Optimization of Pulse Electric Field Treatment and Vacuum Impregnation for Reducing Freezing Injury of Thawed Rucola Leaves

Imelda Christiana Nyoto
Master thesis in Food Engineering 2018
Department of Food Technology, Engineering and Nutrition, Lund University

Supervisors: Federico Gómez and Katarzyna Dymek
Lunds Tekniska Högskola 2018

Abstract

Freezing injuries usually take place in thawed plant-based food products. To reduce freezing injuries, Pulse Electric Field (PEF) treatment and Vacuum Impregnation (VI) of cryoprotectant were used before the freezing process. The aim of PEF is to provoke electroporation to the membranes of the plant cells, thereby allowing the cryoprotective solution impregnated by VI to penetrate the cells.

PEF treatment was optimized to use the minimum possible pulse width in order to obtain a homogeneous electroporation of rucola leaves. In order to achieve this minimum pulse width, several combinations of electric field strengths, pulse numbers, and number of trains were tested. The verification for the electroporation was done first by observing the treated sample with a microscope after it had been stained with Propidium Iodide and then by monitoring the tissue leakage by measuring its conductivity over time. VI treatment was done straight after the PEF treatment and then the sample was stored in a refrigerator to rest before freezing and thawing.

The initial experiment attempted to optimize the PEF parameters, while the second experiment was carried out to determine the optimal concentration of cryoprotectant for VI when combined with an optimized PEF treatment. The leaves were then rested, frozen and thawed before being observed. The initial experiment was done using commercial rucola leaves from Italy. Due to the seasonal availability of commercial rucola leaves, the second experiment was done using rucola leaves planted in the greenhouse at the Biology Department of Lund University.

The results show that the samples treated with PEF and VI with the highest concentration of the cryoprotectant (SC4) had the highest survival after freezing and thawing. This treatment resulted in 88.41 ± 5.23 % of survival 5 min after leaves were taken out from a freezer and decreased to 57.97 ± 5.80 % after 1 hour. The microscopic observations showed that viable cells of frozen and thawed leaves were mostly located near the main veins.

Acknowledgement

My gratitude to my supervisor, Federico Gómez (Lund University) and Katarzyna Dymek (Optifreeze AB) who have never tired of supporting and giving any suggestions for producing this thesis. Hugh of thanks for my examiner Lars Nilsson and my opponent, Panagiotis Manasian for any given input.

Many thanks for my mother, Wartini and my family; my best friends who shared moments: Bobae, Pim, Leo, Uli, Yasmin. Special thanks Andrew especially for watering and accompanying me at night to water my plants at the Greenhouse; my SI family in Lund: Mpi, Tetty, Didi, Ellen and Etha; my proofreader: Victor, Ib, Nabilah, Banne, and Philipp – the little bird; my lab colleague: Amanda and Grant; Friends of Domino, who always gave me support to finish this project.

Special thanks for all staff of Optifreeze AB: Revekka, Stephen, Elena, Ulf, Per, and Eda for continuous support, guidance, training, brainstorming and letting me do my project and using the equipment there.

The greatest thank to you Lord for keeping me on track finishing my thesis. Without your work, I would not be able to finish it.

This thesis has been produced during my scholarship at Lund University, thanks to a Swedish Institute scholarship.

Lund, October 2018
Imelda Christiana Nyoto

Abbreviations and terminology

VI	Vacuum Impregnation
PEF	Pulse Electric Field
PI	Propidium Iodide
FDA	Fluorescein Diacetate
GH	Greenhouse
Recovered	Physical appearance of rucola leaves after resting time which looked like fresh and not collapse
Survival	Viable leaves after freezing and thawing
Dead leaf	Negative control, obtained by freezing and thawing of untreated leaves.

Table of Contents

1. Introduction 1

2. Aims 3

3. Theoretical Background 4

3.1. Rucola 4

3.2. Leaf structure 5

3.3. Freezing and thawing 7

3.4. Pre-treatment strategy for improving freezing tolerance 9

3.4.1. Vacuum Impregnation 9

3.4.2. Glycerol as cryoprotectant 12

3.4.3 Pulse Electric Field 12

4. Materials and methods 14

4.1. Raw material 14

4.1.1. Commercial Rucola Leaves 14

4.1.2. Greenhouse (GH) Rucola Leaves 14

4.2 Characterization of rucola leaves 15

4.3 Treatments 15

4.3.1. PEF treatment 15

4.3.2. Vacuum Impregnation 20

4.3.3. Combination of PEF and VI treatment 21

4.3.4. Resting 21

4.3.5. Freezing and thawing 22

4.4.1. Resistance analysis of thawed leaves 23

4.4.2. Wilting test 23

4.4.3. Viability test - FDA observation 24

4.5. Statistical Analysis 24

4.6. Experimental plan scheme 25

5. Results 26

5.1. Leaves characterization 26

5.2. Optimization of PEF parameters 26

5.2.1. Nuclei staining 26

5.2.2. Resistance measurements 27
5.3. Vacuum impregnation, resting, freezing and thawing 28
5.3.1 Commercial rucola 28
5.3.2. Greenhouse rucola 31
5.4. Influence of the morphology of the leaf on survival 38
6. Discussion 39
6.1. Effect of PEF treatment on conductivity 39
6.2. Effect of treatment on weight loss and recovery 39
6.3. Frozen and thawed survival 40
6.4. FDA observation of viable cells 41
7. Conclusions 42
8. Recommendations and future work 43
References 44

1. Introduction

Rucola has been widely recognized in some parts of the world and became more prevalent in Sweden. It is usually used as a culinary item, primarily as a salad ingredient. However, due to restrictions on the kinds of environments where rucola can be grown, rucola leaves in Sweden are often imported from Mediterranean countries such as Spain and Italy, especially during the winter months. This means that the shelf life of fresh rucola is relatively short since the transportation time has to included. To avoid such issues, freezing is often used as a method for preserving food as well as its nutritional value. However, rucola is primarily consumed as a fresh vegetable, its quality like other plant-based food products greatly decrease after freezing and thawing, especially in terms of texture, colour and taste (Phoon et al., 2007). This problem occurs due to damages to the cell membranes and osmotic dehydration during the freezing process (Pierce, 2001). One of the methods that is widely used for minimalizing freezing injuries is the use of cryoprotectants.

To improve the quality of frozen and thawed products, the cryoprotectant might be infused into the material. Vacuum impregnation can be used to infuse it into the plant tissue (Demir et al., 2018). However, a drawback of using vacuum impregnation is that the cryoprotectant is only infused into the extracellular spaces of the tissue. By applying pulse electric field (PEF) treatment the cell membranes can be electroporated and their permeability increases (Gómez Galindo, 2016). It may enhance mass transfer and allows intracellular penetration (Wu, et al., 2016). Therefore, by using the combination of PEF and VI the cryoprotectant might be distributed in the extra- and intracellular spaces of the tissue. The combination of Pulse Electric Field treatment and Vacuum Impregnation may produce better results in preserving the quality of thawed frozen plant products than only a VI treatment (Galindo et al., 2016).

The impact of PEF and VI on the freezing tolerance of leaves had already been studied. Phoon et al. (2008) observed that spinach leaves had better freezing tolerance when they were treated using a combination of PEF and VI before frozen and thawed. Demir (2012) reported that the order of PEF and VI before freezing and thawing spinach leaves had no significant impact on their survival. It was also reported that most of the viable tissue after being frozen and thawed was located near the centre of the leaves when observed under a microscope with Fluorescence Diacetate staining.

In this thesis, the PEF and VI parameters were both optimized before treatment (freezing and thawing). The effects of the treatment on the survival tissue were also observed over time after the leaves had been thawed.

2. Aims

The aim of this project is to find the optimal PEF and VI parameters for increasing the viability of rucola leaves after freezing and thawing. The first step for reaching this aim is to obtain the PEF protocol with lowest pulse width possible. The second step is to obtain the optimal glycerol concentration of VI protocol when combining it with the optimal PEF protocol. Both steps were evaluated for survival after freezing and thawing.

3. Theoretical Background

3.1. Rucola

Rocket salad (also called Rucola in Italian) has become a trend in gastronomy nowadays due to its special pungent taste, texture and aroma. It commonly appears in mixed salads. Its popularity is boosted with their nutritional benefit such as high content of folic acid, iron and vitamin C (Table 1). Beyond its culinary use, rucola is also used in medicine (Padulosi et al., 1997). Rucola plant is from the *Brassicaceae* family. There are two common species under the same commercial name, *Diplotaxis tenuifolia*, which is grown as a perennial plant, and *Eruca sativa*, which is grown in annual crops (Hall et al., 2012).

Rucola is rapidly growing, can be re-harvested (harvested about 20 - 60 days after sprouting) and is resistant to high temperature. Rucola mostly grows in Mediterranean countries and is susceptible to cold temperature. At low temperature, about 5^{o}C with over watering, the leaves will turn reddish and grow poorly (Padulosi et al., 1997). Thus, in cold temperate zones (like Sweden), rucola is mostly grown in greenhouses to meet demands. However, rucola leaves grow relatively slow in greenhouses. To meet market demands, fresh rucola is imported from countries such as Italy and Spain. As it is used in salads and as pizza topping, rucola is generally demanded as a fresh product (Dolezalova et al., 2013). Therefore, rucola is usually sold as a fresh cut vegetable or mixed with other vegetables for salad mixes.

Table 1. Nutritional value of 100 g of rucola (Livsmedelsverket, 2018)

Nutrient (Unit)	Arugula Rocket salad raw
Carbohydrate (g)	1.99
Fat (g)	0.66
Protein (g)	2.58
Fiber (g)	1.66
Sum of saturated fatty acids (g)	0.09
Sum of monosaturated fatty acids (g)	0.05
Sum of polysaturated fatty acids (g)	0.32
Vitamin C (mg)	15

Folate (µg)	97
Iron (mg)	1.46

3.2. Leaf structure

The leaf structure is illustrated in Figure 1. On both surfaces of the leaf consist of epidermal tissue that is tightly packed and flat. Often, a waxy layer is present on the outer space of epidermal tissue, called cuticle. There are stomata, as gas transporting aperture, on both sides of the epidermal tissue. Below the epidermal tissue the mesophyll tissue is located, which primarily consists of two zones: The first zone is the palisade zone, which consists of pillar-shaped cells under the epidermis layer (Taiz et al., 2010c). The second zone is porous mesophyll, which consists of asymmetrical cell structures that provide empty spaces in the structure of the leaf. Vascular tissue is also present in the leaf structure as transporting tissue. Connected from the roots and stems, it functions as ribbing structure (Dickison, 2000a).

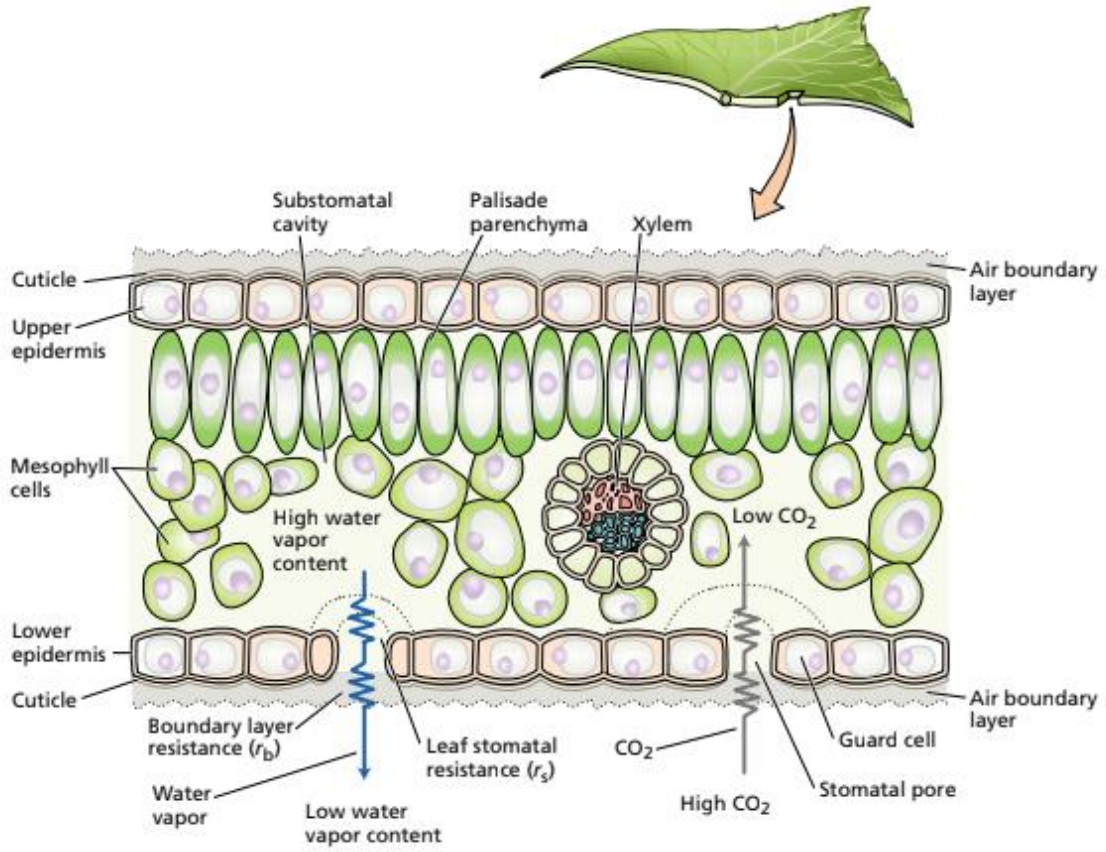


Figure 1. Structural anatomy of the rucola leaf (From Taiz et al., 2010b)

The plant cell has a double layer of cell walls that are glued together by a section called the middle lamella (Figure 2). These layers comprise the outer layer of the cell. There is a membrane that packs the liquid and organelles inside the cell called plasma membrane. This membrane consists of a double layer of phospholipids and controls the elasticity of the cell and the permeability of molecular transport. The cell organelles and cytoplasm are located inside the membrane. The vacuole constitutes a large part of a cell and it could occupy 99% of the cell area. It contains liquid and stores products from the metabolic activities of the cell organelles. The vacuole is wrapped by a membrane called tonoplast, which transports materials to be to the vacuole. This transportation mechanism produces turgor pressure that plays an important role for plant food texture (Dickison, 2000b).

