# PEF-induced cryoprotection of spinach leaves L10

***Katarzyna Dymek, Petr Dejmek, Federico Gomez***
*Lund university*

Duration of the experiments: 90 min
Max. number of participants: 4
Location: Microbiological laboratory
Level: Basic

## PREREQUISITES

Participants should be familiar with Safety rules. No other specific knowledge is required for this laboratory practice.

## THEORETICAL BACKGROUND

There are three main consequences of exposure of plant cells to subzero temperatures such as cellular membrane disintegration, delay of biochemical reactions and lack of available water. Ice crystals are affecting the tissue and cell structure. Trehalose stabilizes the cell membrane and helps to keep its integrity while exposed to subzero temperatures. Using pulsed electric field treatment and vacuum impregnation, trehalose is distributed in the inter- and intracellular spaces and protects the cellular membrane from outside and inside.

The method leading to the protection of plant tissues against freezing injuries consists of two pretreatment steps before freezing is applied: vacuum impregnation and exposure of leaves to pulsed electric field. Vacuum impregnation allows introducing the cryoprotectant in the extracellular spaces of plant tissue while exposure to pulsed electric field treatment is used to reversibly permeabilize cell membrane allowing introducing the cryoprotectant in the intracellular space via reversible permeabilization of cell membranes.

**The aim** of this laboratory practice is to demonstrate how reversible electroporation in combination with vacuum impregnation with a cryoprotectant efficiently protects plant tissues from freezing injuries.

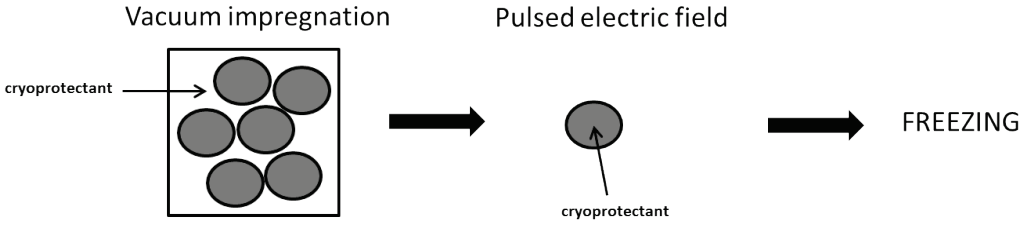


**Figure 1.** Scheme of the process leading to the protection of the plant tissue against the freezing injuries. Vacuum impregnation allows introducing the cryoprotectant into the extracellular spaces of the plant tissue and pulsed electric field is used to introduce the cryoprotectant into the cell interior via pores created reversibly in the cell membrane.

**EXPERIMENT**

We will impregnate spinach leaves with the cryoprotectant (trehalose). The cryoprotectant will be introduced into the leaf's intercellular spaces by vacuum impregnation and into the intracellular spaces by pulsed electric field. After impregnation, the leaf will be frozen and thawed. Leaf's survival will be evaluated by the maintenance of its turgidity. The experiment consists of two pretreatments steps before freezing is applied: vacuum impregnation process and exposure of leaves to pulsed electric field. To achieve vacuum impregnation the leaf is immersed in an aqueous solution of trehalose. The concentration of the solution is isotonic to the leaf, 11% (w/v). The immersed leaf is placed in the chamber and vacuum is slowly applied for around 5 min until the pressure drops to 150 mBar, during this time the air from the extracellular spaces in the leaf tissue is removed. The pressure is kept at constant level of 150 mbar for 1 min and the atmospheric pressure is restored for the next 5 min. During this time the trehalose solution enters the extracellular spaces of the leaf. The volume air fraction of spinach leaf is around 26%, so if the leaf is totally impregnated it increases the weight for around 26%, to evaluate it the leaf is weighted before and after the process.

Electric pulses are applied in a treatment chamber which consists of two parallel plate, electrodes with 5 mm gap in between them. The impregnated sample is placed in the treatment chamber immediately after vacuum impregnation is finished and then pulsed electric field is applied. The protocol which provokes reversible electroporation is as follows: 50 µs (25 µs x 2) bipolar square pulses, 2 ms space between pulses, 300 pulses per train, 4 trains, 10 s between trains, voltage of 600 V and the conductivity of the medium is 250 µS/cm (adjusted with NaCl).

Afterwards the leaf should recover from vacuum impregnation and pulse electric field treatment for 24h at 4oC (in the fridge) in water.

After the resting period, the leaf can be frozen with liquid nitrogen. Liquid nitrogen is used to obtain high freezing rates. The faster the freezing rates, the smaller the ice crystals which are created, which minimizes the injuries provoked by ice crystal expansion. The leaf is immersed in the liquid nitrogen for 1 min to 1 min 15 sec, depending on the leaf size. Thawing is done by immersing the leaf in deionized water at room temperature. Leaf viability can be investigated by wilting test (Figure 2). It is a simple method, which allows evaluating the turgidity of the sample. The leaf is placed on a holder and if turgor is still present in the cells, the leaf will not collapse. The sample will be compared with untreated controls.

**Figure 2.** Wilting test. The leaf which loses turgidity is collapsing.

## FURTHER READING:

Phoon P. Y., Gómez Galindo F., Vicente A., Dejmek P.. Pulsed electric field in combination with vacuum impregnation with trehalose improves the freezing tolerance of spinach leaves. *Journal of Food Engineering* 88: 144-148, 2008.
Cruz R. M. S., Vieira M. C., Silva C. L. M., The response of watercress *(Nasturtium officinale)* to vacuum impregnation: Effect of an antifreeze protein type I. *Journal of Food Engineering* 95: 339-345, 2009.
Pearce R., Plant Freezing and Damage. *Annals of Botany* 87: 417-424, 2001.

## NOTES & RESULTS