Preservation of cell viability in fruit and vegetable tissues after freezing and thawing

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ABSTRACT

Cryoprotection of horticultural products has been a matter of concern for the food industry in its search for efficient ways of improving the quality of frozen-thawed products. Research on the cryoprotection of plant tissues should make progress through a better understanding of the natural protective mechanisms of plant tissues during winter survival. The present study reports results on the use of vacuum impregnation (VI) alone or in combination with pulsed electric field (PEF) to obtain cryoprotectant solutions in the tissues. Spinach samples were treated with a combination of PEF and VI prior to freezing in liquid nitrogen and thawing at room temperature. VI was used to impregnate trehalose (40% w/w solution) as cryoprotectant and PEF was used to distribute the cryoprotectant in the extra and intracellular spaces of the tissue. Strawberries were treated with VI prior to freezing in liquid nitrogen ant thawing at room temperature. VI was used to impregnate a combination of trehalose (12% w/w) and cold acclimated winter wheat extract (AWWE, 0.02% w/w) containing antifreeze protein. Results were evaluated by assessing the maintenance of the texture of the tissue as well as by cell viability analysis using fluorescence microscopy. Results provide evidence that impregnating fruit and vegetables tissues using VI alone or in combination with PEF with the tested cryoprotectants improves drastically the cryoprotection of the treated tissues. Cryoprotection is proved through the maintenance of cell viability and texture after one freezing/thawing cycle. In the case of strawberries, cryoprotection was influenced by the heterogeneity of the tissues in the fruit and the viability of cells close to the surface (epidermal and probably also hypodermal) could not be preserved.

Keywords: cryprotection; cell viability; pulsed electric fields; vacuum impregnation; antifreeze proteins

INTRODUCTION

Freezing is an extensively used method to preserve the quality of food products. However in the case of fruits, this technique may result in textural changes leading to tissue softening. The structural integrity of frozen plant tissue is preserved in a best way using high freezing rates due to small ice crystal formation, less migration of water, less breakage of cell walls and consequently less texture deterioration [1]. However, physical changes such as ice recrystallization during storage at subzero temperatures and thawing may result in deleterious textural changes in fruits and reverse the advantage of fast freezing. Moreover, the fruit structure can collapse during thawing resulting in loss of the cells water holding capacity and leading to drip loss [2]

Attempts have been made to improve the resistance of fruit and vegetables to freezing damage such as reducing the freezable water content by osmotic dehydration prior freezing [3, 4, 5], reinforce cell-cell adhesion by calcium chloride application with or without low methoxyl pectin [6, 7] or improve the textural quality and drip loss of frozen-thawed fruits by application of high fructose corn syrup and high methoxyl pectin [8]. Vacuum infusion is one of the preferred techniques for infusing the fruits with external solutions, filling the porous fractions of the tissue and, therefore, the infused solution is present in the apoplast. However, these methods cannot avoid the freeze-induce damage to cell membranes, turgor loss and the consequent loss of cell vitality, essential factors for the fresh-like characteristics of the product.

Innovation on freezing of fruit and vegetables, aiming at preserving cell vitality after freezing and thawing cycles, should be based on knowledge of the natural cryoprotection mechanisms at the cellular level [9]. Cold-induced stress responses comprise complex metabolic processes regulated at the genetic level (for a review see Gómez Galindo et al. [10]). Among these cryoprotective mechanisms, certain plant tissues accumulate osmotically active substances in the cytoplasm as well as antifreeze proteins (AFPs) in the apoplast during growth in the field in late autumn. The protective role of osmotically active substances such as some amino acids (e.g. proline), quaternary ammonium compounds (e.g. betaine), numerous sugars (e.g. sucrose and trehalose) and several other substances [11] has been widely studied. There is a wealth of information on their stabilizing

effects on biological molecules, cells and organisms, which indicates their functional role in the stress tolerance of many, although not all, organisms [12]. Apart from decreasing the chemical potential of water and the freezing point in the cytosol, the hydrophilic nature of sugars is well-suited for stabilizing the cell membrane through hydrogen bonding between the hydroxyl groups on the sugar and the polar residues in phospholipids. This prevents dehydration effects in membranes [13]. In the apoplast, accumulation of AFPs is very important for cryoprotection. At high AFP concentrations (μM), minimal crystal growth occurs, forming very small, stable hexagonal bipyramids. AFPs are also strong inhibitors of recrystallization [14].