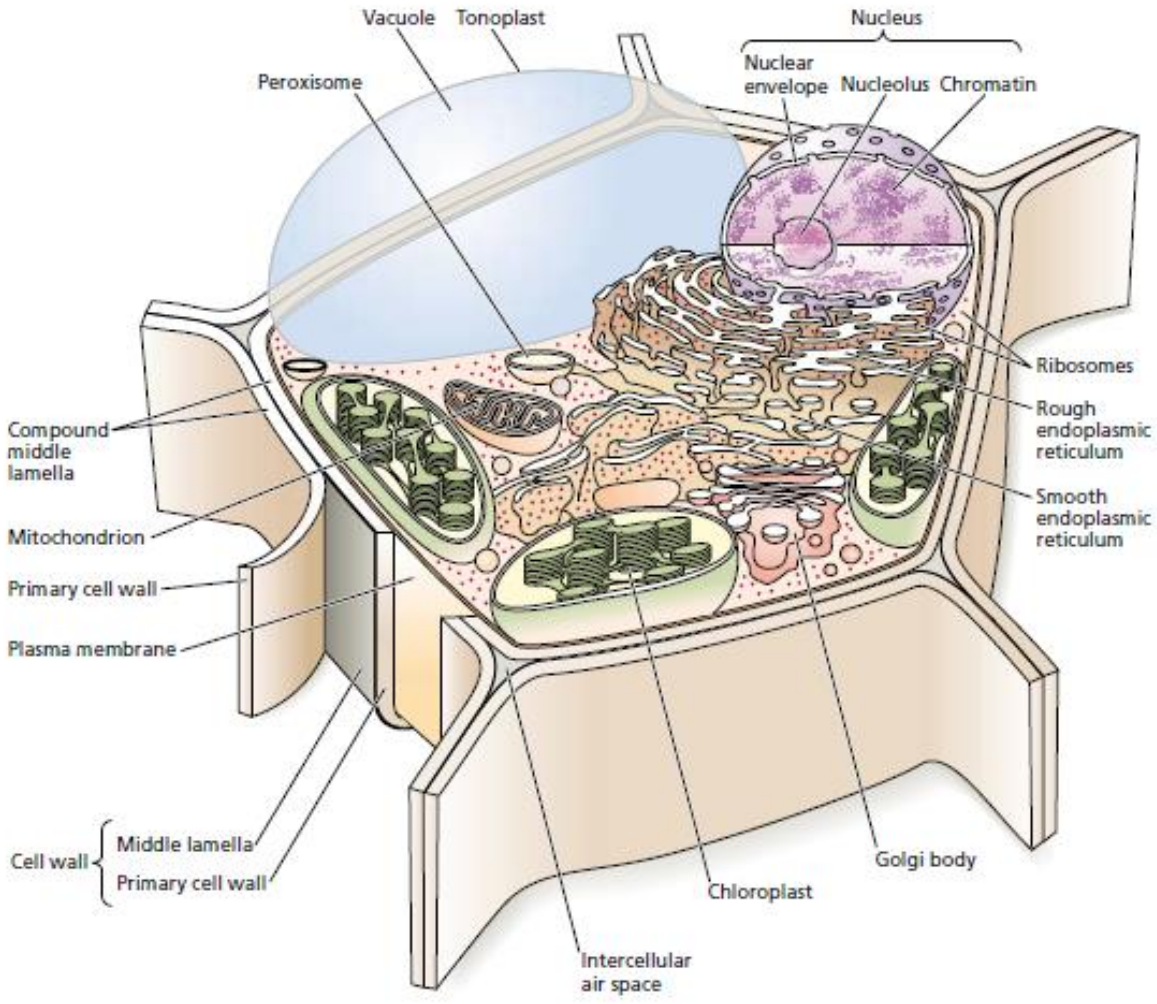


Figure 2. Structure of a plant cell (From Taiz et al., 2010a)

3.3. Freezing and thawing

The shelf life of a product is the most crucial point to consider during food preservation. Shelf life is the period of time when the food is acceptable to consume, usually in terms of quality and safety. One method for food preservation is called inhibition, which aims to prevent the chemical and microbial deterioration of the food. Freezing is one inhibition technique, where the temperature is reduced to convert water into ice. The operation is usually conducted at temperatures below -18^{o}C (Ahmed et al., 2012).

During freezing, plant-based food usually suffers cell damage. An illustration of a freezing process is provided in Figure 3. The plant cell contains solutes at isotonic concentration between inside and outside cell (Figure 3A). Freezing injury is started when the phospholipid plasma membrane solidifies as the temperature falls, affecting the permeability of the cells (Taiz et al., 2010c). If the freezing rate is slow, the extracellular water starts to freeze and produce ice crystals (see Figure 3B). The extracellular ice nucleation produces an osmotic pressure imbalance due to different solute – water concentrations between the extracellular and the intracellular space as a compensation of ice formation. The imbalance of osmotic pressure produces water movement from inside of the cell to the outside of the cells. This dehydration process continues and causes the cell to become deformed (Figure 3C). As the extracellular ice grows, the large ice crystal ruptures the cell membrane, which generates even faster dehydration and leakage of the solutes (Pearce, 2001; Xin et all, 2000; Muldrew et all, 1990). In the other way, when the freezing rate is fast, the temperature drops at very quick time, at this rate, no chance for the dehydration process of intracellular fluid to be occurred (Galindo et al., 2016).

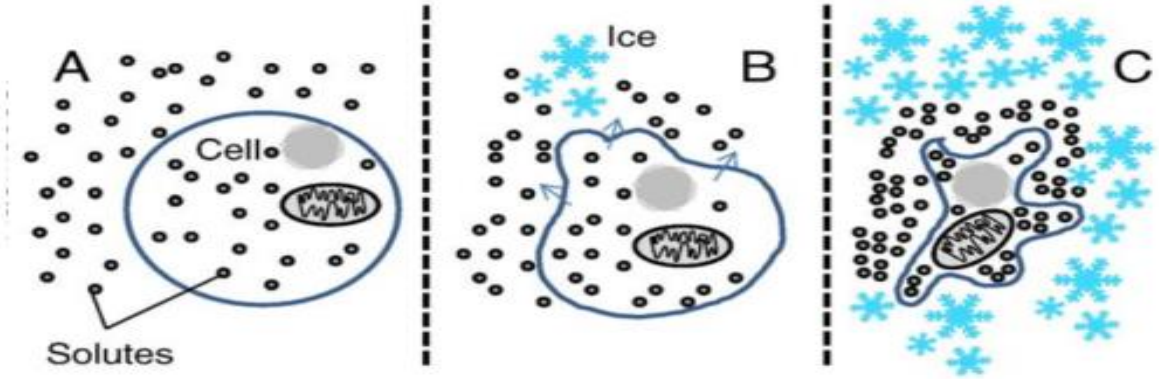


Figure 3. Mechanism of cell freezing; (A) the cell's condition before freezing, (B) movement of intracellular water due to osmotic imbalance caused by different solute concentration as compensation of extracellular ice formation, (C) deformation of the cell due to loss of intracellular water (from Anon, 2016).

Due to the ruptured cell membranes, the plant would not look fresh when thawed. This is due to the fluid leakage from the intracellular to the extracellular space caused by the ruptured membrane. As the damage is permanent, the fluid cannot return to the cell to re-establish the original structure (Hui et al., 2016).

When frozen products are stored, recrystallization often occurs. The main reason of this problem is temperature fluctuations. If the storage temperature is increased, smaller ice crystals will melt. If the storage temperature is then decreased, the water generated from the melted ice will freeze on the surface of large ice crystals and increase the average crystal size (Petzold et al., 2009). This may induce worse freezing injuries and produce an unwanted icy texture.

3.3.1 Evaluation of tissue damage upon freezing and thawing

To evaluate the effect of freezing injuries on plants quantitatively, conductivity measurements can be carried out. As the cytoplasm and vacuole contain large number of ions, and cell leakage occurs because of freezing injuries, therefore, conductivity measurements could describe the actual condition of the cell damage (Hincha et all, 2014). Another method used to evaluate electro-physical effects is measuring the impedance of the biological sample. Membranes of a viable cell could be regarded as capacitors and the plasma and liquids inside the cell are similar to resistors. These combinations form a resistive-capacitive unit that could be measured as the impedance (unit: Ω) (Donsi et al., 2010). In this thesis, resistance (unit: Ω) is used instead of the impedance to calculate conductivity based on the equations below:

$$R = \rho \frac{l}{A} \qquad \text{Equation 1}$$

$$\text{And } \sigma = \frac{1}{\rho} \qquad \text{Equation 2}$$

With R = resistance (Ω); ρ = electrical resistivity (Ωm); l = length of measured system (m); A = area of measured system (m2); σ = conductivity (S/m) (En.wikipedia.org, 2018).

3.4. Pre-treatment strategy for improving freezing tolerance

In order to reduce freezing injuries, cryoprotectants are usually used. A cryoprotectant is a chemical compound that is used to regulate solute concentration of cells, which thus can help the preventing osmotic dehydration occurring during the freezing injury process (Day et al., 2007). One method for introducing substances into food products is vacuum impregnation (VI). VI is known to be more efficient than the common process of mass transfer diffusion at atmospheric pressure (Hui et al., 2016). PEF is often used in combination with VI, to help the cryoprotectant transported to the intracellular area (Galindo et al., 2016).

3.4.1. Vacuum Impregnation

Vacuum Impregnation is a method for infusing liquids to porous structures such as plant tissues. The procedure consists of placing the porous material in a solution while simultaneously also changing pressure levels. The initial step is to reduce the pressure of the system filled with an immersed porous product. By decreasing the pressure, an expansion of the gas volume that is present at the product forces the gas to leave the object. The next step is to increase the pressure levels back to atmospheric pressure, which allows the solution to replace the volume that was left by the gas (Fito et al., 2001).

A schematic of the impregnation phenomena is shown in Figure 4. There are two main events in the VI process: HDM (hydrodynamic mechanism) and DRP (deformation-relaxation phenomena) (Hui et al., 2016). HDM is the process of transporting material in to and out of the porous material by vacuum diffusion (VD) and natural diffusion (ND) by changing the pressure levels. DRP denotes the structural transformation of the product and its pores as an effect of the changes of pressure and HDM. The VI process is described as follows:

At t = 0, the porous material is submerged in the solution. The process has not started and the value of pi (internal pressure of the pore) and pe (external pressure) is equal to pat (atmospheric pressure). At this state, vg (pore volume) is 1.

At $0 < t < t'$ (step 1 – A), at the initial stage, p1 (vacuum pressure) = pe, but pi > pe, which induces an expansion of the gas volume (Xc1). Due to pressure differences, the gas leaves the pore. This process proceeds until equilibrium is reached. Equilibrium ($t = t'$), is reached pi = pe < pat, gas movement stops, (vg1A = 1 + Xc1).

At $t' < t \leq t1$ (step 1 – B), no more gas flows and capillarity pressure (pc) is induced. This produces mass transfer (Xv1) from the solution medium to the pore (HDM commences). Equilibrium is reached when pi = pe + pc = p1 + pc, then vg1B = 1 + Xc – Xv1.

At $t1 < t \leq t2$, the vacuum pressure (p2) is returned to the atmospheric pressure (pat). As pressure increases, pi is lower than pe, producing more capillarity pressure (pc) due to the pressure difference. This condition induces more liquid transfer from the solution to the pore (Xv). An increase in pressure may also produce compression of the gas volume of the pore (Xc). The equilibrium state can be formalized as vg2 = 1 + Xc – Xv.

Frequently during VI process, the impregnated solution firstly fills porous spaces, such as the extracellular spaces of the tissue. The solution will enter the cell due to the changes in pressure and the concentration difference inside and outside of the cell. In a hypertonic condition, the liquid from the cells will flow outside and cause a loss of water. Severe water loss will disturb the permeability of plasma membrane and may kill the cell (Hui et al., 2016).

VI has been studied for many purposes. By infusing different solutions to porous food products, the quality of the food product can be improved. Impregnation with cryoprotectant to avoid or reduce freezing injuries and impregnation with vitamins and minerals to improve nutritional values and health benefit are relevant examples. VI could also be used for other processes as well, for instance impregnating with inhibitors of enzymatic activity, which could prolong the shelf life of cut fruits and vegetables (Betoret et al., 2016). VI also could reduce nitrate content of baby spinach leaves by infusing sugar solution (Yusof et al., 2016).

