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Influence of vacuum impregnation and pulsed electric field on the freezing temperature and ice propagation rates of spinach leaves



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ABSTRACT

Efforts are currently directed towards improving the quality of sensitive tissues of fruits and vegetables after freezing and thawing. One of the methods under investigation is the combination of vacuum impregnation (VI) with cryoprotectants and pulsed electric field (PEF), applied to the plant tissue prior exposure to freezing. The influence of these processes on the freezing temperature and ice propagation rate of spinach baby leaves are here studied. Leaves impregnated with trehalose, sucrose, glucose and mannitol exhibited significantly lower ice propagation rate and higher freezing temperatures in comparison to non-treated controls. Leaves subjected to PEF also showed increased freezing temperatures compared to the non-treated leaves; however the ice propagation rate was not influenced by PEF for the cryoprotectants used in the study, except for leaves impregnated with trehalose, where it was significantly increased and water, where it was significantly decreased. The combination of VI and PEF resulted in comparable freezing temperatures and ice propagation rates as the leaves subjected only to VI.

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1. Introduction

Freezing is a widely used and well established preservation method for food products, including fruits and vegetables (Gómez Galindo & Sjöholm, 2004). Even though freezing possesses significant advantages, there are still aspects of the freezing process that require improvement, such as preservation of color, texture and flavor (Jeremiah, 1996), especially in sensitive plant tissues such as leaves which are extremely difficult to protect against freezing injuries.

The quality of frozen-thawed food products may be improved by compositional and/or structural modifications of the plant tissue before freezing. One of the methods to change the structure and composition of plant tissues is vacuum impregnation (VI) (Derossi & Severini, 2012; Fito, Chiralt, Barat, Spiess, & Behnlian, 2001), which has been shown to improve the quality of frozen-thawed fruits and vegetables by incorporating stabilizers such as pectin (Reno, Prado, & Resende, 2011; Xie & Zhao, 2004) or zinc and calcium salts (Xie & Zhao, 2004) into the structure of the commodity prior to freezing. Impregnation of cryoprotectants, such as antifreeze proteins (Velickova et al., 2013), and sugars, such as sucrose (Ursachia, Segalb, & Dicu, 2009) and trehalose (Velickova et al., 2013), have been

reported to have a positive effect on the quality of the food products after freezing and thawing. Another method is the use of a pulsed electric field (PEF) which has shown to improve the texture of frozen-thawed carrots (Shayanfar, Chauhan, Toepfl, & Heinz, 2014) and potatoes (Jalté, Lanoisellé, Lebovka, & Vorobiev, 2009). It was also reported that improvement of visual quality of frozen potato tissue was observed after PEF and osmotic pre-treatment (Ben Ammar, Lanoiselle, Lebovka, Van Hecke, & Vorobiev, 2010). Remarkably, PEF treatment used in combination with VI resulted in the preservation of cell viability in spinach leaves after a freeze/thaw cycle (Phoon, Galindo, Vicente, & Dejmek, 2008).

Even though VI and PEF have been reported to have an advantageous effect on the quality of frozen-thawed fruits and vegetables, no studies are available on the influence of these treatments on the freezing process itself. Therefore, the objective of the present investigation was to examine the influence of these treatments on the freezing temperature and ice propagation rate of spinach leaves, factors which describe the severity and velocity of the freezing process and, consequently, may be related to the quality of the final product.

High resolution infrared thermography (HRIT) was employed in the current study to observe ice nucleation (freezing) and propagation in spinach leaves that were subjected to VI and PEF with various sugars prior to exposure to freezing temperatures. These

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processes can be viewed using HRIT due the heat released when water in the tissue undergoes a phase transition from a liquid to a solid. HRIT is an excellent tool to visualize the process of freezing and ice propagation in plants (Wisniewski, Lindow, & Ashworth, 1997) and has been demonstrated to be a novel method for analysis the effect of various compounds on these parameters (Gusta, Wisniewski, Nesbitt, & Gusta, 2004).

