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Investigation of the metabolic consequences of impregnating spinach leaves with trehalose and applying a pulsed electric field

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ABSTRACT

The impregnation of leafy vegetables with cryoprotectants using a combination of vacuum impregnation (VI) and pulsed electric fields (PEF) has been proposed by our research group as a method of improving their freezing tolerance and consequently their general quality after thawing. In this study, we have investigated the metabolic consequences of the combination of these unit operations on spinach. The vacuum impregnated spinach leaves showed a drastic decrease in the porosity of the extracellular space. However, at maximum weight gain, randomly located air pockets remained, which may account for oxygen-consuming pathways in the cells being active after VI. The metabolic activity of the impregnated leaves showed a drastic increase that was further enhanced by the application of PEF to the impregnated tissue. Impregnating the leaves with trehalose by VI led to a significant accumulation of trehalose-6-phosphate (T6P), however, this was not further enhanced by PEF. It is suggested that the accumulation of T6P in the leaves may increase metabolic activity, and increase tissue resistance to abiotic stress.

1. Introduction

The impregnation of leafy vegetables with cryoprotectants using a combination of vacuum impregnation (VI) and pulsed electric fields (PEF) has been proposed by our research group as a method of improving their freezing tolerance [15]. However, achieving a uniform distribution of the cryoprotectant in the highly heterogeneous structure of the leaves is challenging. Furthermore, the mechanisms involved in freezing tolerance are not yet fully known. It is thus important to broaden our knowledge on these two aspects for process optimization, and possible application of the technology to a wider range of plant tissues.

Complete removal of the air from the spongy mesophyll and intercellular spaces of the leaves and its replacement with the solution containing the cryoprotectant (e.g., an isotonic solution of trehalose) can only be achieved by optimizing the VI parameters [5]. Subsequent application of PEF should lead to homogeneous electroporation of the different cells distributed in the different tissues through the cross section of the leaf, ensuring that the cryoprotectant is located in both the intra- and extracellular spaces [15]. The technological challenges of electroporation have been investigated through mathematical modelling by Dymek et al. [2] using both fresh and impregnated spinach leaves as model systems. The discrepancies found between the model and experimental data were attributed to air remaining in the structure of the leaves after VI [19]. Total replacement of the air by the solution may not be possible, as recently speculated by Panarese et al. [14], who showed that oxygen-dependent metabolic processes such as photorespiration still took place in the impregnated spinach leaf after impregnation.

To the best of our knowledge, no other studies have been carried out to investigate the metabolic responses of plant tissues to a combination of VI and PEF. Remaining air in the spinach tissue after VI is believed to cause an increase in metabolic activity [14]. Interestingly, the catabolism of the impregnating sugars taken up by the cells through the corresponding cell membrane transporters and/or endocytosis has also been described [14,20]. The metabolic consequences of a possible increase in the concentration of sugars in the intracellular space and/or stress responses due to electroporation after impregnation remain to be explored.

In this study, we evaluated the efficiency of the VI process in spinach leaves by visualizing any remaining air in their structure with X-ray

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microtomography. We then used isothermal calorimetry to investigate the effects on the metabolic activity of the leaves caused by the combination of VI and PEF. These measurements were complemented with the analysis of trehalose-6-phosphate (T6P) as a marker of increased trehalose concentration in the cells, and a possible signalling molecule for metabolic response to abiotic stress [3,7,8,10,23].

2. Material and methods

2.1. Raw material and sample preparation

Baby spinach leaves (*Spinacia oleracea*) were grown in a greenhouse at a temperature of 21 °C with a photoperiod of 14 h. Seeds were planted 1.5 cm deep in the soil, in trays with 1.5 cm between the plants in a row and 3 cm between the rows. The plants were watered daily. Leaves 6.0 ± 0.5 cm long and 2.5 ± 0.3 cm wide were harvested in the morning (always at the same time of the day). Leaves from the centre of the plant, which were not shaded by any other leaves, were chosen. The leaves were transported to the lab packed in plastic bags. They were then rinsed with deionized water, and samples 3 cm long and 8 mm wide were immediately cut from the central part of the leaf parallel to the main vein (but not including the vein) with a scalpel. These samples were used for the calorimetric measurements.