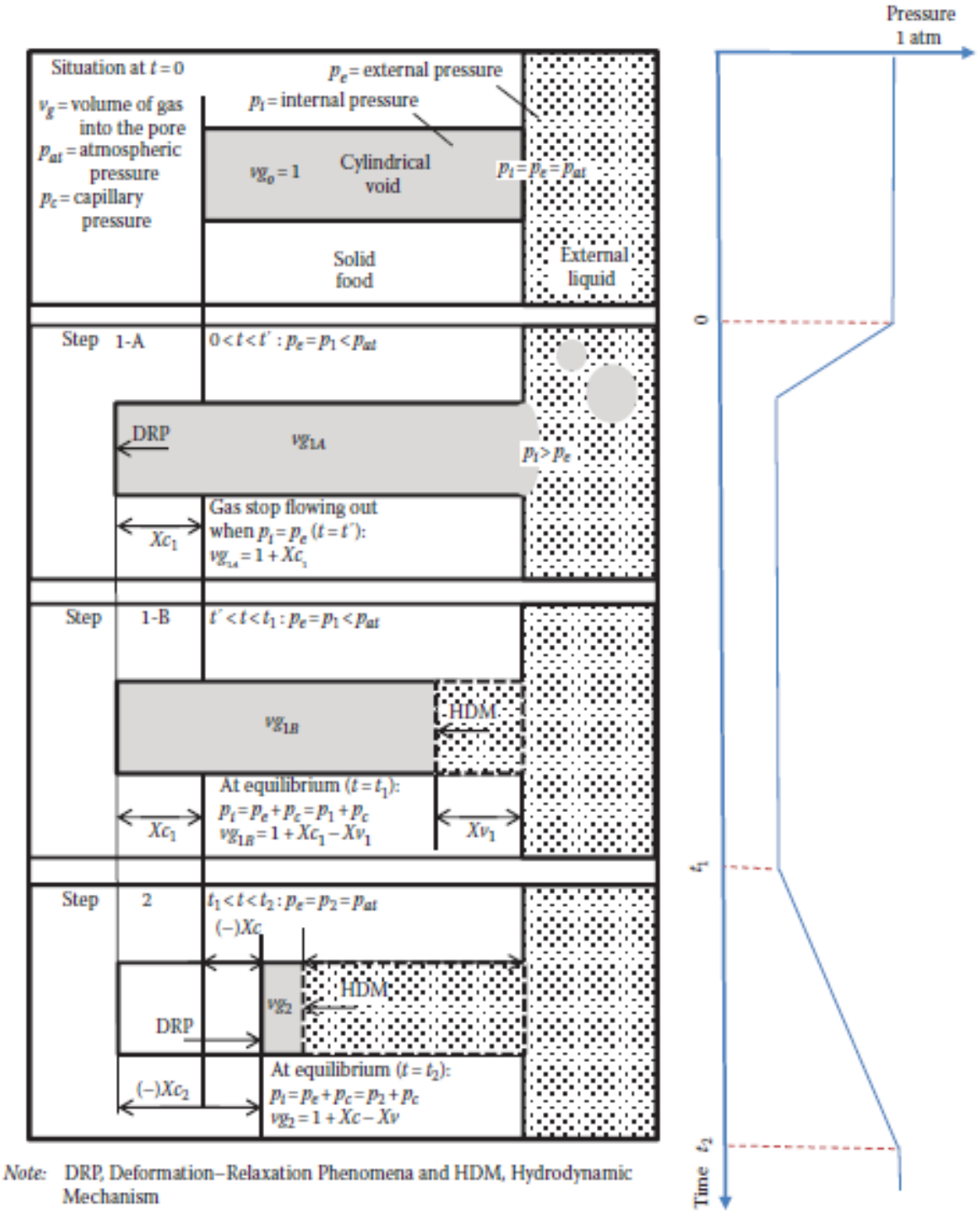


Figure 4. VI design, volume deformation and phenomena from pressure and time interaction (From Hui et al., 2016)

3.4.2. Glycerol as cryoprotectant

Studies of plants naturally surviving winter promoted the idea of freezing plant-based food. It was observed that in specific plant tissues, some substances (such as sugars and antifreeze proteins) were accumulated in their cells during the end of fall season (Velickova et al., 2013). This indicates that these substances have important roles as cryoprotective agents. Later studies outlined some characteristics are needed for a compound to be qualified as a cryoprotective agent: It needs to have a high solubility in water, be easily permeate inside cells and have a low perniciousness as it needs to be used at high concentration (Day et al., 2007).

Glycerol has permeability towards cell membranes, which allows it to be transported inside the cells. The presence of glycerol in the cells increases solute concentrations and prevents the withdrawal of the water to extracellular spaces. Other advantages of using glycerol as cryoprotectant is that the hydrophilic group of this poly-alcohol could establish a firm interaction with the polar head of lipids as material of the cell membrane. It will therefore reduce the deformation of plasma membranes during freezing processes (FAO, 2012.; Day et al. 2007).

3.4.3. Pulse Electric Field

Pulse Electric Field (PEF) has been used for more than five decades and its initial commercial purpose was to eliminate microorganisms (Donsi et al., 2010). The use of PEF is expanding to other fields and lately it has been used to intensify mass transfer process involving living cells. PEF is commonly used for drying, extraction and freezing (Gómez Galindo, 2016).

The detailed mechanisms behind PEF are still being investigated. However, the experimental studies have shown that when an electrical force is applied to an intact cell, the cell membrane is charged, and the electric membrane potential is increased. If an electric force (E) is applied and it is lower than the critical potential of the membrane (Ec), the force has no effect on the membrane. In the contrary, if $E > Ec$, the membrane will be disturbed by the electric force and it will provoke an electroporation on the membrane (Figure 5). This electroporation can be fatal for the cell, depending on the electric strength applied (Donsi et al., 2010).

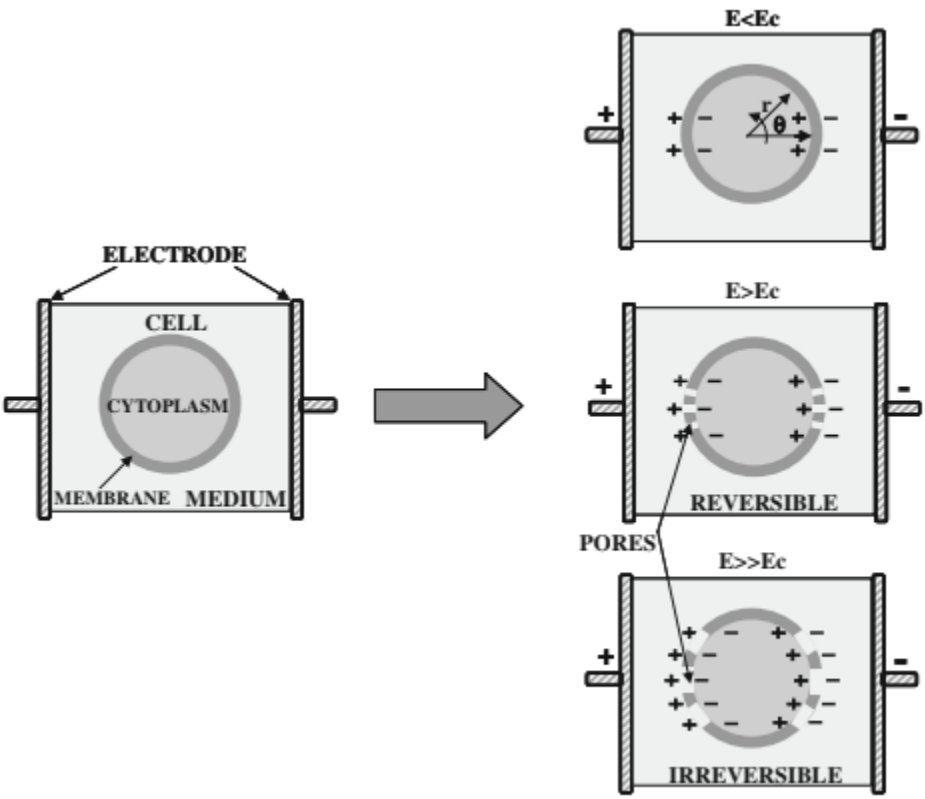


Figure 5. Mechanism behind PEF (from Donsi et al., 2010)

In increasing viability of cells as result of freezing and thawing, reversible permeability of PEF electroporation in combination with VI is aimed (Gómez Galindo, 2016). PEF could increase the amount of impregnated cryoprotectant inside the cell, which may result in a more effective cryoprotection (Phoon et al., 2007). However, the reversibility of electroporation is dependent on several factors, such as type of polarization, electric field strength, pulse shape, pulse number, pulse width and pulse space (De Vito et al., 2007; Gómez Galindo, 2016).

4. Materials and methods

4.1. Raw material

4.1.1. Commercial Rucola Leaves

The commercial rucola leaves were obtained from Cater Grönt, a local supplier in Malmö. The leaves were transported from Italy and arrived at the distributor warehouse (Everfresh AB, Sweden) in 4 days. They were stored at 2 – 6 ^{o}C, then washed and packed by the distributor within 4 days after the arrival day (Cater Grönt, 2018). Packages of 500 g with a shelf life of 9 days were distributed to the suppliers and to our laboratory on the same day they were packed.

In our laboratory, the leaves were stored immediately at 4^{o}C (± 2^{o}C). To control the quality of the experiment, the age of the leaves used in experiment were set to 4 - 6 days before their expiration date stated on the package.

4.1.2. Greenhouse (GH) Rucola Leaves

The leaves were planted in a greenhouse in the Biology Department of Lund University, using a growing medium that consisted of plant soil mixed with clay ("Krukväxtjord med Lera & Kisel" from Weibulls). The medium was mixed with expanded perlite with volume composition of medium: perlite as 90:10. The rucola seeds (Diplotaxis tenufoila sp) were bought from Enza Zaden under market name PRUDENZIA F1. The seeds were planted in 56 x 25 x 6 cm (L x W x H) trays (with perforation for drainage) with 2.5 kg mixed growing medium per tray, with 7 x 3 planting point holes per tray and 4 – 8 seeds per hole. The plants were watered daily with about 2 litres for three trays. A fertilizer (Kristalon, Yara Vlaardingen B.V. Netherlands) with the composition N:P:K = 14 : 9 : 25 + 3.3 MgO and traces of other minerals was used. 7 grams for every litre of water was used. Two litres of fertilizer for three trays were added once every two weeks. The greenhouse was maintained at 16-18^{o}C and 45-55 % RH and the leaves were harvested after 5 - 8 weeks. The harvested leaves were kept in a humid box, stored in a refrigerated room at 4^{o}C ± 2^{o}C and used the same day as they were harvested. The leaves were washed with tap water before used in the experiments.

4.2. Characterization of rucola leaves

Both commercial and GH rucola (20 leaves each) were measured using a calliper for their length and width and a micrometer for their thickness. Only the blades of the leaves were measured, excluding the petiole (see Figure 6). The thickness of the leaves was only measured on the commercial rucola.

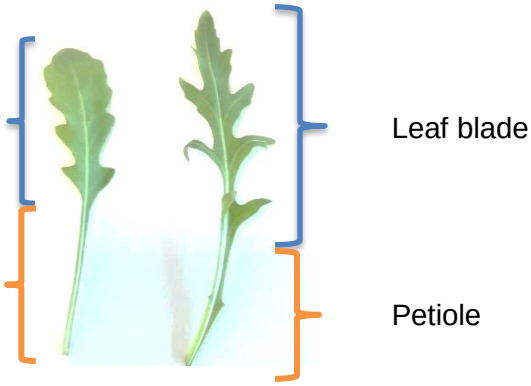


Figure 6. Illustration of rucola leaves. Only the leaf blades were measured.

During the growth of the rucola plants in the green house, the morphology of the leaves was identified and classified according to its morphology's group. Each group was treated as specified below:

4.3. Treatments

The treatment was conducted as described in the scheme below:

PEF → VI → resting → freezing (overnight, 16 to 24 hours) → thawing

Each step in the procedure will be explained in further detail below.

4.3.1. PEF treatment

Electric pulses were applied with a CEPT pulse generator (Arc Aroma Pure AB, Lund, Sweden). The PEF parameters were controlled using software (ARC CEPT HM13). Pulses were delivered by placing the samples between a pair of stainless steel electrodes with a 1.6 cm gap between them. The sample placed between the electrodes was immersed in a medium with 250 µS conductivity (made by diluting NaCl in deionized water). The conductivity of the medium was measured using a conductivity meter (Eutech Instruments, US). The electric pulses given were monitored using a handheld oscilloscope (HANTEX, China). Twenty-five leaves were treated in each batch.

4.3.1.1. Optimization of the PEF treatment

During this experiment, the PEF parameters were varied with the aim of finding a homogenous electroporation using the lowest pulse width possible. Several values for pulse field strength, pulse number and pulse train were tested as shown in Figure 7.