2. Materials and methods

2.1. Plant material

Baby spinach leaves (*Spinacia oleracea*, cv. Big Ruffles and Bloomsdale) were grown in a greenhouse using 14 h photoperiod and a temperature of 21 °C. Plants were watered daily. Leaves 5–6 cm long were harvested, rinsed with deionized water and used within 30 min in the described experiments.

2.2. Vacuum impregnation (VI)

In preliminary experiments, five leaves of baby spinach were immersed in a series of different concentrations of different sugars and changes in weight were recorded over a period of 16 h (Panarese et al., 2014). The concentrations leading to neither weight loss nor weight gain, namely trehalose 110 g/L, sucrose 130 g/L, glucose 100 g/L and mannitol 80 g/L were chosen for vacuum impregnation.

Immersed leaves were placed in a vacuum chamber connected to a vacuum pump. Based on preliminary experiments to establish a maximum weight gain of spinach, a minimum absolute pressure of 15 kPa was chosen. The pressure was decreased from atmospheric pressure to 15 kPa mbar gradually over 5 min. Pressure was then kept at a constant 15 kPa for 1 min. Atmospheric pressure was subsequently gradually restored over a period of 5 min. The entire cycle was repeated twice. The relative increase in leaf weight after vacuum impregnation was $37 \pm 4.75\%$.

2.3. Pulsed electric field treatment (PEF)

A pulse generator (Bio Rad Gene Pulser Generator Model 1652076, Bio Rad Laboratories, Hercules, CA, USA) was connected to the treatment chamber, which contained two stainless steel electrodes placed in parallel with a 0.5 cm gap between them. A solution with an electrical conductivity of 380 $\mu\text{S}/\text{cm}$, adjusted with NaCl, was placed in the gap between the electrodes. A leaf was placed in the chamber, parallel to the electrodes, and 25 exponential decay pulses of 100 μs time constant and amplitude of 350 V were applied.

Electroporation conditions, aiming at obtaining uniform electroporation of the leaf surface (which is representative of the electroporation of the whole leaf cross section at more than 300 V (Dymek, Rems et al., 2014)) were established in preliminary experiments where leaves were immersed in 250 μM propidium iodide (Dymek, Dejmek, & Gómez Galindo, 2014) and pulses were subsequently applied. Propidium iodide enters only the electroporated cells and binds to the cell nuclei resulting in fluorescence, which was then detected under the microscope (Zeiss Microscopy, Germany). The viability of the electroporated leaves was determined using a wilting test (Phoon et al., 2008), that was administered at least 16 h after the pulses were applied.

2.4. Ice-nucleating (*Ice*⁺) bacteria

In samples where significant differences in the freezing temperature were detected, Cit7 *Ice*⁺ bacteria were used as ice

nucleator allowing a comparison of ice propagation rates without the problem of freezing occurring at different temperatures in different leaves. The Cit7 strain of *Ice*⁺ bacteria (*Pseudomonas syringae*), courtesy of Dr. Steven Lindow, University of California, Berkeley, were grown for 48–96 h at 20 °C in Petri plates containing King's medium B (King, Ward, & Raney, 1954). Before the experiments, bacterial cells were scraped from the surface of the petri dish and suspended in distilled water. A 10 μL drop of the bacterial cell suspension was placed on the leaf surface to initiate freezing at a warm sub-zero temperature (−1 °C), thus preventing the plant tissue from supercooling.

2.5. Freezing

A high resolution infrared camera (FLIR SC660, FLIR Systems, Inc., Wilsonville, OR, USA) was placed in an environmental chamber (model T20S, Tenney environmental, White Deer, PA, USA) to visualize and record freezing events in the plant samples. The camera was connected to a laptop computer and controlled with ExaminIR Max FLIR (FLIR Systems, Inc., Wilsonville, OR, USA) software.