The leaves used in the X-ray microtomography experiments were collected from a greenhouse of a local grower (Mechelen, Belgium) 1 h after harvesting, and stored at 4 °C in sealed plastic bags. Leaves of 5.5 ± 0.5 cm length were selected and used in the experiments within 3 days of harvesting.

2.2. X-ray microtomography

A square sample measuring 5 mm × 5 mm was cut close to the main vein half-way along the main vein's length. The sample was removed from the leaf just before the X-ray scan. Three replicate samples were prepared and analysed.

Each sample was placed on a polystyrene foam mounting stage with the adaxial side of the leaf in contact with the stage. A layer of Parafilm® was gently wrapped around the lower surface of the sample and the stage to avoid sample dehydration, and to keep the sample in a vertical position during the scan. Samples were scanned 15 to 60 min after the end of VI.

The samples were scanned using a SkyScan1172 high-resolution X-ray micro-CT system (Bruker, microCT, Kontich, Belgium), operating at 60 kV and 167 μA. The experimental conditions were optimized to obtain high-quality radiographic projection images, while considering the contrast and resolution, as well as manageable scanning times (15 min per sample). The X-ray shadow projections from each angular views obtained with a pixel size of 4.9 μm were captured on a 2000 × 1048 CCD X-ray camera and averaged from 3 frames as the object rotates on a high precision stage with 0.4° rotation step up to a rotation angle of 180°. Cross section images (slices) were obtained using NRecon 1.6.2.0 (Bruker microCT, Kontich, Belgium) tomography reconstruction software. Contrast of the images was enhanced using beam hardening correction, ring artefact reduction and smoothing values of 35%, 8 and 2 respectively. To standardize the greyscale range of the 8-bit bitmap output images, the linear attenuation coefficient range (dynamic range) was set to 0–0.122. This resulted in a 3D stack of 950 virtual sections, each consisting of 1244 × 344 pixel images with an isotropic voxel size of 4.9^3 μm^3.

A segmentation procedure was applied to separate solid voxels from gas filled spaces or voids. The most straightforward technique to segment a grey scale CT image is to define a global threshold value located at an obvious and deep valley in the histogram of grey level frequencies. Voxels with a grey value lower and higher than that threshold value are considered to be background or air spaces, such as pores and object (tissue) respectively [6]. A global threshold of 49 was decided from the Otsu threshold of different datasets. This was checked visually and applied to all the datasets recorded. The segmentation procedure was implemented in CTAn 1.9.1.0 (Bruker, microCT, Kontich, Belgium). In order to exclude the damaged cells as a result of the cutting of the sample, the image datasets were virtually cropped, removing about 100 pixels (0.5 mm) from each cut edge, as shown in Fig. 1. The porosity of the leaf was quantified as the % total volume of pores with respect to the total volume of the sample.

2.3. Vacuum impregnation

The concentration of the trehalose solution isotonic with the leaves was experimentally determined by immersing the leaves in a series of solutions of different concentrations. The concentration leading to neither weight loss nor weight gain, namely 11% trehalose, was used. Based on preliminary experiments to establish the maximum weight gain and to avoid visible damage to the spinach tissue, an impregnation protocol with a minimum absolute pressure of 15 kPa was chosen. Samples cut from the spinach leaves were immersed in the trehalose solution and placed in a vacuum chamber connected to a vacuum controller (S.I.A., Bologna, Italy) and a vacuum pump. The pressure was decreased to 15 kPa over 3.5 min, after which it was kept constant at 15 kPa for 1 min, and subsequently increased to atmospheric pressure over 4.5 min. This cycle was carried out twice.

2.4. Pulsed electric field treatment

The parameters providing reversible electroporation have been determined in preliminary experiments with fluorescence microscopy [1], using propidium iodide as an electroporation indicator and fluorescein diacetate as a cell viability indicator. The parameters were optimized for untreated leaves and for leaves previously vacuum impregnated with trehalose.