The present study represents one step forward in the quest for procedures aiming cell survival after thawing. We used spinach and strawberries for impregnation of cryoprotectants with two methods that differ in the general procedure but have the same principle. This is to impregnate and distribute in the tissues natural cryoprotectants that allow winter survival of plant tissues in nature. Spinach samples were treated with a combination of pulsed electric fields (PEF) and vacuum impregnation (VI) prior to freezing in liquid nitrogen and thawing at room temperature. VI was used to impregnate trehalose (40% w/w solution) as cryoprotectant and PEF was used to distribute the cryoprotectant in the extra and intracellular spaces of the tissue. Strawberries were treated only with VI prior to freezing in liquid nitrogen ant thawing at room temperature.

MATERIALS & METHODS

Raw materials handling

Rectangular samples, 3.0 cm long, 0.5 cm wide and 0.06 cm thick, were cut from the spinach leaves using a sharp blade. To ensure that the thickness of each sample was even, the thickness was measured with a caliper in 2 - 3 different regions. All leaf samples were free from major portions of conductive tissue.

Strawberries (10.5° ± 1.6°Brix, 11.6 ± 0.95% dry weight and ρ = 0.896 ± 0.004 g/cm^3) grown in the south of Sweden in the summer of 2009 were purchased from the local market. The fruits were washed, hand stemmed and selected according to size (height: 30 ± 2 mm; width: 27 ± 3 mm), firmness and similar visual ripening. For the experiments, fresh strawberries were purchased daily from the local market. Maximum storage time was one day at 20^{o}C.

Cryoprotectant solutions

The following cryoprotectants were prepared as aqueous solutions: Trehalose (Cargill*Ascend 16400, Denmark) and spray dried, unpasteurised cold-acclimated winter wheat grass extract (AWWE) as a source of (AFP) (Microstar Biotech Ltd., Zhuhai, China; AWWE contained 12 % proteins). Four different solutions were prepared: 12% (w/w) trehalose solution, 40% (w/w) trehalose solution, 0.2 % (w/w) AWWE solution and a third solution containing 12% (w/w) trehalose and 0.2 % (w/w) AWWE.

Treatments

Spinach samples were treated with a combination of PEF and VI prior to freezing and thawing. The various procedures and their corresponding processing steps are shown in Figure 1, where spinach leaves were treated with

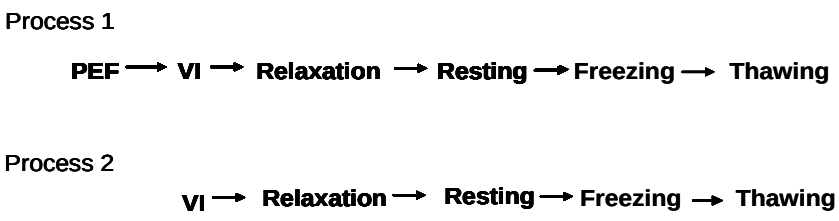


Figure 1. A combination of treatments for improving the freezing tolerance of spinach leaves.

For the PEF treatment, the rectangular spinach samples were placed in between two flat stainless steel electrodes (36 mm long and 9 mm wide) were they received electric pulses at room temperature. The sandwich structure of electrodes and sample was wrapped with scotch tape (Scotch MagicTM) to minimize sample evaporation. The electric pulses were delivered by a CythorLabTM electroporator (ADITUS AB, Lund, Sweden), and the parameters of the treatment were programmed by a computer software package (PulseEditTM, ADITUS AB, Lund, Sweden). Ten trains of bi-polar, rectangular electric field pulses with a nominal electric field strength of 580 V/cm (the nominal field strength is here defined as the applied voltage divided by the separation between the

electrodes, i.e. 0.06 cm) were used. Each train lasted 20 ms and consisted in 25 μs pulses. The resting period between the trains was 10 s to avoid heating of the samples (Figure 2).