Leaves that were either vacuum impregnated, PEF treated, or vacuum impregnated and PEF treated, as well as untreated controls, were rinsed with deionized water and blotted with a tissue before placing them in the environmental chamber. Leaves were subjected to freezing directly after the treatments or 16 h later. In the latter case, leaves were stored at 4 °C in saturated air, in the dark, until they were subjected to the freezing protocol. The environmental chamber was set to 0 °C and leaves were allowed to equilibrate for 15 min in the chamber. The temperature of the environmental chamber was reduced at a rate of approximately 0.5 °C per minute until the sample was frozen. Leaves had equilibrated with the air temperature in the chamber prior to each decrease of a 0.5 °C. The freezing process was recorded at a rate of 10 frames per second. The temperature at which freezing of an individual sample was initiated was defined as the temperature at which the first exothermic event was observed with infrared thermography to be initiated and propagated. The exothermic event represented the initiation of the freezing of extracellular water, while the time it took for the exothermic event to travel throughout the sample represented the rate of ice propagation. The time it took for the exothermic event to dissipate and reach equilibrium with the air temperature represented the time required for all the extracellular water to completely freeze and for the system to be in equilibrium. The freezing temperature was defined as the temperature of the environmental chamber when the first sign of an exothermic event (freezing) was visually detected. The ice propagation rate was determined from the digital recordings by measuring the time and distance from the initial freezing point to the most distal freezing point within the leaf sample.

2.6. Statistical analysis

Statistical significance ($p < 0.05$) of the treatments was tested by means of a one way ANOVA analysis using Excel (Microsoft Office, Redmond, WA, USA). The Tukey–Kramer multiple comparison test was used to analyze differences between treatments.

3. Results

3.1. Effect of vacuum impregnation and pulsed electric field on the freezing temperature of baby spinach leaves

Fig. 1 shows the influence of vacuum impregnation (VI) of spinach leaves with water, trehalose, glucose, sucrose, and

mannitol on the freezing temperature of the leaves. VI treatment resulted in leaves freezing at a significantly warmer temperature relative to the untreated control, regardless if the VI treatment was done with water or one of the tested sugars. Non-treated leaves supercooled to -14.5 ± 0.9 °C, while leaves vacuum impregnated with water, trehalose, mannitol, sucrose and glucose froze at -10.4 ± 1.8 , -5.9 ± 2.1 , -8.9 ± 0.9 , -8.7 ± 1.1 , -9.7 ± 1.7 °C, respectively. Leaves vacuum infiltrated with trehalose exhibited the warmest freezing temperatures compared to all of the other tested compounds. Though significantly different than the non-treated controls, no significant differences were observed in the freezing temperature between leaves vacuum infiltrated with water, mannitol, glucose, or sucrose.

PEF by itself induced the freezing of spinach leaves to occur at a significantly warmer temperature compared to non-treated leaves (Fig. 2). The combination of VI and PEF with sugars induced the freezing to occur at even warmer temperatures than PEF alone. A comparison of Figs. 1 and 2 indicates that VI with water combined with PEF did not influence the freezing temperature of spinach leaves to any greater degree than VI with water alone. Leaves subjected to both treatments froze at approximately -11 °C.

3.2. Effect of vacuum impregnation and pulsed electric field treatment on the ice propagation rate in spinach baby leaves

Fig. 3 shows typical infrared photographs showing drastic differences of ice propagation rate between the untreated (1s, Fig. 3a) and the trehalose-impregnated leaf (16 s, Fig. 3b). Table 1 and Figs. 4 and 5 show the results of the calculated rates for the different tested conditions.

To evaluate the ice propagation rate in impregnated leaves with the tested sugars, *Ice⁺* bacteria were used to avoid the influence of different freezing temperatures (Fig. 1). When the freezing temperature of the leaves was standardized at -1 °C, the rate of ice propagation was significantly slower in spinach leaves vacuum impregnated with any of the tested sugars and mannitol than the rate in non-treated and water impregnated leaves (Table 1).