A PEF was applied to the sample inside the calorimetric ampoule by equipping the ampoule with two stainless steel electrodes separated by 0.5 cm. The leaf was placed between, and parallel to, the electrodes, as shown in Fig. 2. Deionized water (18 mL), adjusted with NaCl to a conductivity of 600 μS/cm, was injected into the ampoule with a syringe before the PEF treatment was applied. The electrodes were then connected to the pulse generator (Arc Aroma Pure, Lund, Sweden). Fifty, square, monopolar pulses of 250 μs duration and 200 V amplitude, with an

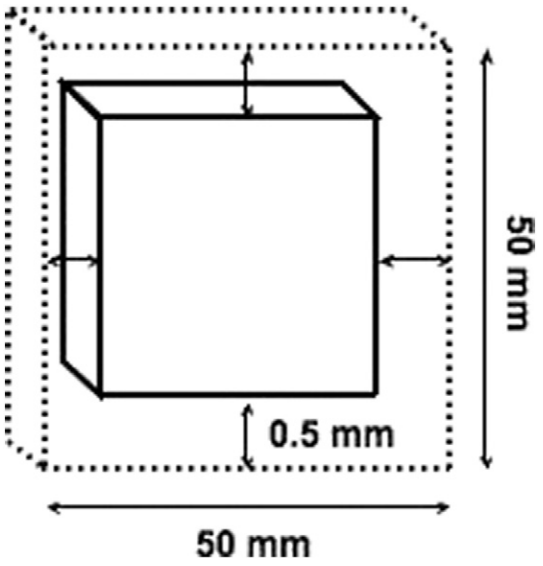

Fig. 1. Trimming operation in the samples. To exclude cells possibly affected by sample preparation, a portion of 0.5 mm was virtually cropped from the sample boundaries for every dataset prior to image analysis.

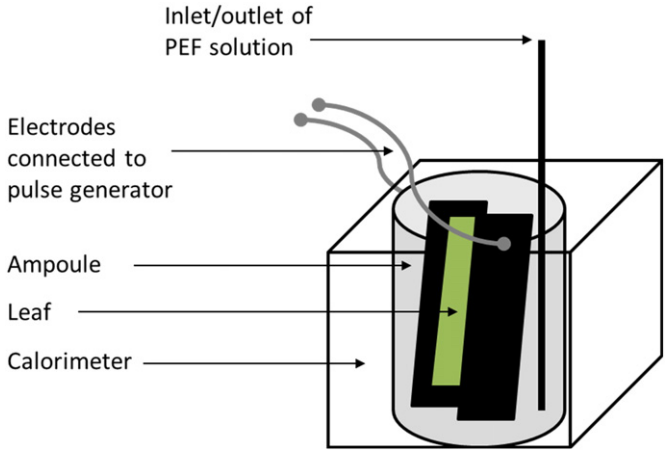

Fig. 2. Schematic illustration of the calorimetric ampoule adapted for the application of PEF to the leaf sample.

interval of 1 ms between pulses, were applied to the leaf sample. Immediately after PEF treatment the medium was withdrawn from the ampoule using a syringe.

2.5. Calorimetric measurements

The calorimetric measurements were performed with a microcalorimeter (Thermometric AB, Järfälla, Sweden). The calorimeter was placed in a home-built thermostated water bath maintained at 25 °C. The output voltage from the heat flow sensors was amplified with an instrument amplifier (A22, EM Electronics, UK) and recorded by a data logger (ADC-24, Pico Technology, Cambridgeshire, UK) every 10 s. The corresponding thermal power was then calculated from the expression:

$$P = \varepsilon \frac{V_S - V_{BL}}{M} \quad (1)$$

where P (mW kg^{-1}) is the specific thermal power, ε (mW V^{-1}) is the calibration coefficient of the calorimeter, V_S (mV) is the voltage output of the calorimeter with the sample, V_{BL} (mV) is the baseline voltage output (without the sample) and M (g) is the mass of the sample. The sample was placed in a 20 mL stainless steel ampoule, which was placed inside the calorimeter in an ampoule holder. An empty stainless steel ampoule was used as a reference. The leaf samples were subjected to three kinds of treatment, described below.

(i) *A combination of VI and PEF*. The leaf sample was placed in the ampoule for 4500 s (the time was measured from the time at which the signal peak came into the measurement range). The leaf was then removed from the ampoule and VI was applied causing a signal disturbance for 40 min. The leaf was subsequently placed back in the ampoule and the signal was recorded for 4500 s, before PEF treatment was applied to the sample inside the ampoule. The PEF treatment caused a 20 min disturbance in the signal. The signal was recorded for another 4500 s after PEF treatment.