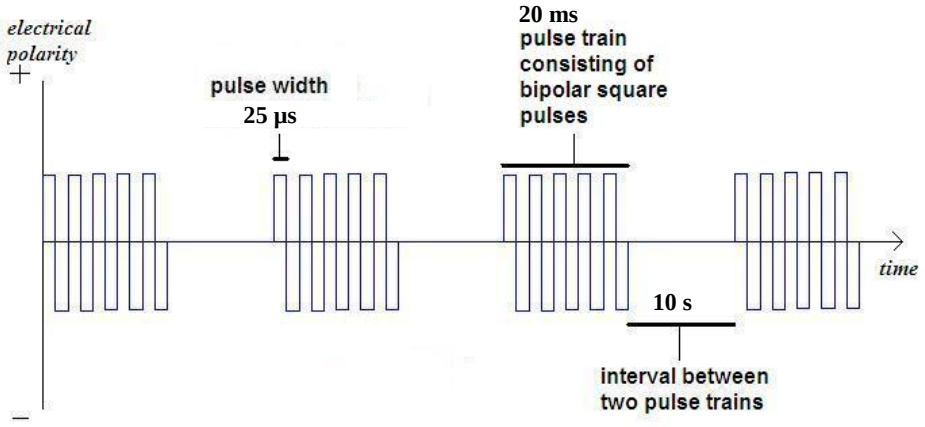


Figure 2. A simplified diagram of the PEF treatment design. Ten trains of bi-polar, rectangular electric field pulses with a nominal electric field strength of 580 V/cm were applied as described in the Section 2. Pulse characteristics are shown in the Figure.

The VI treatment was carried out at 20°C in a chamber connected to a vacuum pump. The spinach and strawberry samples were immersed in the treating solutions for 25 min. This duration comprised a gradual increase of the vacuum for 3 min, a holding time of 20 min at -86 kPa (man) and a gradual release of the vacuum for 2 min. After the impregnation, the samples were kept in the trehalose solution for 2.5 h (relaxation time).

The other processing steps were carried out in spinach samples as follows: the resting step was performed by submerging the samples in deionised water overnight at 4°C to regain turgor. Freezing was done by submerging the individual samples in liquid nitrogen for 7 s. The samples were then immediately thawed in water at room temperature.

Strawberry samples were only vacuum impregnated with the different cryoprotectant solutions.

Analysis

The viability of the cells was evaluated by using fluorescein diacetate (FDA, Sigma-Aldrich, USA), as described by Gómez Galindo et al. [15]. Samples were incubated for 5 min in a 0.5M sucrose solution 10^{-6} M FDA in the darkness at room temperature. Stained sections were rinsed thoroughly in distilled water for 1 min and examined under fluorescent light in a Nikon upright microscope (Eclipse Ti-U, Nikon Co, Japan) equipped with a Nikon digital video camera (digital sight DS-Qi1Mc, Nikon Co, Japan). Undamaged, viable cells could be easily identified by a bright fluorescence.

Figure 3 shows how strawberry fruits were sampled for microscopic examinations.

The turgidity of the treated spinach samples was evaluated by simply holding the centre of the sample with a small pincer and observing whether the leaves would bend or not.