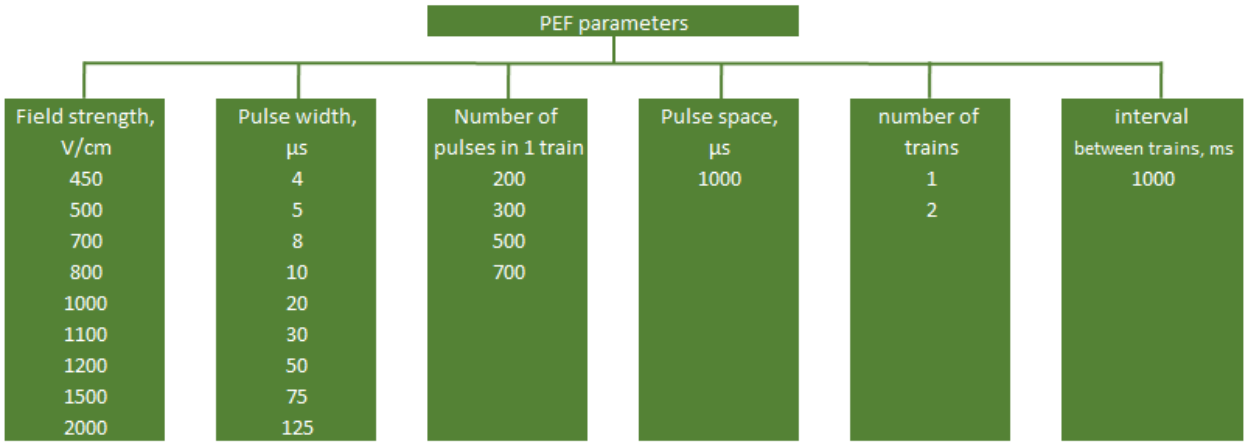


Figure 7. PEF parameters tested in experiments

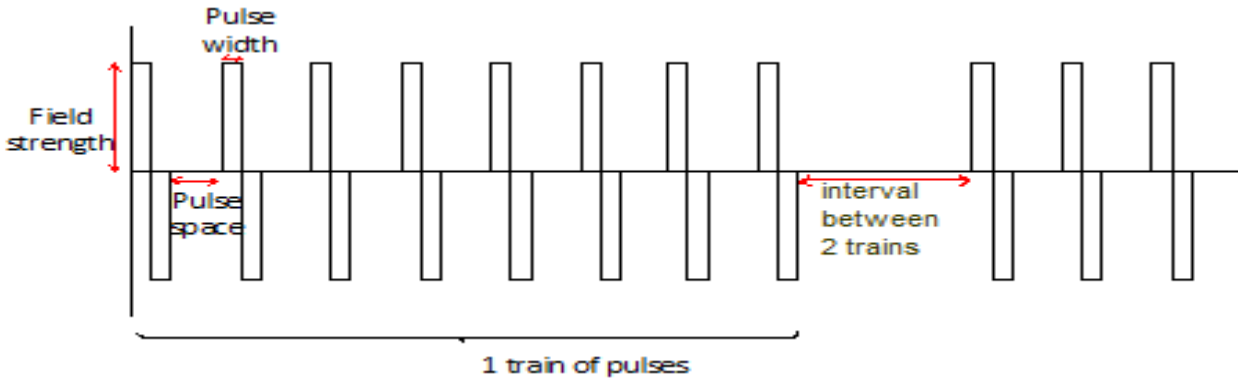


Figure 8. Schematic for the applied PEF parameters

A schematic of the applied PEF parameters is given in Figure 8. A detailed account of how the PEF results were evaluated is presented in the following sections.

4.3.1.2. Influence of raw material on PEF parameters

The preliminary experiments in this thesis for determining the PEF parameters with lowest pulse width possible was performed using commercial leaves. This result was then purposed to be used for the GH rucola. According to Gómez Galindo (2016) and Gómez Galindo et al. (2017),

homogeneous electroporation is very much dependent on the plant tissue and its structure, including factors such as cell size, wax layer and the air ratio of the plant tissue. Therefore, the optimal PEF parameters used for the commercial rucola might not be the optimal one for the GH rucola. Therefore, another optimization of the PEF parameter was done using the GH rucola with the same procedure, by using the same lowest pulse width obtained from the commercial leaves.

4.3.1.3. Microscope observation for electroporation evaluation

The homogeneous electroporation of the leaf surface, which has been reported as good indicator of the electroporation of the whole leaf at voltages higher than 100 V/cm (Dymek et al., 2015), was observed by observing stained nuclei under the microscope. 250µM Propidium Iodide (PI) solution was used as the electroporation media to stain the nuclei. NaCl was added to the solution to obtain 250µS of conductivity. The electric pulse was applied in a dark chamber because of its sensitivity to light in PI solutions. After PEF treatment, the leaves were observed under a microscope (MMT, MILMEDTEK AB, Sweden) using a filter that generates yellow light (maximum excitation of PI obtained at 535 nm wave length, Thermo Fischer Scientific, 2018) and with a 10X 1/0.3 Plan fluor bottom lens magnification. Three repetitions were done to determine the extent of the electroporation.

4.3.1.4. Electrolyte Leakage analysis (Conductivity measurements)

A dead cell usually indicated by the leakage of intracellular fluid due to the cell membrane damaged while an intact cell will not produce any leakage. This leakage often observed by measuring the tissue's electrical resistance.

The resistance of the leaves was measured using a 4192A LF IMPEDANCE ANALYZER (Hewlett Packard, US) at frequencies of 1, 10 and 100 kHz. The electrodes were made from stainless steel with an area of measurement of 9 mm x 5 mm (Figure 9a), filter papers MN 713 (MACHEREY NAGEL, Germany) with thickness 0.28 mm covered the electrodes (Figure 9b). They were wetted using 10 µl of conductive solution (Figure 9c). The conductive solution was made using a deionized water mixed with NaCl to achieve 250µS of conductivity. The leaf was then placed on the wetted filter paper (Figure 9d) between the electrodes and clamped (Figure 9e). The measurement was not done in the whole leaf but in a portion of it, illustrated in Figure 10. The leaf was then placed in a humidified styrofoam box and connected with cables to an impedance

analyzer (Figure 9f). The box contained 7.5 grams of wet tissue to create saturated atmosphere. The complete setup can be seen in Figure 9g. The resistance measurement was taken 5 minutes after the sample was attached to the analyzer. Fresh, untreated leaves were used as a measurement for intact cells while frozen-and-thawed untreated leaves were used for measuring dead cells.

The cells behaved differently while exposed with electrical current at different frequencies. At lower frequencies, cell membranes behave as capacitors, absorbing the electrical current. At higher frequencies, the cell membranes became less resistive, but at very high frequencies, they act like they had no resistance at all. Therefore, the measurement might not detect intact or dead cell (Donsi et al., 2010). Therefore, the frequency of the measurement must be selected for describing the actual condition of observation.

By plotting resistance vs frequency, the frequency used for the measurements was selected as the greatest resistance difference between intact and dead cells at the lowest possible frequency, avoiding the plateau (constant part) of the curve (Zimmermann, 1982). The measured resistance was then used for calculating the **conductivity** of the sample using equations 1 and 2. The length of the measured system was calculated with l=2 $x\ df\ +\ dl$

Equation 3 below:

$$l = 2\ x\ df\ +\ dl \qquad \textit{Equation 3}$$

With l = length of measured system (mm), df = thickness of the filter paper (mm), dl = thickness of the leaf (mm).

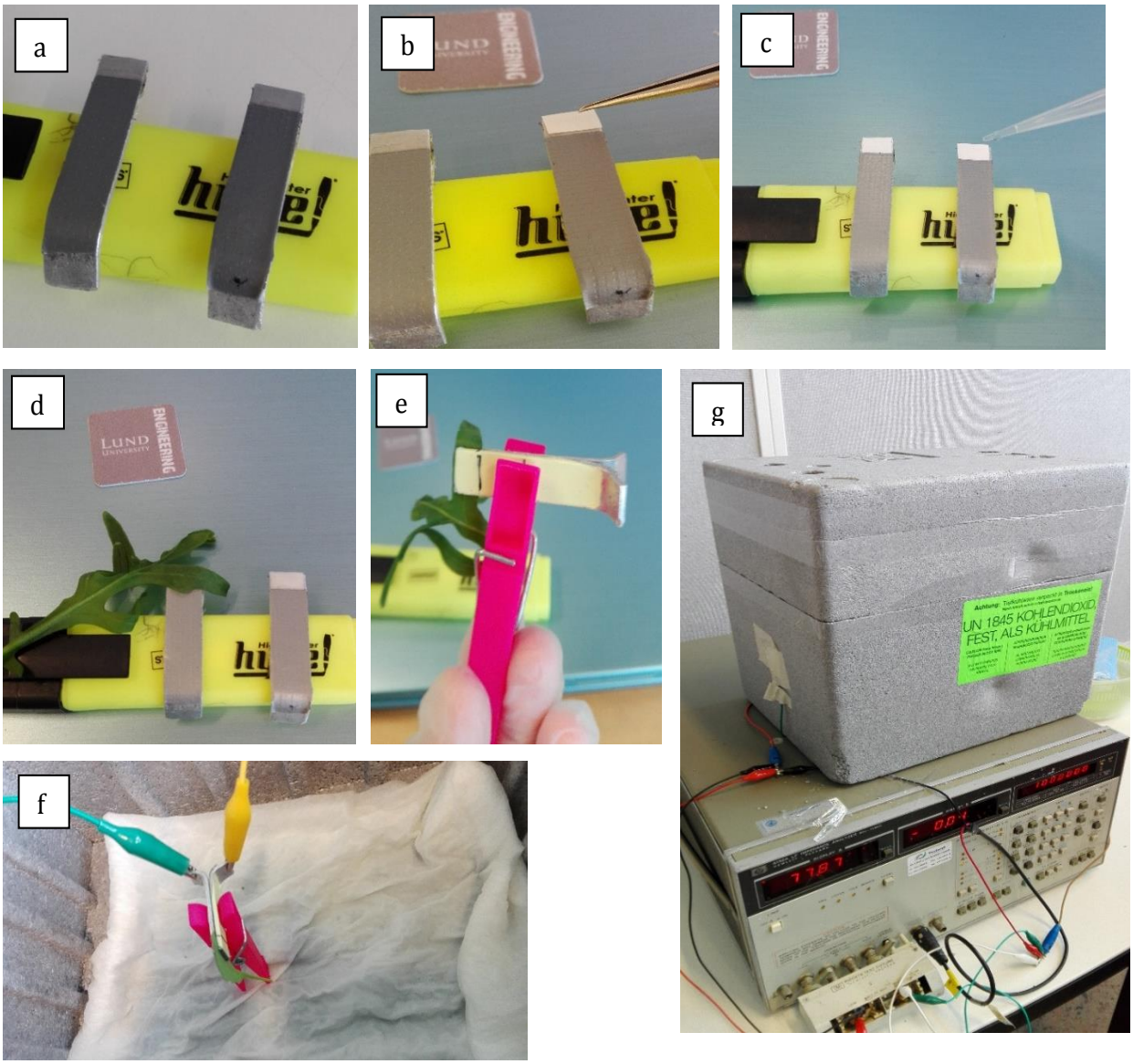


Figure 9. Setup for resistance measurements; (a) Electrode preparation, (b) filter papers were placed on the electrode, (c) the filter papers were wetted using conductive solution, (d) leaf sample settlement, (e) the sample was clamped before measurement, (f) the clamped sample was placed inside a humidified box and connected to the machine through cables, (g) complete setup.

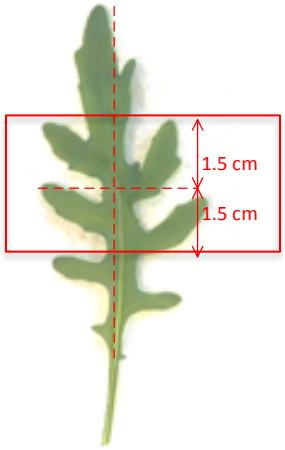


Figure 10. Tissue resistance area measured on the leaves as indicated by the red box.

4.3.2. Vacuum Impregnation

After the PEF treatment, the leaves were immediately immersed in the cryoprotectant solution (glycerol hypertonic solutions) and placed into a chamber for the vacuum impregnation process. The vacuum impregnation process was done with a minimum pressure of 90 mbar, with 10 minutes of falling pressure, 1 minute of holding pressure and 15 minutes of raising pressure, as illustrated in Figure 11. The weight change of the leaves after the vacuum impregnation was evaluated by weighing 5 leaves before and after the VI process.

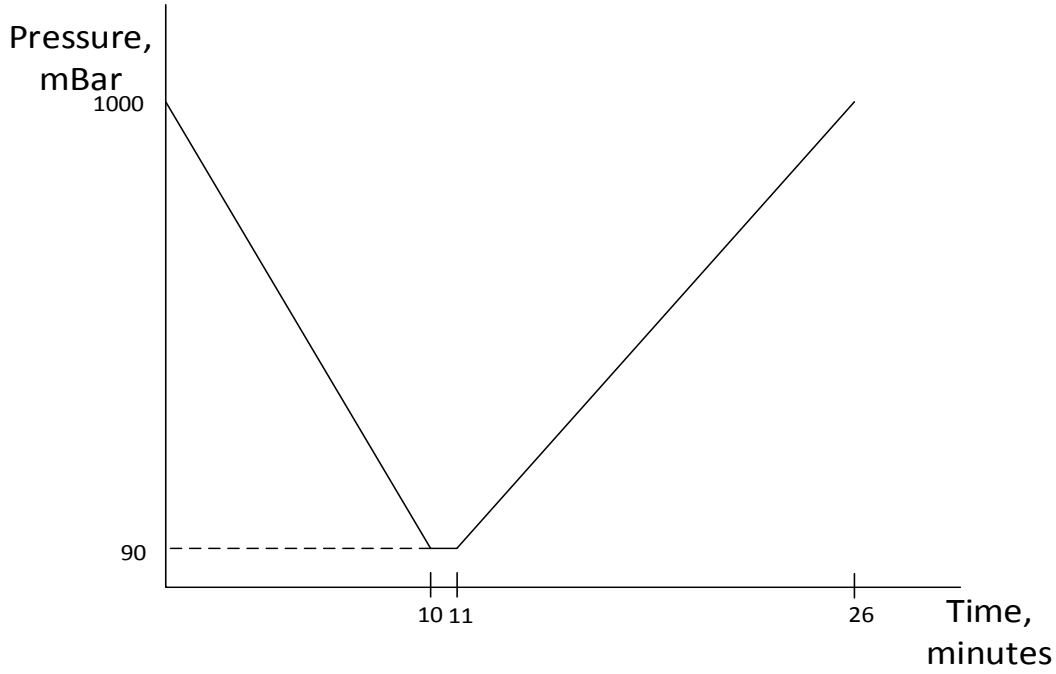


Figure 11. Vacuum impregnation protocol

4.3.3. Combination of PEF and VI treatment

The optimal PEF parameters obtained from the trials were then subjected to VI (Figure 10) with different concentrations of glycerol, as specified below, before resting, freezing and thawing. The glycerol concentrations are not reported due to industrial confidentiality. In each of the tests, the concentration of each cryoprotectant solution was increased in 4 % w/v. The combinations were also compared to treatments consisting only of VI, as described below.