The short and the long term effect of VI on the ice propagation rate was also investigated (Fig. 4). Since no difference between the freezing temperatures of leaves frozen immediately after VI (Fig. 1) and 16 h later was detected, and consequently, this temperature had no influence on the ice propagation rate, this experiment was conducted without the *Ice⁺* bacteria as nucleator. Leaves

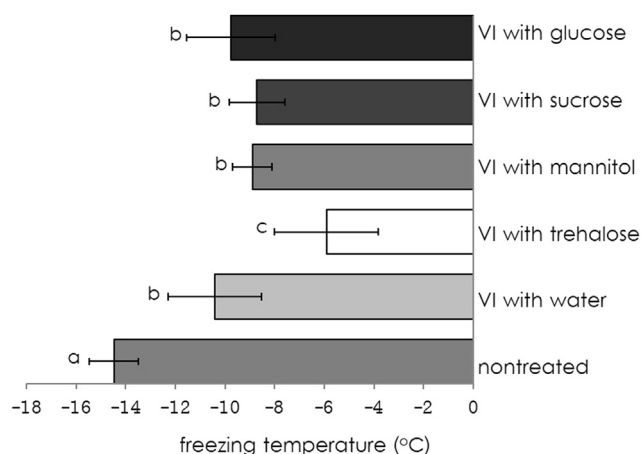


Fig. 1. Freezing temperature of spinach leaves. Leaves were either untreated or vacuum impregnated (VI) with water, or isotonic solutions of trehalose, mannitol, sucrose or glucose. Data represents the mean \pm sd, $n = 6$. Different letters indicate a significant difference between the means ($p < 0.05$).

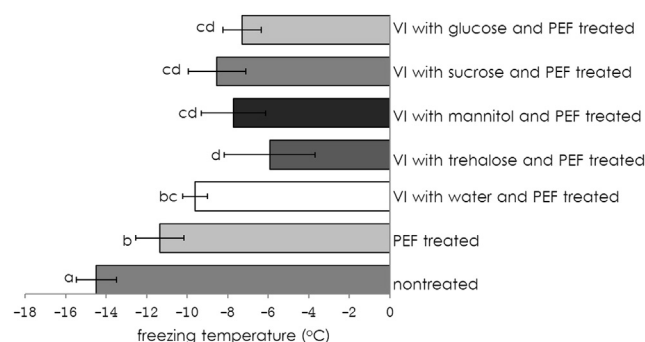


Fig. 2. Freezing temperature of spinach leaves. Leaves were either untreated, treated with a pulsed electric field (PEF) treated or vacuum impregnated (VI) with water, or isotonic solutions of trehalose, mannitol, sucrose or glucose. Data represents the mean \pm sd, $n = 6$. Different letters indicate a significant difference between the means ($p < 0.05$).

impregnated with water and subjected to freezing 16 h later exhibited a dramatic decrease in the ice propagation rate compared to leaves subjected to freezing directly after impregnation (Insert Fig. 4). In contrast, leaves impregnated with sucrose, glucose, or mannitol, exhibited no difference in the ice propagation rate when frozen 16 h after impregnation compared to leaves frozen immediately. A significant difference was observed, however, in leaves vacuum impregnated with trehalose. Leaves frozen 16 h after vacuum impregnation exhibited a faster ice propagation rate compared to leaves frozen immediately after vacuum impregnation. A similar tendency was observed in leaves vacuum impregnated with trehalose and subjected to PEF prior to freezing. PEF treatment resulted in an increase in the ice propagation rate only in leaves vacuum impregnated with trehalose (Fig. 5). Leaves vacuum impregnated with water (Insert Fig. 5) exhibited a significant decrease in the ice propagation rate decreased when subjected to PEF prior to freezing.