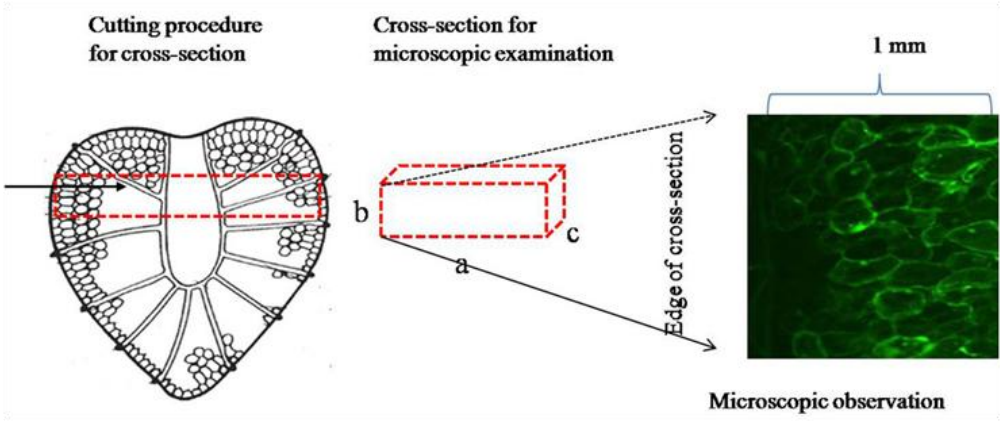


Figure 3. Preparation of cross-section for microscopic observations

RESULTS & DISCUSSION

The FDA and the wilting tests demonstrated that the application of ‘‘Process 1” (Figure 1) resulted in viable, turgid spinach leaves (see Figure 4c for a typical result). These results strongly suggested that the applied processing steps allowed the uptake of trehalose by the tissue, thus dramatically increasing the freezing resistance of the samples. The results from ‘‘Process 2” (Figure 1) where the FDA and the wilting tests showed non-viable cells and totally wilted leaves (see Figure 4d for a typical result) was considered evidence that the impregnation of the trehalose in the extracellular space alone, as expected after the VI process, was insufficient for cryoprotection and that the electroporation provoked by PEF, probably facilitating the distribution of trehalose between the intra and extracellular spaces, was a key event providing freezing resistance to the leaves.

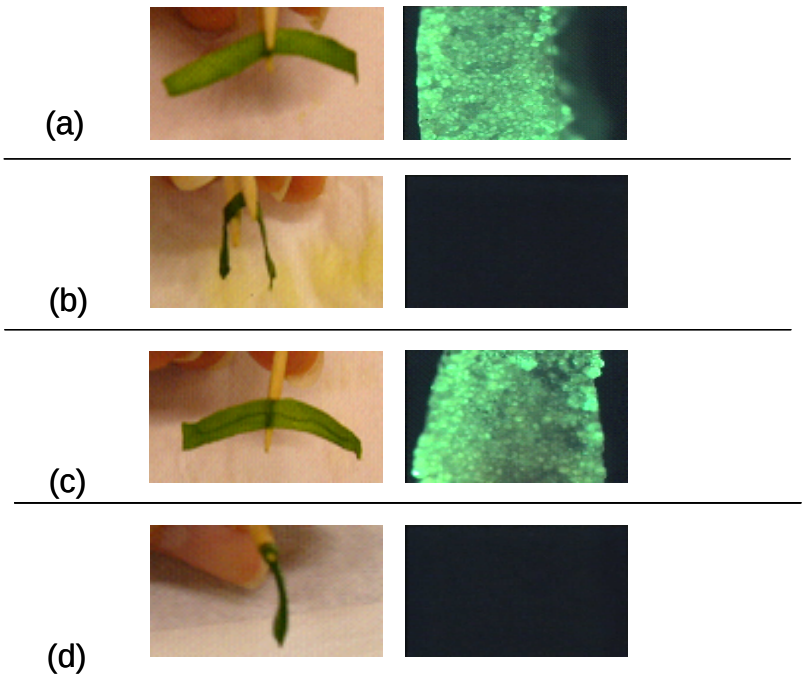
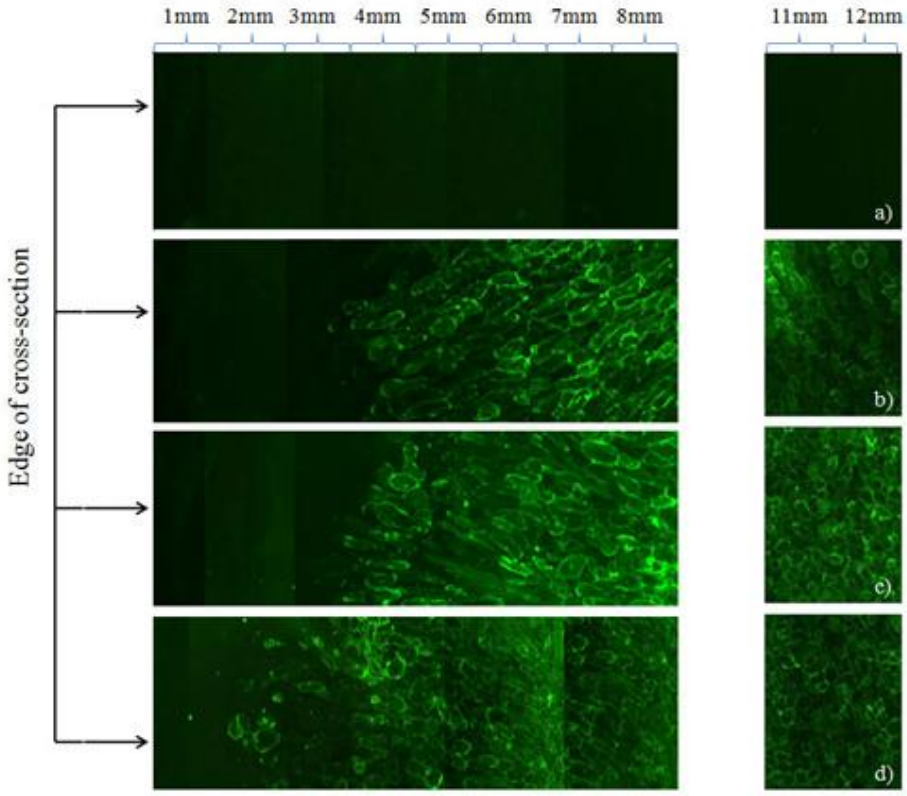