(ii) *VI only*. The leaf sample was placed in the ampoule for 4500 s. It was then removed from the ampoule and VI was applied causing signal disturbance for 40 min. The leaf was subsequently placed back in the ampoule and the signal was recorded for a further 4500 s. To mimic PEF treatment, the NaCl solution was injected to the ampoule causing a 20 min signal disturbance, but PEF was not applied. The signal was recorded for another 4500 s.

(iii) *No treatment (control)*. The untreated leaf was placed in the ampoule and the signal was recorded for three periods of 4500 s with two signal disturbances representing the times taken to apply VI and PEF.

Three replications were made for each of the treatments. The entire sequence of experiments took approximately 5.5 h.

2.6. Determination of trehalose-6-phosphate

Leaves were either treated with VI with trehalose and PEF, VI only with trehalose, or untreated (controls). The samples were kept for 2 h at room temperature in the light in saturated air to avoid leaf dehydration. Fifty milligrams (fresh weight) of the sample was placed in 1.5 mL Eppendorf tubes. The tubes were immersed in liquid nitrogen and stored at −80 °C until analysis. T6P was determined as described by Sastre Toraño et al. [16]. Briefly, 50 mg (fresh weight) of frozen, ground spinach leaf samples was spiked with lactose-1-phosphate as an internal reference, and then extracted with acetonitrile/chloroform and back-extracted in water. The water phase was then further purified over solid phase extraction before injection into a HILIC/MS system. Four replicates of each of the treatments were analysed.

2.7. Statistical analysis

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

3. Results

3.1. Influence of VI on the porosity of spinach leaves

Typical X-ray microtomography images of an untreated leaf and a leaf impregnated with trehalose are shown in Fig. 3, where the grey areas correspond to tissue structure and the black areas to air. The quantified porosity of the untreated leaf was 48.7 ± 1.3% (Fig. 3A). VI reduced the porosity of the leaf to 2.1 ± 1.7% (Fig. 3B). Although the leaf maximum weight gain was achieved, VI did not completely replace the leaf gas fraction with the liquid phase by VI, as can be seen from the small black areas showing pockets of air in Fig. 3B.

3.2. Influence of VI and PEF on the metabolic activity of spinach leaves

Typical raw calorimetric data are shown in Fig. 4. The calorimetric signal was disturbed each time the ampoule was placed in the calorimeter, and when VI or PEF treatment was applied. When the signal returned to the recording range after the application of VI or PEF, a further 2000 s was required for the signal to stabilize.

To enable a comparison between different treatments the thermal power curve was normalized by setting the initial thermal power of the sample to 100, corresponding to an average value of 2078 ± 239 mW kg^{-1}. Typical normalized calorimetric results of the thermal power generated by the untreated leaf, the leaf subjected to VI only and the leaf subjected to both VI and PEF are shown in Fig. 5.

The untreated control sample exhibited a decrease of 78 ± 18% in the thermal power generated during the total treatment time (5.5 h). At 8900 s, the leaves subjected to VI with trehalose exhibited more than twice the thermal power of the untreated leaves at the same time point (137 ± 18% compared with 52 ± 17%). At 15,000 s, samples subjected to VI alone showed approximately 4 times higher thermal power than the non-treated control samples (98 ± 23% compared with 22 ± 8%) ($p < 0.05$), while the leaf samples subjected to the combination of VI and PEF showed more than 6 times higher thermal power than the untreated leaf (149 ± 16 compared with 22 ± 8%) ($p < 0.05$), and 1.5 times higher than the leaf subjected to VI alone (148 ± 16% compared with 99 ± 23%) ($p < 0.05$).

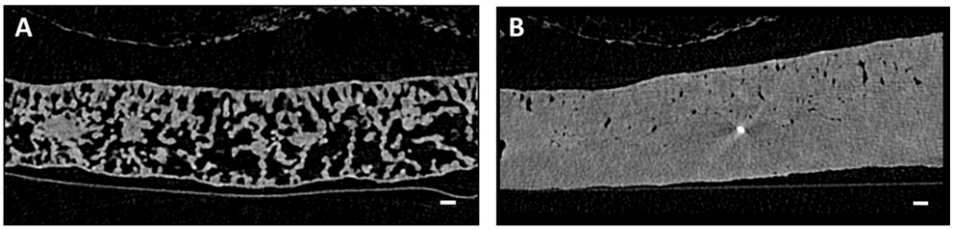

Fig. 3. Typical X-ray microtomography images where grey areas represent the cellular structure and black areas represent air spaces. A. The cross section of an untreated spinach leaf and B. the cross section a spinach leaf vacuum impregnated with isotonic trehalose.