- SC 1: Optimal PEF parameter + VI glycerol hypertonic solution 1 + resting + freezing + thawing
- SC 2: Optimal PEF parameter + VI glycerol hypertonic solution 2 + resting + freezing + thawing
- SC 3: Optimal PEF parameter + VI glycerol hypertonic solution 3 + resting + freezing + thawing
- SC 4: Optimal PEF parameter + VI glycerol hypertonic solution 4 + resting + freezing + thawing
- SC 5: VI glycerol hypertonic solution 1 + resting + freezing + thawing
- SC 6: VI glycerol hypertonic solution 2 + resting + freezing + thawing
- SC 7: VI glycerol hypertonic solution 3 + resting + freezing + thawing
- SC 8: VI glycerol hypertonic solution 4 + resting + freezing + thawing

4.3.4. Resting

After VI, the samples were stored in a plastic container (size 24 cm x 17 cm x 5.5 cm – 2.1L) for resting. Four layers of tissue paper (5 g) were placed at the bottom of the plastic container and 8.75 g of water was poured on it. A net was placed on the wet tissue to avoid direct contact between the samples and the wet tissue (*Figure 12*). The plastic container was then stored in a refrigerator under 4 ^{o}C (± 2 ^{o}C). Storage conditions were monitored using a USB temperature and humidity data logger (Clas Ohlson, Sweden). The humidity levels were monitored to be in the range of 94 – 99.9 % RH while the temperature was in the range of 3.2 – 5.6 ^{o}C. The resting process duration will not be reported due to confidentiality. The recovered leaves were then separated from those that did not recover. The physical appearance of the leaves was used to determine which of the

leaves had successfully recovered. The recovered leaves must appear fresh and cannot have a collapsed structure.

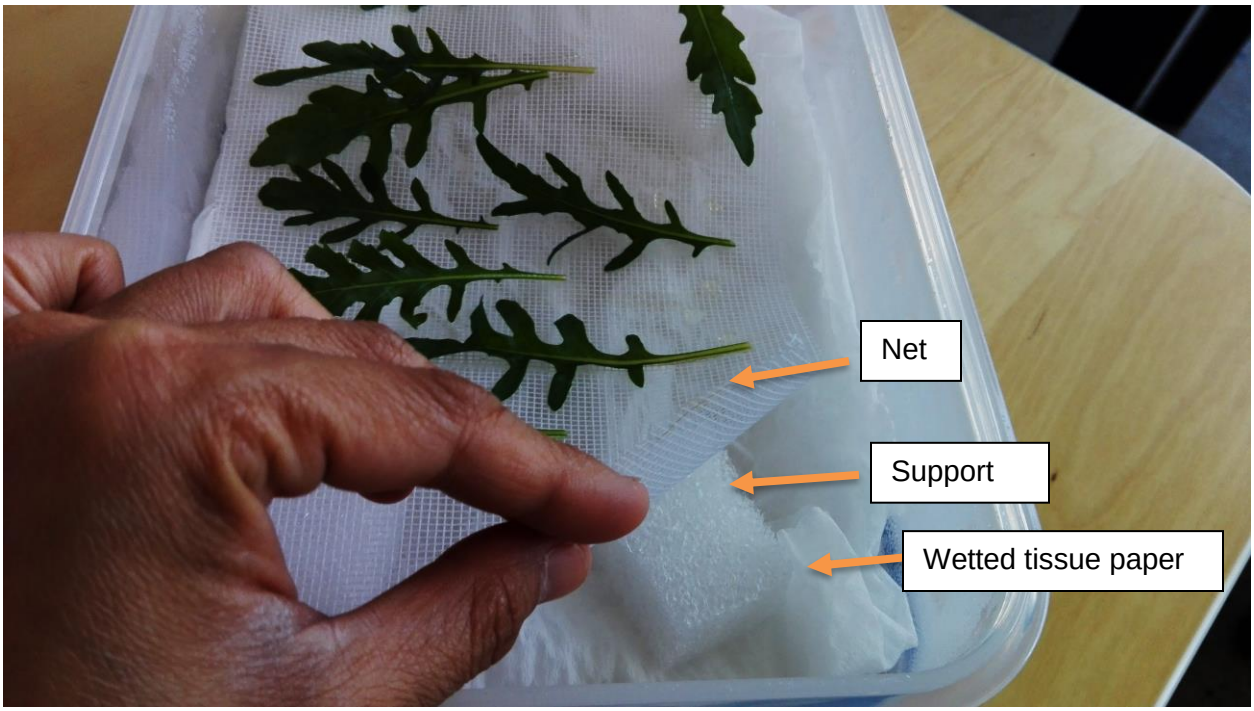


Figure 12. Setup for resting of the leaves.

4.3.5. Freezing and thawing

After resting, the recovered leaves were blotted to remove any excess liquids. The leaves were then divided into two parts, for about 22 leaves was subjected for wilting test evaluation and three leaves for the conductivity measurement per batch (both of analysis tests will be explained in section 4.4). If the treatment has low number of recovered leaves, the quantity of wilting test leaves was reduced. The leaves subjected for the conductivity analysis, were measured for its resistance right before they were frozen. All of the leaves were then frozen in a chest freezer (Electrolux, Italy) at -22^{o}C for 16 - 24 hours. The frozen leaves were thawed on a tissue paper at room temperature for 5 minutes.

4.4 Evaluation of treatments

Thawed leaves were evaluated in order to determine the percentage of leaves that had survived by using three different methods. The methods used were wilting test, conductivity, and microscopic observations using Fluorescein Diacetate (FDA) to observe the viability of the cells. For FDA

observation, 24 hours storage time after frozen and thawed leaves from the wilting test were used. A detailed account of the different methods is given below.

4.4.1. Wilting test

First determination of survival leaves and the one which not survived was done by wilting test. The turgidity of the thawed leaves was evaluated by placing them on a bamboo rod. The bamboo rod was 3 mm in diameter and 30 cm in length (Figure 13). Fresh leaves did not wilt, whereas leaves that had lost their turgor would wilt. The thawed leaves were observed and compared to the dead leaf (negative control) and fresh leaves (positive control). The dead leaf was untreated leaves that had been frozen and thawed. The leaves that did not wilt or were similar to the fresh leaf after thawing were counted as surviving leaves. The survived leaves were stored in a humid box and stored under refrigeration at 4^{o}C (± 2^{o}C). The survival leaves were evaluated and observed from time to time (5 min, 30 min, 1 hour, 2 hours, 5 hours and 24 hours of storage time after they were thawed).

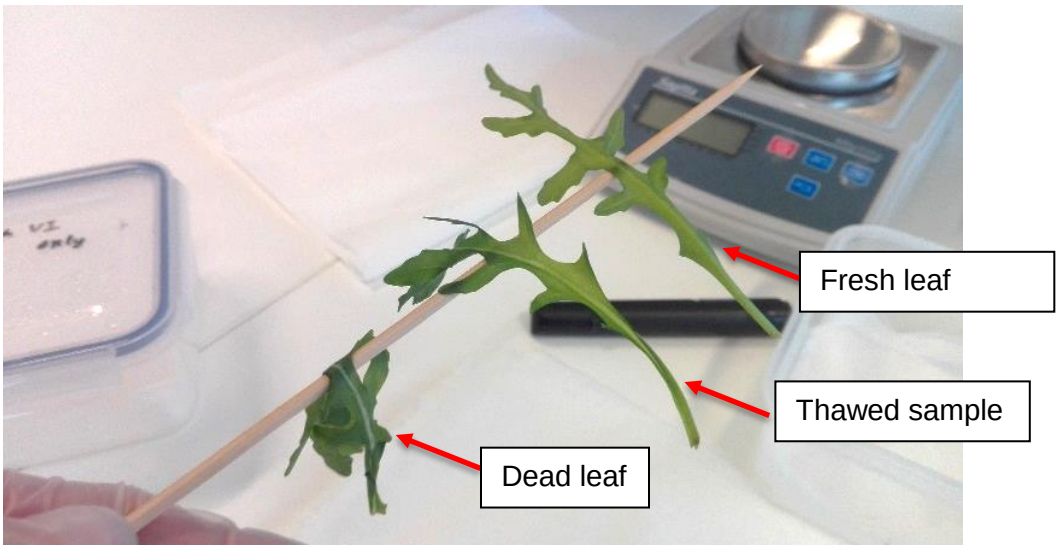


Figure 13. Wilting test method

4.4.2. Resistance analysis of thawed leaves

The three leaves per batch, first were evaluated using wilting test for its survival. Then the survivability was validated by measuring the tissue's resistance using impedance measurements (described in Figure 9). After the first measurement, the samples were stored in a box (12 cm x 12 cm x 6 cm – 0.83 L) with 4 layers of tissue paper (2 g) placed at the bottom of the box and wetted with 3.75 g of water. The box was stored under refrigeration at 4^{o}C (± 2^{o}C). The same sample was

analysed every 30 minutes for one hour. At least three repetitions were done for the resistance measurements. The value of resistance was calculated as conductivity.

4.4.3. Viability test - FDA observation

Another validation of the wilting test was the observation under microscope using FDA staining. The FDA stock solution was made by mixing 0.125 g FDA in 25 ml of acetone. For treating the leaves, 1ml of FDA stock solution was mixed with 100 ml deionized water for a final concentration of 0.12 mM FDA. The thawed samples were dipped in the final FDA solution for 10–15 minutes and then observed under a microscope (MMT, MILMEDTEK AB, Sweden) with a filter that generate blue light (excitation of FDA obtained at 488-494 nm wave length, Sigma-Aldrich, 2018) and with 10X 1/0.3 Plan fluor bottom lens magnification. Viable cells got a bright green colour, while the dead cells remained dark.

4.5. Statistical Analysis

A statistical analysis of the results was performed using ANOVA (single factor) and 2t-test with 95% Confidence Interval (p value < 0.05) using MINITAB statistical software, (Minitab Inc, US).

4.6. Experimental plan scheme

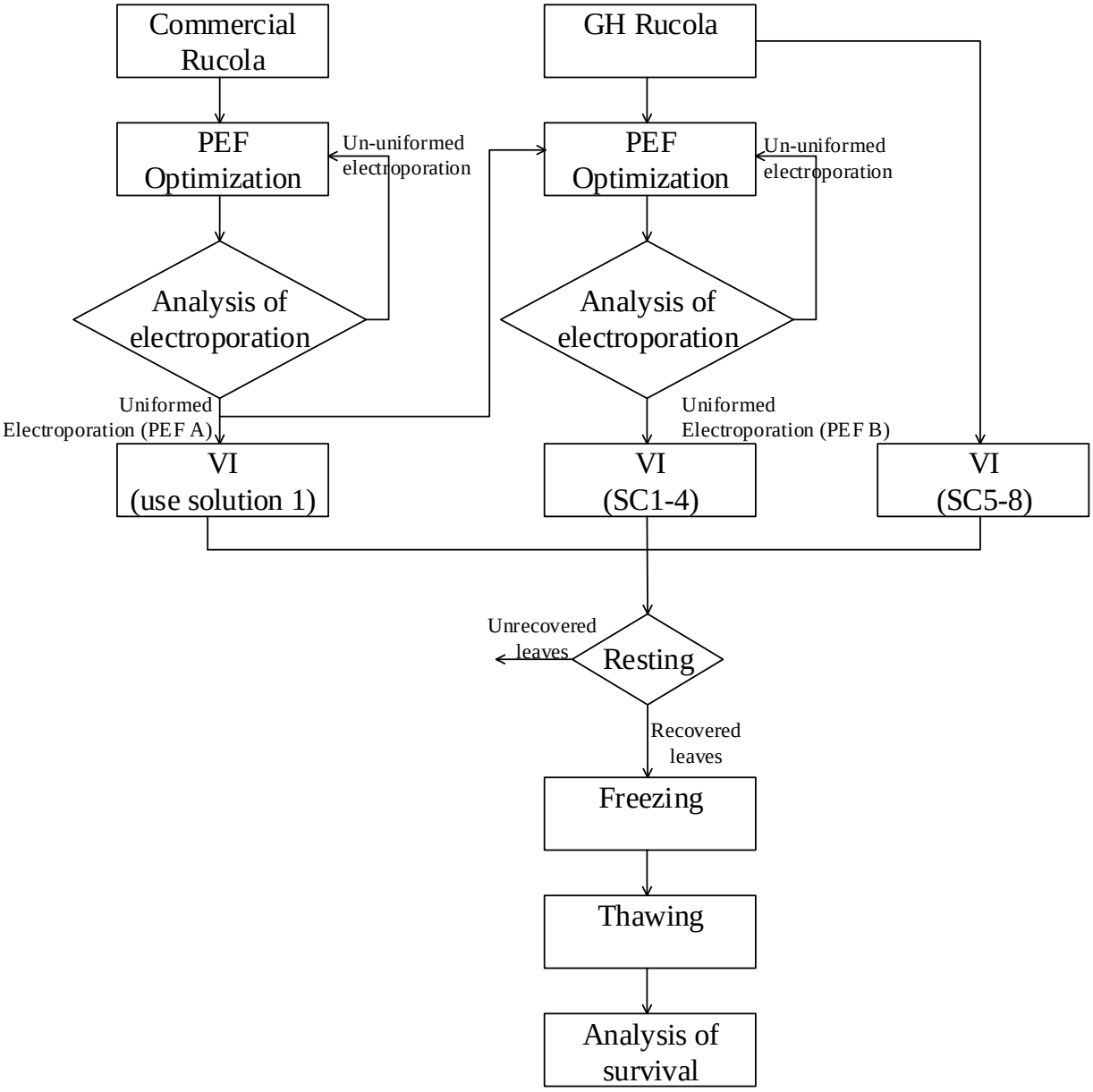


Figure 14. Scheme of experimental plan

5. Results

5.1. Leaves characterization

The measurement of the dimensions of the leaves, both from the commercial and the GH leaves, are reported in
Table *2*.