Figure 4. The freezing tolerance of spinach leaves after different treatments. Left panel: Typical results (10 measurements) from the wilting test, performed as described in the Materials and methods, showing the turgidity of the samples. Right panel: Typical results (10 measurements) from microscopic observations using fluorescein diacetate to identify viable cells. Viable cells are distinguished by a bright fluorescence. (a) A fresh spinach leaf. (b) A leaf frozen in liquid nitrogen for 7 s and immediately thawed in water at room temperature. (c) Spinach leaves subjected to ‘‘Process 1”, as schematised in Figure 1. (d) Spinach leaves subjected to ‘‘Process 2”, as schematised in Figure 1.

In the case of strawberries, Figure 5 shows the micrographs of frozen/thawed samples. As expected, freezing and thawing of fresh, untreated strawberry caused the loss of cell viability in all tissues of the fruit (Figure 5a). When trehalose and AWWE were infused alone into the samples prior freezing (Figure 5b,c), some improvement of

cell viability was observed, starting 3.5-4th mm from the surface, showing cryoprotection of the cortical tissue and the pith. The best results in terms of viability of the cells were obtained using the combination of trehalose and AWWE during vacuum infusion (Figure 5d) where the viable cells after freezing and thawing appeared already from the 2nd mm from the surface, confirming better cryoprotection of the cortical and vascular tissue, right bellow the hypodermal layer, and the pith.



Figue 5. Cell viability test of strawberries after one freezing/thawing cycle. (a) untreated strawberry, (b) vacuum infused with trahalose, (c) vacuum infused with AWWE, (d) vacuum infused with trahalose and AWWE.

As seen in Figure 5d, dead cells from the epidermal and probably also hypodermal tissue could not be preserved. This result produces strawberry fruits that are still soft to the mouth feeling at the surface, even if the general structure of the fruit is better preserved.

CONCLUSIONS

The results presented herein provide evidence that the impregnating spinach leaves with the cryoprotectant trehalose with the aim of improving the freezing tolerance of spinach leaves could be achieved only when the VI process was used in combination with PEF. The combined unit operations probably allowed trehalose to be present in both the extracellular and intercellular spaces.

The combined effects of trehalose and AFP significantly improved the freezing tolerance of strawberry fruits

The cryoprotection effect was influenced by the heterogeneity of the tissues in the fruits. Tissues close to the surface of the fruits did not survive thawing. Cortical tissue, vascular tissue and pith survived the freezing/thawing cycle.

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