4. Discussion

The freezing point in herbaceous plants is a complex event influenced by the presence of extrinsic and intrinsic ice nucleation agents (Ashworth & Kieft, 1995), the permeability and thickness of the cuticle (Wisniewski & Fuller, 1999), the osmotic potential of cells, and anatomical/morphological characteristics of the plant that act as a physical barrier to ice propagation (Carter, Brennan, & Wisniewski, 2001). The presence of sugars and proteins in the apoplast and extracellular space can also have a great impact on both the nucleation temperature and the rate of ice propagation (Gusta et al., 2004). The effect of sugars was clearly demonstrated in our study, however, rather than depress the freezing temperatures as would be expected due to their osmotic effect, they induced the tissue to freeze at a warmer temperature and prevented the tissues from supercooling (Fig. 1). The presence of the impregnated sugars, however, did dramatically reduce the rate of ice propagation (Table 1). Trehalose had the greatest impact on the freezing temperature among the sugars and sugar alcohol (mannitol) tested (Fig. 1). Another evident effect of the impregnated trehalose that was not seen with the other tested sugars was the dramatic increase in the ice propagation rate when leaves were frozen 16 h after impregnation (Fig. 4). In freezing experiments using simpler systems, such as filter paper strips, trehalose was shown to result in the greatest decrease in the rate of ice propagation compared to other equimolar tested substances, such as sucrose and betaine (Gusta et al., 2004). This is similar to what was observed in Fig. 4. Therefore, the dramatic increase in the rate of propagation

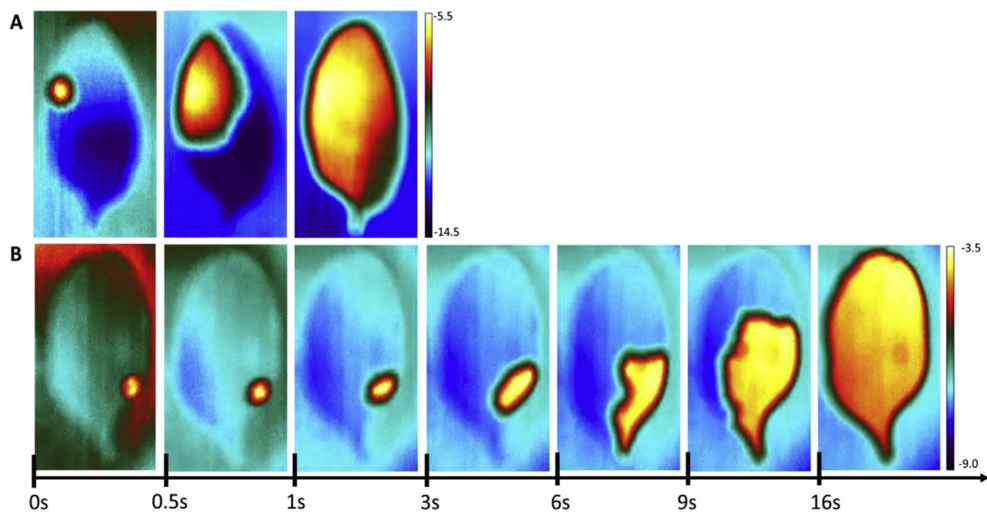


Fig. 3. Typical infrared photographs of the freezing of spinach leaves (A) untreated leaf, (B) the leaf was vacuum impregnated with isotonic trehalose prior to freezing. This example is shown in leaves where *Ice*⁺ bacteria were not used.

Table 1

Ice propagation rate in young spinach leaves. Experiments utilized a drop of water containing *Ice*⁺ bacteria that was placed on the adaxial leaf surface in order to prevent variations in the level of supercooling in different leaves subjected to different treatments. The initial freezing took place at -1°C . Leaves were either untreated or vacuum impregnated (VI) with water, isotonic solutions of trehalose, mannitol, sucrose or glucose. Data represents the mean \pm sd, $n = 6$. Different letters indicate a significant difference between the means ($p < 0.05$).

	Ice propagation rate (mm/s)
Nontreated	1.87 ± 0.9^a
VI with water	0.22 ± 0.04^b
VI with trehalose	0.04 ± 0.004^c
VI with mannitol	0.03 ± 0.003^c
VI with sucrose	0.04 ± 0.005^c
VI with glucose	0.04 ± 0.008^c

observed after 16 h with impregnated trehalose is difficult to explain. In the case of metabolically active plant material, such as the young spinach leaves used in this study, the physics of this effect remains unknown. Factors that may contribute to the obtained results may include the chemical interaction of the sugars

with the cell membrane, the capacity of cells to metabolize the impregnated sugars (Panarese et al., 2014) or possible structural changes that might be induced as a result of the impregnation process over a period of hours, or as a result of changes in gene expression, and its concomitant effect, induced by the impregnated sugars (Delatte et al., 2011).