Table 2. Dimensions of the leaves

Leaves	Length	Width	Thickness
Commercial	7 cm (± 1 cm)	3 cm (± 1 cm)	Fresh leaf: 0.30 mm (± 0.03 mm) Dead leaf: 0.13 mm (±0.02 mm)
Greenhouse	6.95 cm (± 0.73 cm)	2.74m (±0.39 cm)	

5.2. Optimization of PEF parameters

5.2.1. Nuclei staining

During the experiment, it was found that the PEF generator could not apply electric pulses lower than 4µS pulse width. Moreover, with 4µS pulse, uniformed electroporation was not possible even when using very high electric field, 2000 V/cm. Thus, the pulse width was adjusted to apply the lowest possible pulse width. Uniform electroporation for the commercial rucola was achieved at 20µS, 1200V/cm, 500 pulses using 1 train. Typical uniform and non-uniform electroporated leaves are shown in Figure 15 A and B.

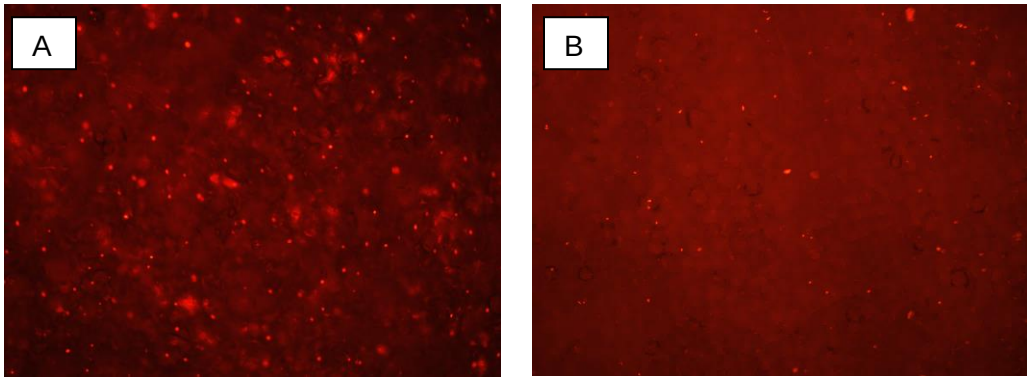


Figure 15. Observation under microscope for PI staining; (A) uniformly electroporated cells, (B) non-uniformly electroporated cells. Electroporated cells are seen as bright spots.

For GH leaves, the electroporation was successful at 1100 V/cm, 500 pulses and 1 train. The summary of optimized PEF parameters is reported in Table 3.

Table 3. Summary optimization of PEF parameters

Name	Pulse width, µs	Electric field strength, V/cm	Number of pulses	Number of trains	Pulse space, µs	Material
PEF A	20	1200	500	1	1000	Commercial rucola
PEF B	20	1100	500	1	1000	GH rucola

5.2.2. Conductivity measurements

The resistance was measured at 1, 10, and 100 kHz of frequency during the test. The chosen frequency was 10 kHz. As it can be seen in Figure 16, at the frequency of 10 kHz, the curve has not reached the plateau, moreover the difference of resistance's value between fresh and dead leaves at this selected frequency was the highest, which made distinguishing viable and nonviable leaves much easier. According to Zimmermann (1982), at higher frequency, the cell membrane performs as less resistive when it is exposed to an electrical current. This means the cell membrane is 'transparent', allowing the electrical current to pass through the membrane, behaving like a conductor.

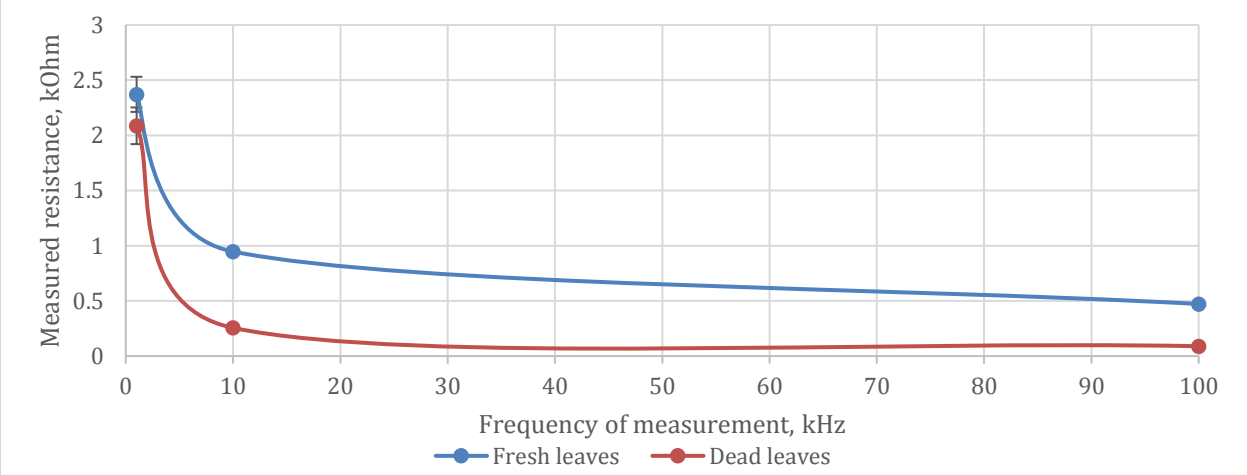


Figure 16. Resistance measured on positive (fresh leaves) and negative (dead leaves) controls. Dead leaves refer to the untreated fresh leaves after freezing and thawing. Error bar was generated from standard error of the mean.

From Figure 17, it appeared that with the set of chosen parameters, the leaves survived, as there were no significant differences of conductivity of the samples; before, straight after PEF and 24 hours after PEF execution. This indicated that the electroporation did not significantly affect the tissue.

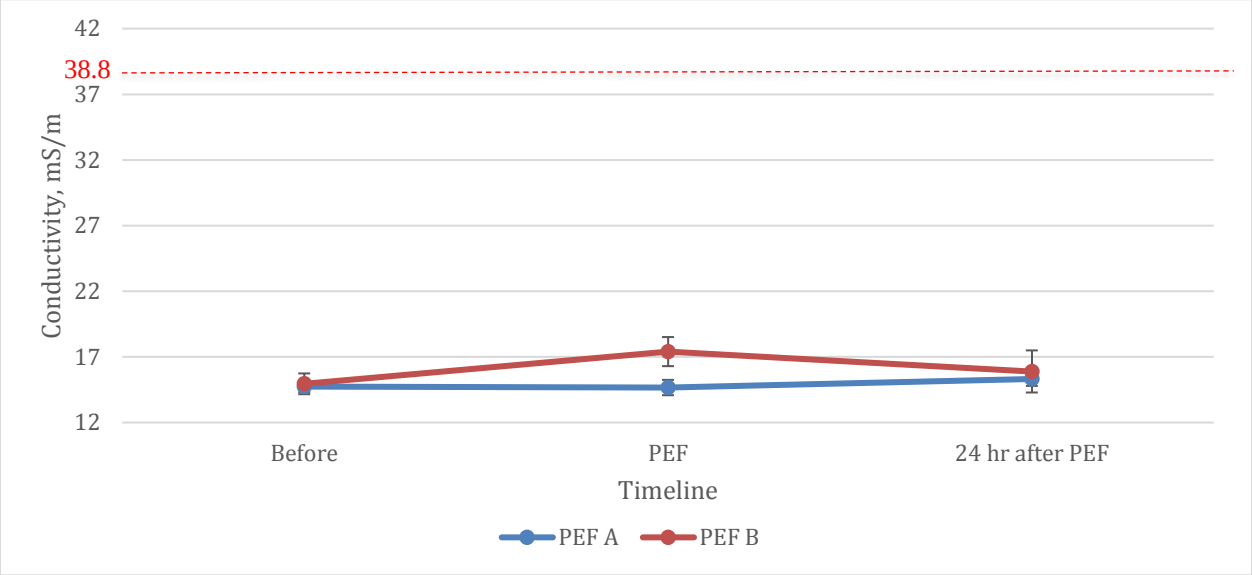


Figure 17. Conductivity of samples before PEF, directly after PEF and 24 hours after PEF. Different PEF parameters for PEF A and PEF B are reported in Table 3. Error bars represent the standard error of the mean. The reference value of 38.8 (± 0.93) mS/cm, Y axis, is the average conductivity from the negative control (from frozen and thawed of untreated leaves)

5.3. Vacuum impregnation, resting, freezing and thawing

5.3.1 Commercial rucola

5.3.1.1 Weight loss after vacuum impregnation and recovery

As the glycerol concentration used for VI process was quite high (hypertonic solution), the cells were drained out which resulted in a collapsed structure (Figure 18 A). The leaf blades were often wrinkled and less turgid, especially when compared to fresh leaves (Figure 18 B). After the impregnation, the collapsed leaves also lost weight. As seen in Table 4, the weight loss of samples treated with a combination of PEF and VI was lower than that of the leaves treated only with VI.

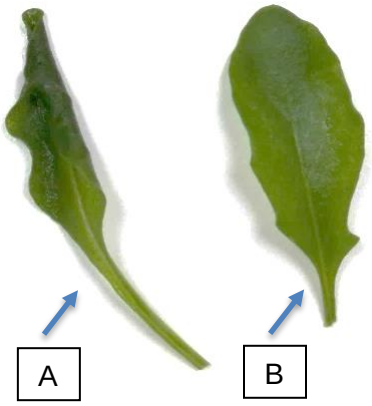


Figure 18. Typical picture of vacuum impregnated rucola leaf with glycerol hypertonic solution shows collapsed structure (A) compared to the untreated fresh leaf (B).

After resting, the amount of commercial leaves that recovered was quite low (Table 4). The average recovery of all treatments was lower than 50%, which means that more than 50% of the treated leaves with PEF and VI could not recover their turgidity and were therefore discarded for further experiments. Leaves were rejected after resting time. However, the treatments that used PEF in combination with VI had a higher recovery rate compared to the treatment using only VI, highlighting the importance of PEF.

Table 4. Weight loss and % recovery of different treatment of the commercial leaves. P-value (*) were generated from one-way ANOVA of the treatments.

Treatment	Weight loss, %	% recovery	p-value (weight loss) *	p-value (% recovery) *
PEF A	25.76	38	0.001	0.027
VI only (glycerol solution 1)	35.15	19		

5.3.1.2 Wilting test

The result of the wilting test using PEF A is described in Figure 19. The percentage of viable leaves was decreasing over time after thawing. Only about 17% of frozen thawed leaves survived after 2 hours and no leaves survived 24 hours after thawing.

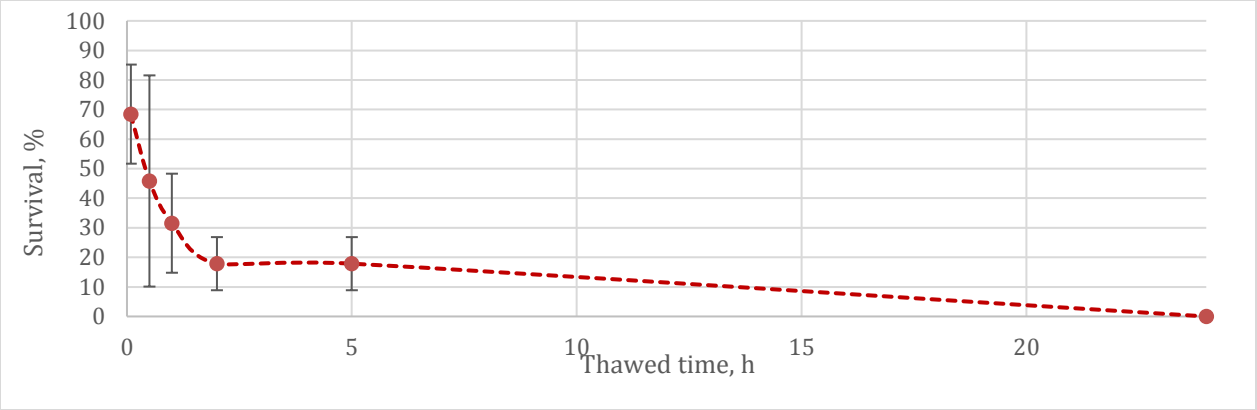


Figure 19. The result of the wilting test on commercial rucola leaves using PEF A treatments. The interval bar shows the standard error mean.

5.3.1.3 Conductivity measurements of frozen and thawed sample

The measured conductivity of survival leaves (from the wilting test) over time after thawing was quite low (showed by light blue, light brown and dark brown solid line, Figure 20), indicated by the conductivity value of 17.45 to 19.00 mS/m compared to the negative control, 38.8ms/m (horizontal dashed line, Figure 20). When the leaves were measured for a longer period, a constant conductivity reading was obtained from first hour up to 24 hours. These conductivity values indicated that when the conductivity measurement for the first hour was low (showed by light blue, light brown and dark brown solid line in Figure 20), the leaves would survive and not suffer for extreme leakage after 24 hours. Meanwhile, when the measured conductivity value for the first hour was high (closer to the conductivity of the negative control), the leaves were not survived.