3.3. Influence of VI and PEF on the accumulation of trehalose-6-phosphate

The concentration of T6P in the various leaves is shown in Fig. 6. The concentration in untreated leaves was 0.7 ± 0.1 mmol/kg. After the application of VI, a significant ($p < 0.05$) increase in the concentration of T6P was detected (9.5 ± 0.3 nmol/g). Samples subjected to the combination of VI and PEF did not show a significant ($p < 0.05$) increase in T6P concentration (11.4 ± 3.3 mmol/kg) compared with samples subjected to VI only.

4. Discussion

In this study, we investigated the metabolic response of spinach leaves after the application of VI and PEF. Calorimetric measurements provided evidence that VI with trehalose leads to a significant increase in metabolism. In contrast, the metabolic thermal power of untreated spinach leaves decreased during the time frame of the experiment (Fig. 5), possibly due to changes in the rate of respiratory substrate mobilization or down-regulation or damage to the respiratory pathways [11]. The thermal power of the impregnated samples also decreased over the time scale of the experiments. Since the leaves were exposed to light before the calorimetric measurements, light-enhanced dark respiration may have contributed to the decrease in metabolic activity of the leaf [13], even in the metabolically more active impregnated leaves.

When vacuum is applied to the tissue, the air is removed from the highly interconnected spaces forming the complicated network of tortuous paths and clusters in the tissue [12,21,22]. The flow of the impregnating liquid into the tissue will be strongly influenced by the topology and geometry of this network [19]. In the spinach samples used in these experiments, the porosity was considerably reduced at maximum weight gain; however, the tissue was not completely filled with the liquid. The small pockets of air remaining in this complex cellular network (Fig. 3) may account for the activity of the oxygen-consuming pathways, as demonstrated by our previous study on vacuum impregnated spinach [14], where it was also demonstrated that, under anaerobic conditions, the metabolic activity of the leaf was not stimulated by impregnation with sugar. However, as the porosity is rather low, we cannot rule out localized fermentation in the tissue.

Once the extracellular space is impregnated, trehalose is known to enter the cells through non-specific mannose transporters [18], and this may lead to a high accumulation of T6P, possibly by feedback inhibition of T6P phosphatase, as has been demonstrated when impregnating *Arabidopsis* leaves with trehalose [9]. This accumulation has been found to take place rapidly, within 30 min in *Arabidopsis* [9]; a similar time to that taken to measure the metabolic activity with calorimetry after impregnating the sample (Fig. 5). The question of whether there is a direct link between T6P and increased metabolic activity cannot be answered based on the results of this study. However, it is an attractive subject for further research as it has been demonstrated that in young tissues (as was the case for the baby spinach leaves used in our experiments) T6P inhibits Snf1-related kinase activity, increasing carbon utilization and thus metabolic activity [17].

After being impregnated, the leaves were subjected to reversible electroporation, which further increased their metabolic activity. Cell membrane electroporation and resealing involve much more than structural changes in the lipid matrix. This is a complex metabolic response that may involve several processes, such as energy release by the movement of ionic species, ATP hydrolysis to re-establish charge gradients across the cell membranes, and/or other physiological events taking place after electroporation and long after resealing [4]. The application of PEF did not further increase the concentration of T6P. There is no hard evidence that electroporation of the impregnated tissue leads to the transport of trehalose to the cytoplasm, thus increasing its intracellular concentration. The results of the present study indicate that either trehalose is not further accumulated in the cytosol as the result of PEF, or that any further increase in the concentration of trehalose does not lead to further accumulation of T6P after that induced by VI.