A rupture or dysfunction of the plasmalemma due to the direct or indirect effects of freezing results in a mixture of water, sugars and organic solutes migrating to the apoplast. These solutes would then have a direct effect on freezing rates. It has been shown that small M_r compounds (M_r cutoff of 3500) retard the rate of freezing of cell extracts (Gusta et al., 2004). In our experiments, the application of PEF, which destabilizes the plasmalemma and promotes the leakage of solutes to the extracellular space, significantly decreases the ice propagation rate when the tissue has been previously impregnated with water but not in non-impregnated tissue (insert Fig. 5). The PEF conditions used in this study were selected to induce reversible permeabilization of the cells, meaning that the cells would have the capacity to recover from the treatment and even re-absorb some of the leaked solution (Gómez Galindo, Wadsö, Vicente, & Dejmek, 2008). Our results suggest that the presence of water may have contributed to the leaked solutes

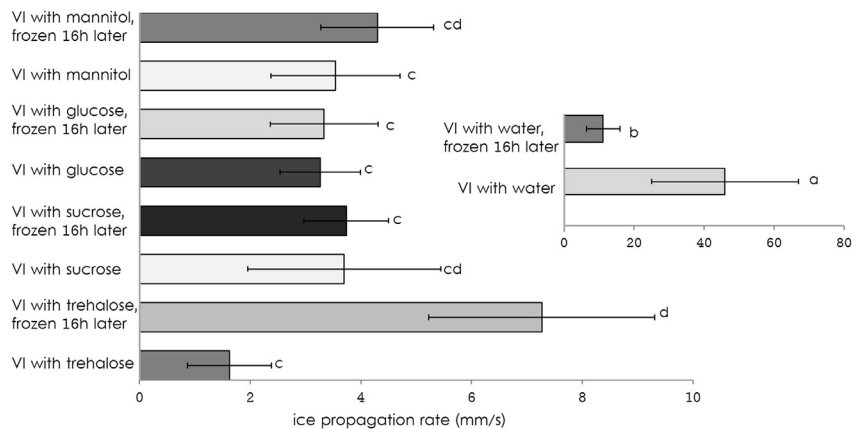


Fig. 4. Ice propagation rate in leaves vacuum impregnated (VI) with different solutions without *Ice*⁺ bacteria. Leaves were frozen immediately after VI and 16 h after VI. Insert shows the ice propagation rate for leaves vacuum impregnated with water. Leaves were frozen immediately or 16 h after VI. Data represents the mean \pm sd, $n = 6$. Different letters indicate a significant difference between the means ($p < 0.05$).