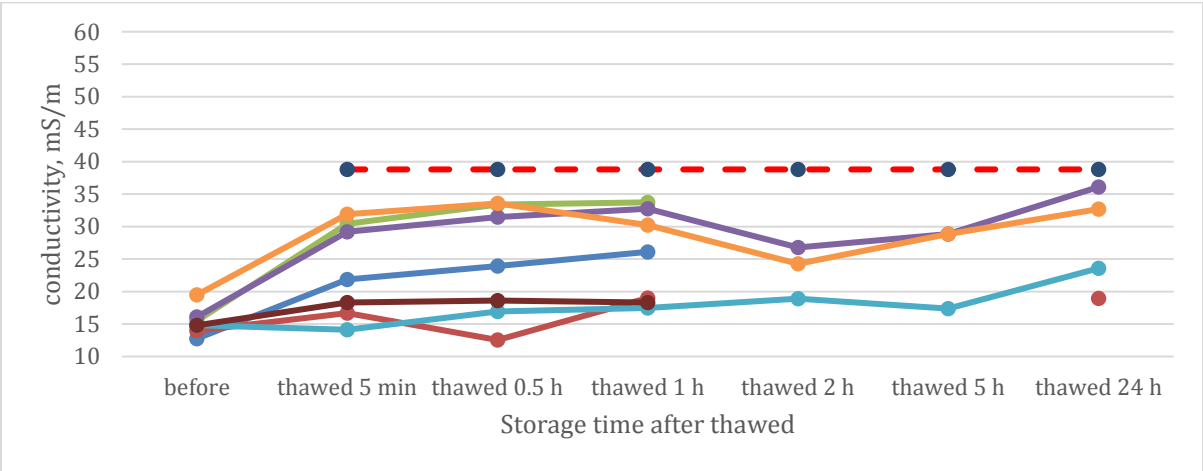


Figure 20. Conductivity of thawed leaves versus time using PEF A treatments. The dashed-red horizontal line represents the conductivity of the negative control: 38.8 mS/cm (± 0.93). Each solid line represents one leaf (sample).

5.3.2. Greenhouse rucola

5.3.2.1. Weight loss after vacuum impregnation and recovery after resting

No significant differences in weight loss were detected between samples SC1 - SC4 (Combination of PEF B and VI, Figure 21). In contrast, samples SC5 - SC8 (treated only with VI) show less weight loss at lower glycerol concentration compared to higher concentrations. In contrast to the commercial leaves, less weight loss was found from all GH samples.

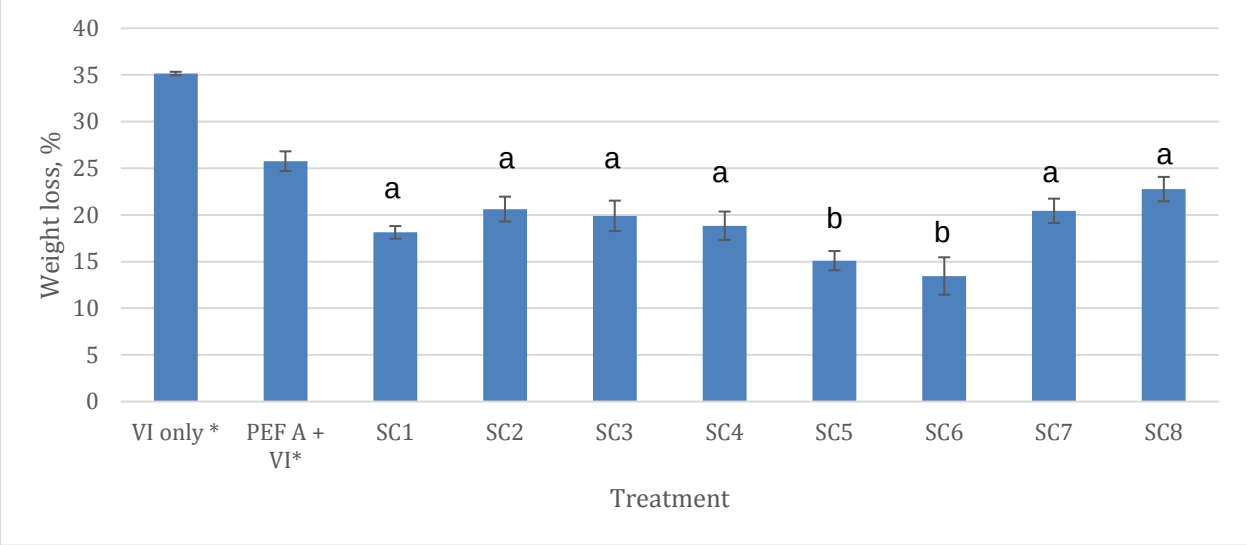


Figure 21. Weight loss of samples at different glycerol concentrations treated with PEF + VI and VI only. The interval bar shows the standard error of the mean. (*) mark indicates treatment using commercial rucola leaves with glycerol hypertonic solution 1 for comparison. Different letters above the error bar indicate statistical differences.

Recovery among the GH leaves after resting was much higher (close to 100% recovery) compared to the commercial leaves (Figure 22). Treatments with only VI and with PEF and VI showed no significant differences in recovery for the GH leaves.

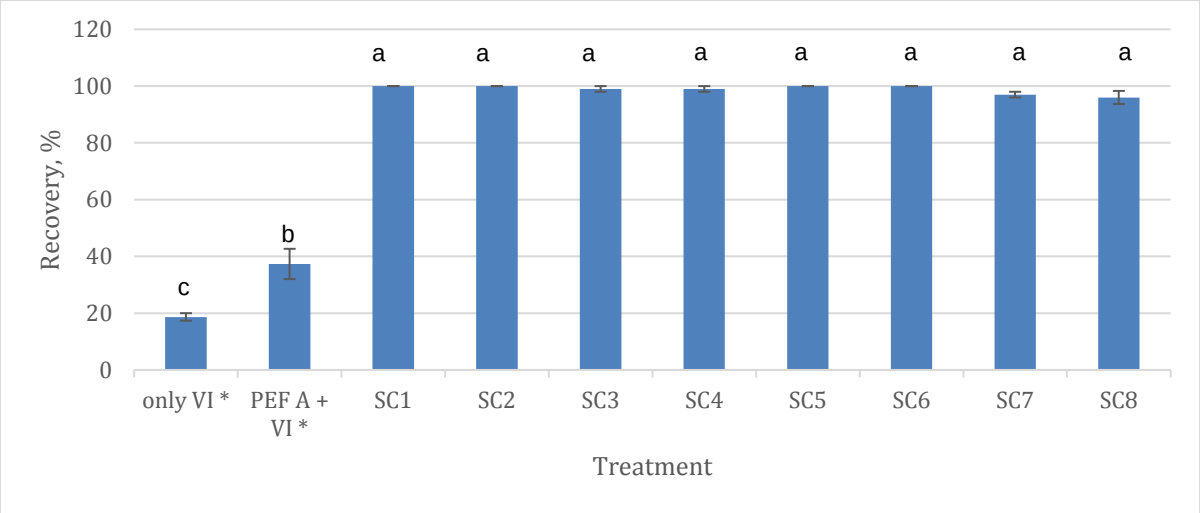


Figure 22. Percentage of recovery among rucola leaves after resting using different treatments. The interval bar shows the standard error of the mean. (*) mark indicates treatments using commercial rucola leaves with glycerol hypertonic solution 1 for comparison. Different letters above the interval bar indicate statistical differences.

5.3.2.2. Wilting Test

Results of the wilting test analysis are shown in Figure 23. At a same point in time, the percentage of survival was reduced as lower glycerol concentration was used both for treatments using PEF + VI (SC1 – SC4) and for treatments with only VI (SC5 – SC8). This condition also occurred by the duration of storage time after thawing, the longer storage time, the lower survival obtained for each treatment. When the samples were thawed for 5 minutes (0.08 h), the result of treatment with PEF and VI at the highest glycerol concentration (SC4) showed a very good rate of survival (about 88%). The results obtained from the SC3 and SC4 treatments were significantly different from those of the SC7 and SC8 treatments (treatments with only VI at the same glycerol concentration) before they had been thawed for no more than two hours.

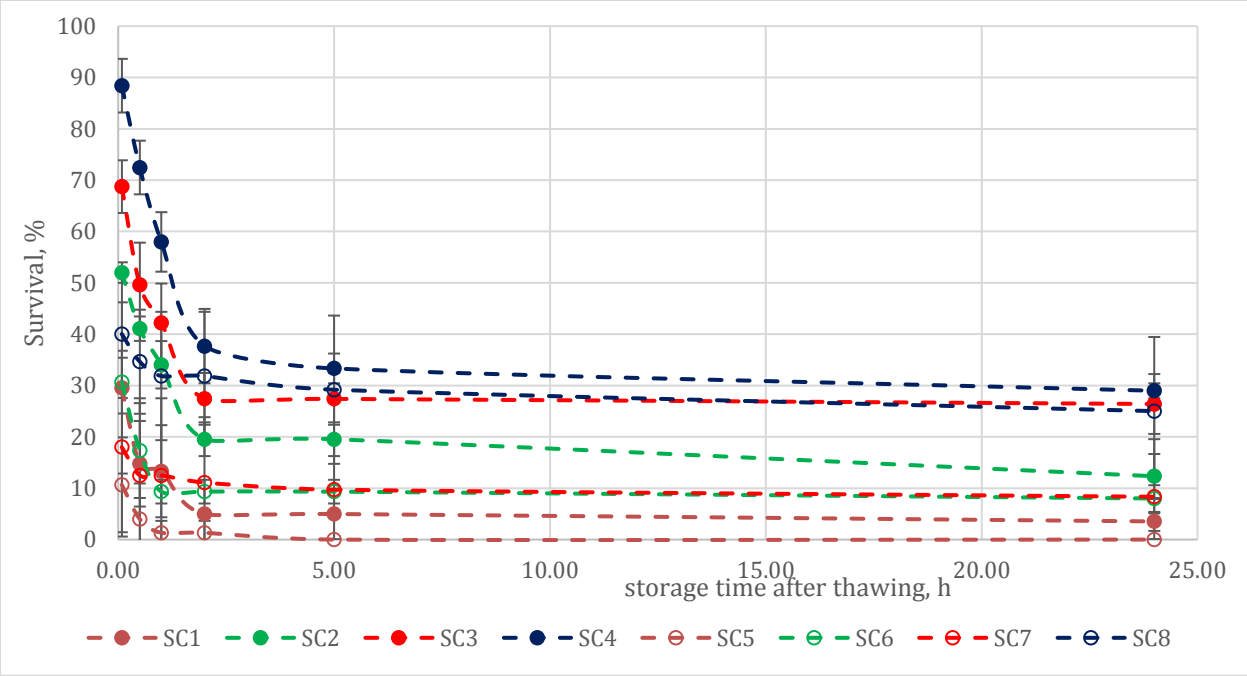


Figure 23. Percentage of survival using wilting test, solid marks denote treatments with PEF and VI (SC1 – SC4); open marks denote treatments with only VI (SC5 – SC8). The average and standard error were calculated from 3 replications consisting of at least 20 leaves per batch.

When the highest concentration of glycerol (SC8) was used, the leaves treated only with VI kept a survival of around 30 % throughout the measuring time (without the need of PEF). At the same concentration, PEF contributed to a better survival only for the first hour storage time after thawing. Interestingly, after this first hour there are no significant differences between the survival of VI treated samples and PEF + VI treated samples. The PEF contribution at each time point for each of the studied glycerol solution was calculated as:

PEF contribution = % survival (PEF + VI) - % survival (VI) Equation 4

Results are reported in Figure 24. Figure 24A shows that after frozen and thawed for 0.08 hours (5 minutes), treatment with PEF and VI showed much better result with concentrations 3 and 4 than with concentrations 1 and 2. Slightly different results were obtained when for 0.5 h storage time after the samples were thawed (Figure 24 B), which showed similar result for concentrations 3 and 4, but showed that concentration 2 had better results than concentration 1. Figure 24 C – F show minimal contribution of PEF.

