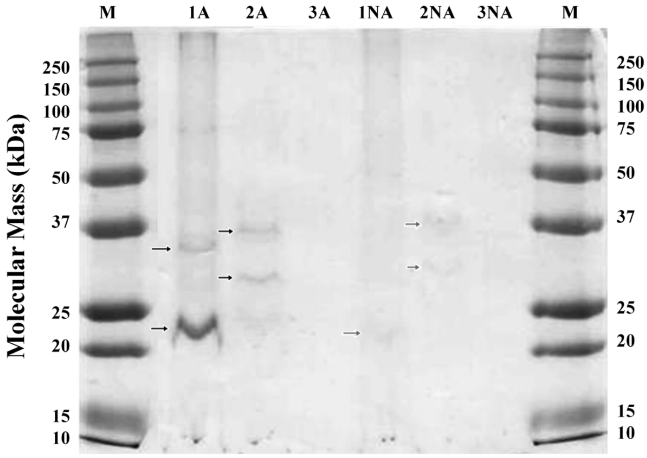

Figure 3. 12% SDS-PAGE of apoplastic extracts from cold acclimated (A) and non-acclimated (NA) leaves. Lanes 1A, 2A and 3A: extracts from cold acclimated leaves of (1A) *Drimys angustifolia*, (2A) *Senecio icoglossu* and (3A) *Eucalyptus*; lanes 1NA, 2NA and 3NA: extracts from non-acclimated leaves of (1NA) *Drimys angustifolia*, (2NA) *Senecio icoglossu* and (3NA) *Eucalyptus;* lanes M: 250-10 kDa Precision Plus Protein Standards. Protein bands are indicated by arrows.

1999; ZHANG et al., 2008). Huang and Duman (2002) isolated three AFPs from *Solanum dulcamara* leaves with molecular weights of 29, 47 and 67 kDa. The AFP isolated from *Lolium perenne* showed an apparent molecular weight of 29 kDa (PUDNEY et al., 2003).

Therefore, the proteins detected in the apoplastic extract of *Drimys angustifolia* follow the general pattern of plant AFPs, which are generally small peptides that have low molecular weight. This is the first time that the presence of antifreeze proteins with recrystallization inhibition activity is being described in samples of naturally acclimated leaves of this species.

4 Conclusion

Several studies have demonstrated that antifreeze proteins may represent an important alternative for controlling ice crystal growth, which is interesting to several fields of knowledge. For the Food Technology field, many studies have demonstrated the application of these proteins in various foods, although the cost is still a major obstacle to their commercial use.

This study evaluated apoplastic extracts from leaves of three plants in relation to their recrystallization inhibition activity and one of them, the *Drimys angustifolia* extract, showed this activity at a relative low concentration. This species is widely found in southern Brazil, principally in areas of higher altitude and lower daily temperature. It is important to note that this activity was observed only in acclimated samples, showing the importance of the cold period for a higher expression of these proteins. The acclimation process was carried out naturally and, therefore, the cultivation of this plant under controlled conditions is not a precondition for the expression of AFPs.

Clearly, there is a need for further researches, involving the characterization of these proteins, the details of their action mechanism and their application in various food matrices, such as pasta, fruit, and ice-cream. In addition to the application AFPs itself, the appreciation of native species may represent an important future alternative for local agriculture.

Once available in abundance, in a safe manner and at low cost, these proteins may represent a new alternative to minimize impacts on texture after thawing and to extend the shelf life of frozen food products.

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