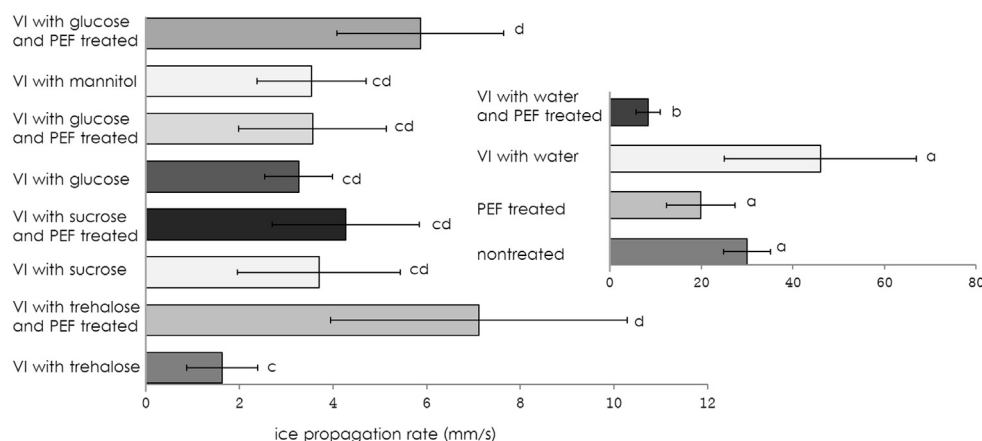


Fig. 5. Influence of pulsed electric field (PEF) on the ice propagation rate of vacuum impregnated leaves without Ice^+ bacteria. Comparison of ice propagation rate for leaves vacuum impregnated with different solutions and leaves vacuum impregnated and PEF treated. Data represents the mean \pm sd, $n = 6$. Different letters indicate a significant difference between the means ($p < 0.05$).

forming a fluid zone surrounding the cells. Then as freezing progressed sugars would have become more concentrated, protecting the plasma membrane from the encroaching ice and retarding the rate of freezing. However, when PEF is applied to the impregnated tissue with the other sugars and mannitol, the induced leakage from the cells does not seem to have an additional effect on the propagation rate, with the interesting exception of the trehalose (Fig. 5). These discrepancies and anomalies need to be explored in more comprehensive experiments.

When PEF was solely applied, the leaked solutes induced the spinach leaves to freeze at a significantly warmer temperature compared to non-treated tissue (Fig. 2). Impregnation with water prior to PEF rose the freezing temperature even more, however, the increase was not statistically significant. These results highlight the importance of even a small concentration of solutes in the extracellular space on the freezing response of tissue. Again, while one would have expected the sugar solutions in the apoplast to decrease the freezing temperature of the leaves (i.e. promote supercooling), the exact opposite was observed in that freezing occurred at a warmer temperature. Clearly, the presence of sugars in copious amounts of water surrounding the cells and filling up extracellular spaces has a completely different effect than when sugars and water are distributed unevenly and in small amounts in untreated tissues. While this study has provided data that is, in some cases, problematic to interpret, it does open up a host of questions that need to be addressed in order to understand the freezing process in complex systems, and highlights the utility of using infrared thermography to actually visualize the freezing process rather than relying solely on thermocouple data or post-thaw observations. Additionally, of the sugars and sugar alcohol examined in the present study, trehalose produced results that were unique compared to the results obtained with the other compounds. Therefore, the properties of trehalose, relative to the freezing process in plant tissues and in general, should be explored in more detail.

5. Conclusions

This study has focused on the effect of vacuum impregnation and pulsed electric field treatment, with and without the addition of various sugars, on the freezing temperature and ice propagation rate in young spinach leaves. The following points highlight some of the important findings as well as interesting questions derived from this study:

- 1 Vacuum impregnation with water, sugars (trehalose, sucrose, glucose) and mannitol increases the freezing temperature of spinach leaves, making them freeze at a warmer temperature, compared to non-treated leaves. These results run contrary to the expected effect of solutes on freezing point depression and promotion of supercooling in plant tissues. Sugars present in copious amounts of water filling up extracellular spaces (as after VI) may have caused this effect on supercooling.
- 2 Vacuum impregnation with sugars (trehalose, sucrose, and glucose) and mannitol significantly lower the ice propagation rate compared to non-treated leaves and leaves impregnated with water. This result was expected.
- 3 PEF treatment induced a significant leakage of solutes and other cytoplasmic constituents into extracellular spaces in the tissue that the ice propagation rate of leaves impregnated with water decreased approximately four-fold as a result of the PEF treatment.
- 4 The ice propagation rate in spinach leaves vacuum impregnated with trehalose increased as a result of the PEF treatment and when leaves were subjected to freezing 16 h after they were impregnated with water compared to freezing immediately after impregnation with trehalose. Of the sugars and sugar alcohol (mannitol) tested, trehalose produced effects that were much different than the other sugars. The properties of trehalose relative to the freezing process need to be further explored.

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