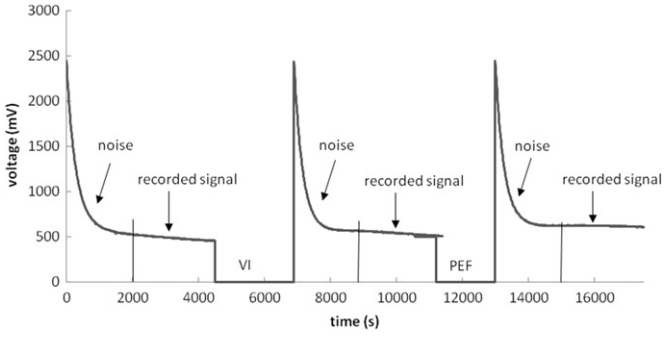

Fig. 4. An example of the raw calorimetric data obtained using the following measurement sequence. The leaf sample was placed in the ampoule for 4500 s, the first 2000 s were needed to stabilize the signal and the signal from the metabolic activity of the sample was recorded for the next 2500 s. The leaf was then removed from the ampoule and VI was applied causing a signal disturbance for 40 min. The leaf was subsequently placed back in the ampoule and the signal was recorded for 4500 s before PEF treatment was applied to the sample inside the ampoule. Again the first 2000 s was needed to stabilize the signal and the metabolic activity of the sample was recorded for the next 2500 s. The PEF treatment caused 20 min disturbance in the signal. The signal was recorded for another 4500 s after PEF treatment. Again the first 2000 s was needed to stabilize the signal and the metabolic activity of the sample was recorded for the next 2500 s.

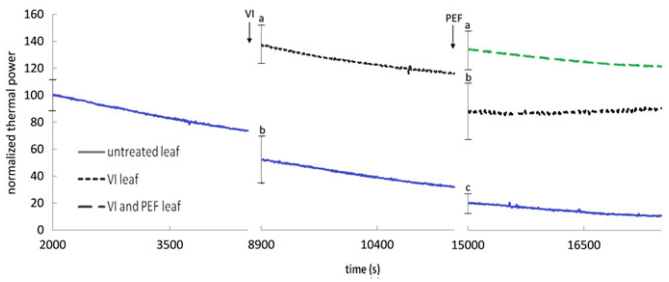

Fig. 5. Typical normalized calorimetric data showing the thermal power generated by the control samples and samples subjected to VI only and to VI and PEF. Error bars represent the standard deviation of three replicate experiments at 2000 s, 8900 s and 15,000 s, which are the first measurement points after VI and PEF. Letters above the error bars indicate statistical differences ($p < 0.05$) between the values at specific time point.

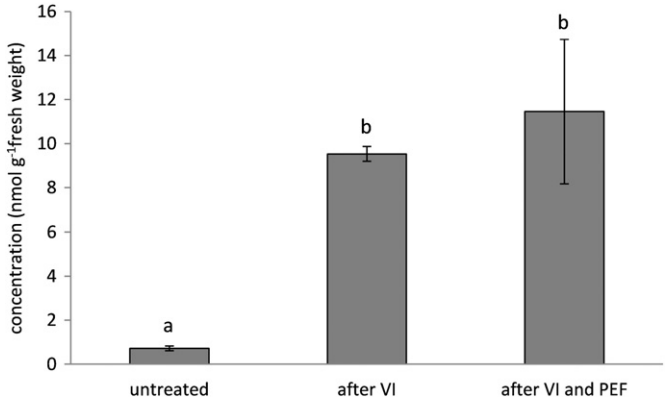

Fig. 6. Concentration of T6P determined in untreated spinach leaves, leaves subjected to VI only and to a combination of VI and PEF. Error bars represent the standard deviations of four replicates. Letters above the error bars indicate statistical differences ($p < 0.05$).

5. Concluding remarks

The following remarks highlight the most important findings.

1) VI leads to a considerable reduction in the porosity of the leaves. However, all the air is not removed at maximum weight gain, which may account for the activity of oxygen-consuming pathways in the cells.
2) VI with trehalose drastically increased the metabolic activity of the leaves. The accumulation of T6P in the cells might lead to an increase in carbon utilization and higher metabolic activity.
3) Subjecting the impregnated leaves to PEF further increases their metabolic activity. This would be expected if cell recovery mechanisms were acting, even if T6P is not involved.

This explorative study provides the basis for further investigations on the accumulation of T6P following VI and its role in defence responses to abiotic stress. Such investigations my provide opportunities to study ways of improving the tolerance of delicate plant tissues to freezing.

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