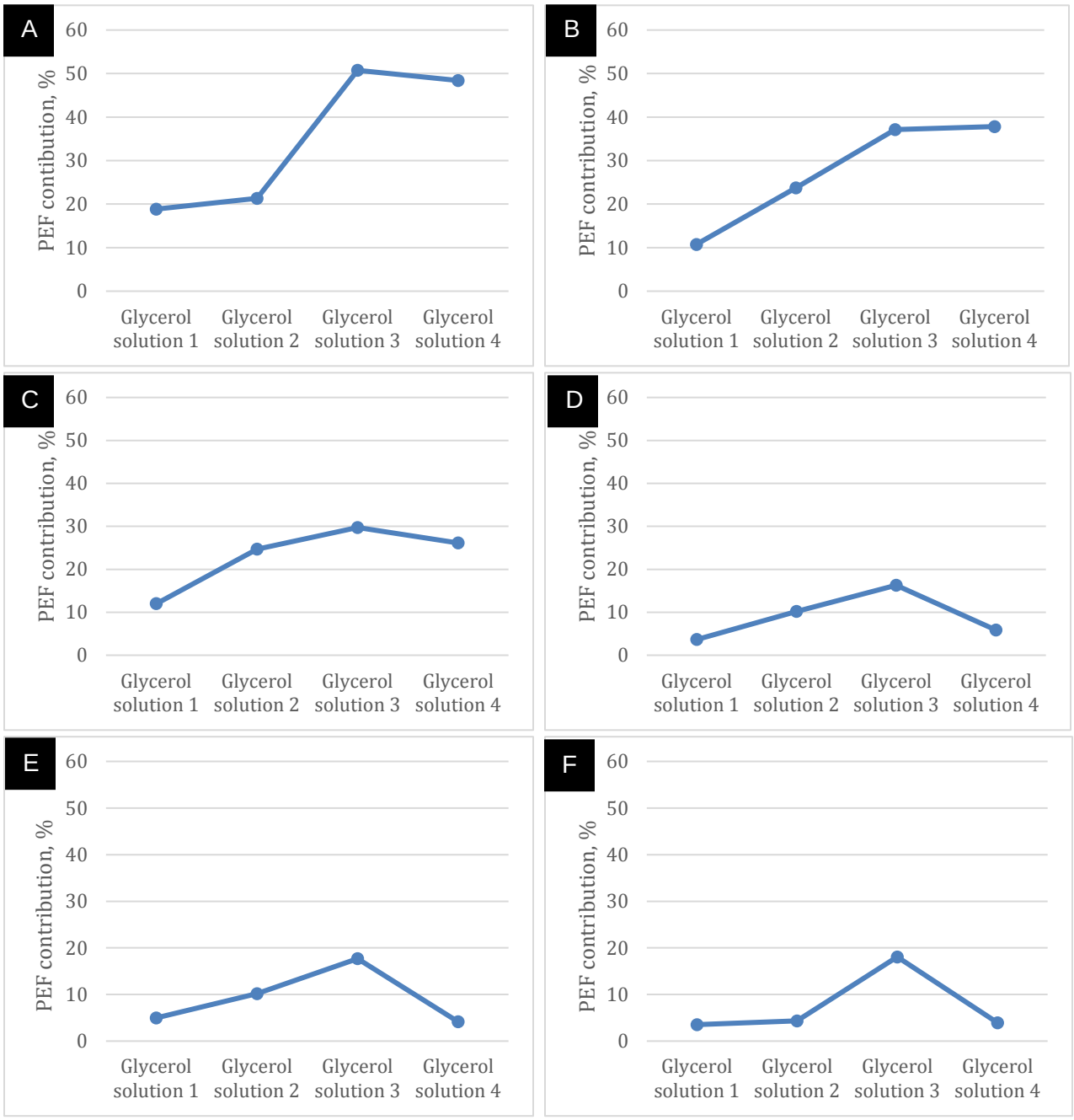
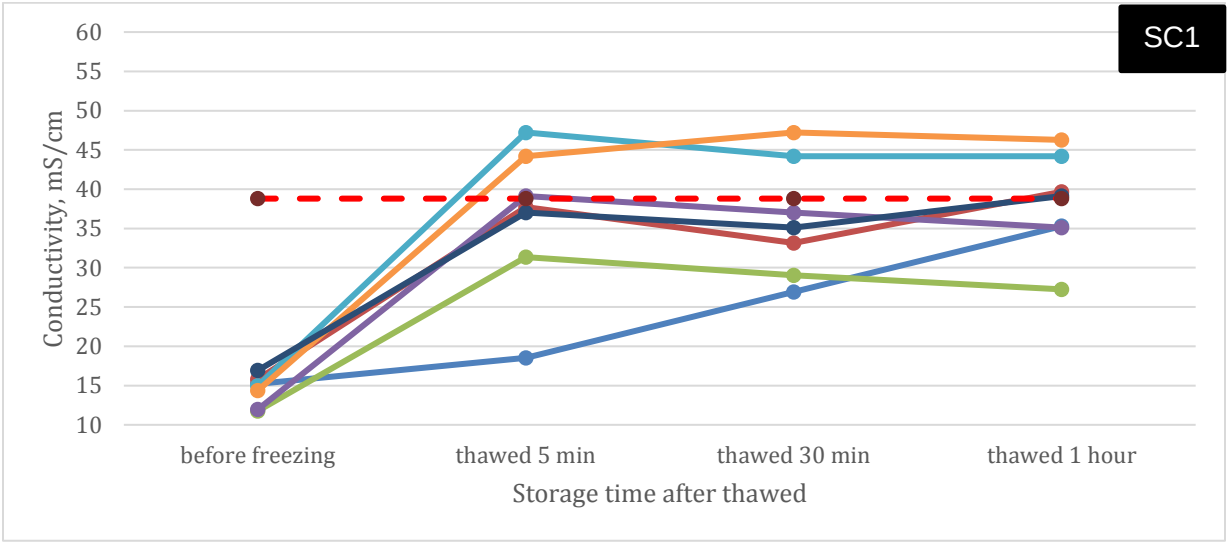
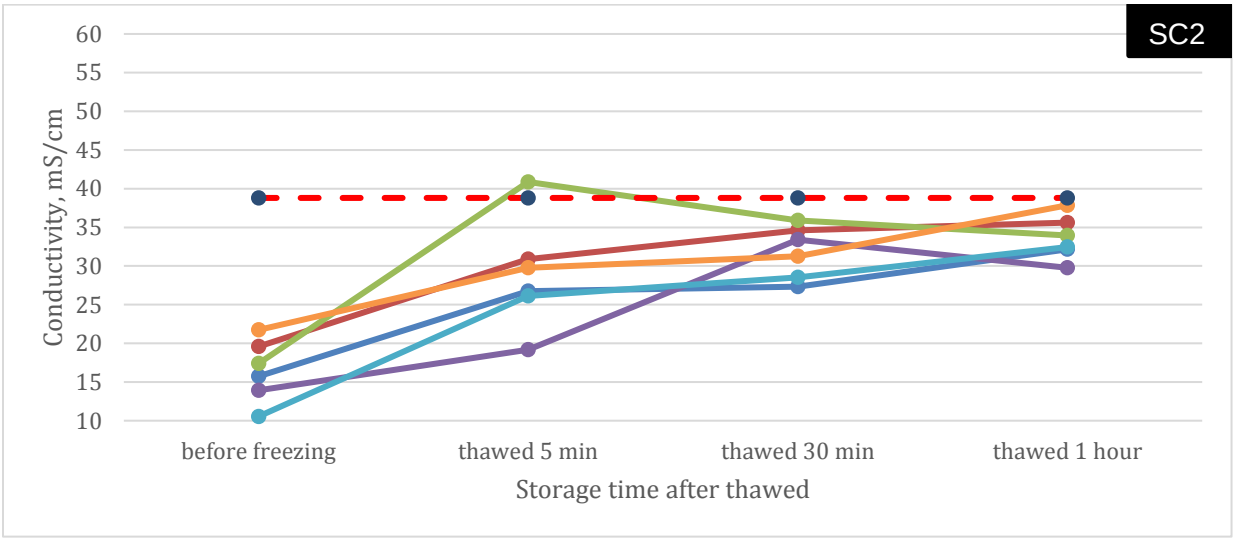


Figure 24. The differences of survival when using a combination of PEF + VI and when only VI. PEF contribution was calculated by subtraction of the value of survival of leaves treated with only VI from the survival of leaves treated with the combination of PEF+VI (Equation 4). The subtraction value was taken from the average survival from both treatments. In Figures A – F PEF contribution to survival 0.08 hours (5 minutes), 0.5 hours, 1 hour, 2 hours, 5 hours, and 24 hours storage time after thawing is represented.

5.3.2.3. Conductivity measurements of frozen and thawed samples

The measured conductivity of survived leaves treatments using SC3 and SC4 over time after thawing was much lower than the conductivity of the negative control (Figure 25, green box). Meanwhile the result of treatments using SC1 and SC2 suffered more leakage (most of the samples were not survived) as has been showed in Figure 25 where the solid lines are closer to the negative control line. This result showed that with higher concentration of cryoprotectant, there was more survival. The measurement was taken for the first hour only, as explained before that the curve pattern was relative constant when it was measured for longer period up to 24 hours as represent in Figure 20.





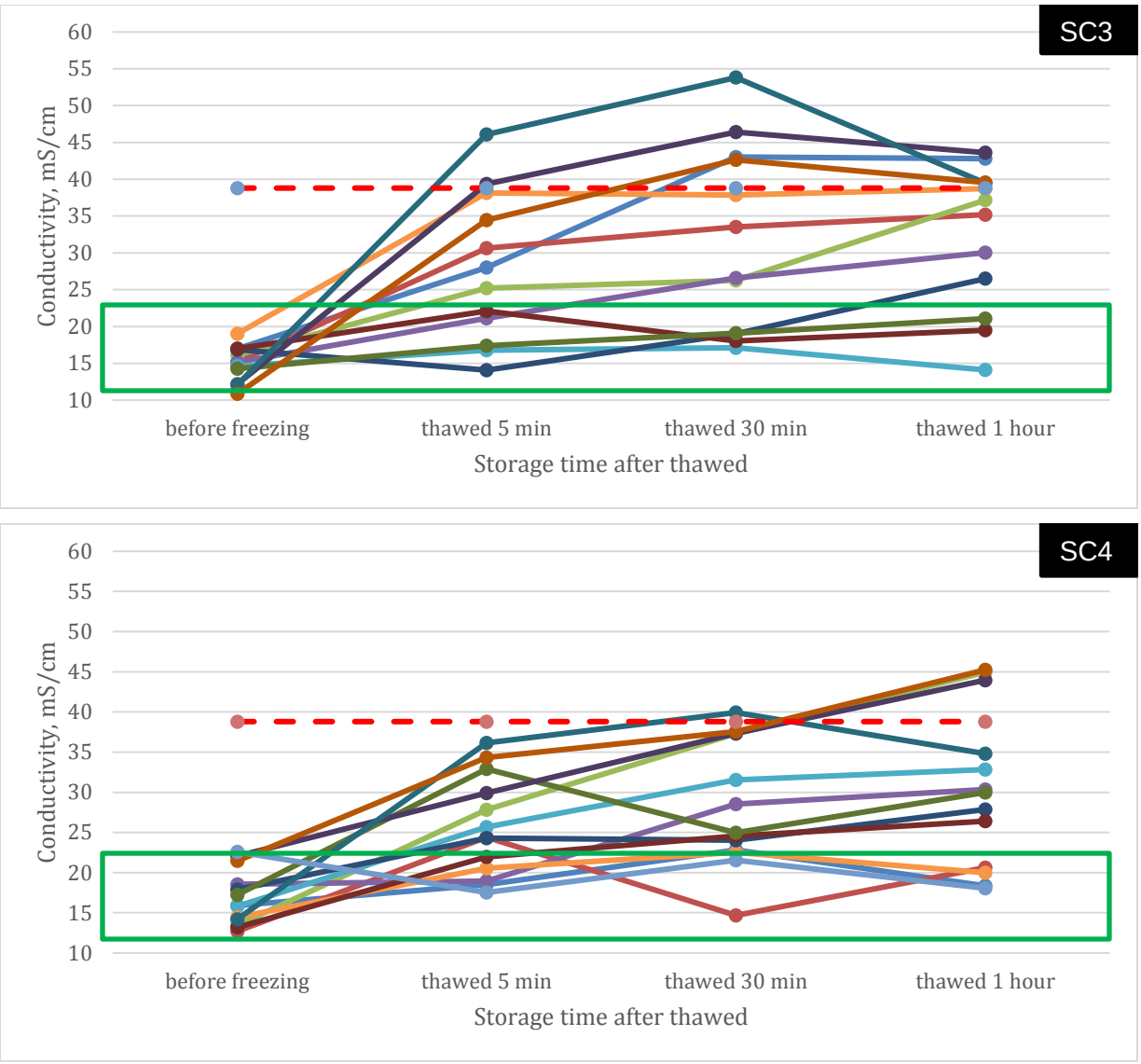


Figure 25. Conductivity of SC 1, SC 2, SC 3 and SC 4 versus time. The dashed-red horizontal line represents the conductivity of the negative control: 38.8 (± 0.93). Each solid line represents one leaf. The green box indicated survived leaves by the wilting test.

5.3.2.4. FDA observation of frozen and thawed sample

Microscope observations of the leaves are shown in Figure 26. Figure 26A shows a fresh leaf and Figure 26B shows a dead leaf (obtained by freezing and thawing an untreated leaf). Viable cells are indicated by a green yellowish glowing colour, while dead cells remain dark. Figure 27 shows a leaf, which 24h after thawing was classified as having "survived" by the wilting test. The figure

indicates that cells that are located far from the main vein were not viable (Figure 27A and Figure 27B). In the contrary, the cells near the main vein showed viability (Figure 27C).

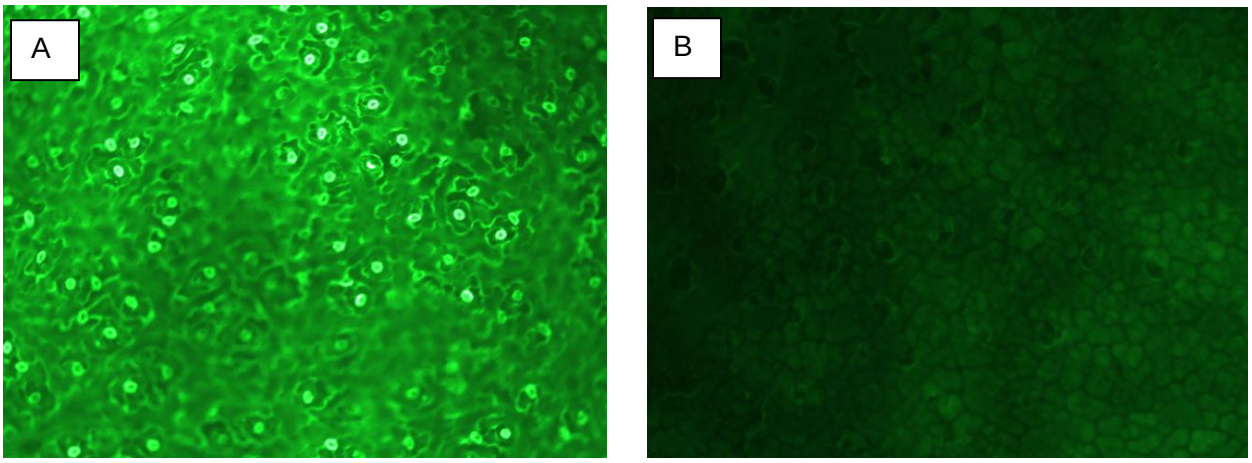


Figure 26. Typical microscopic observations of rucola leaves stained with FDA (A) fresh and (B) dead leaf.

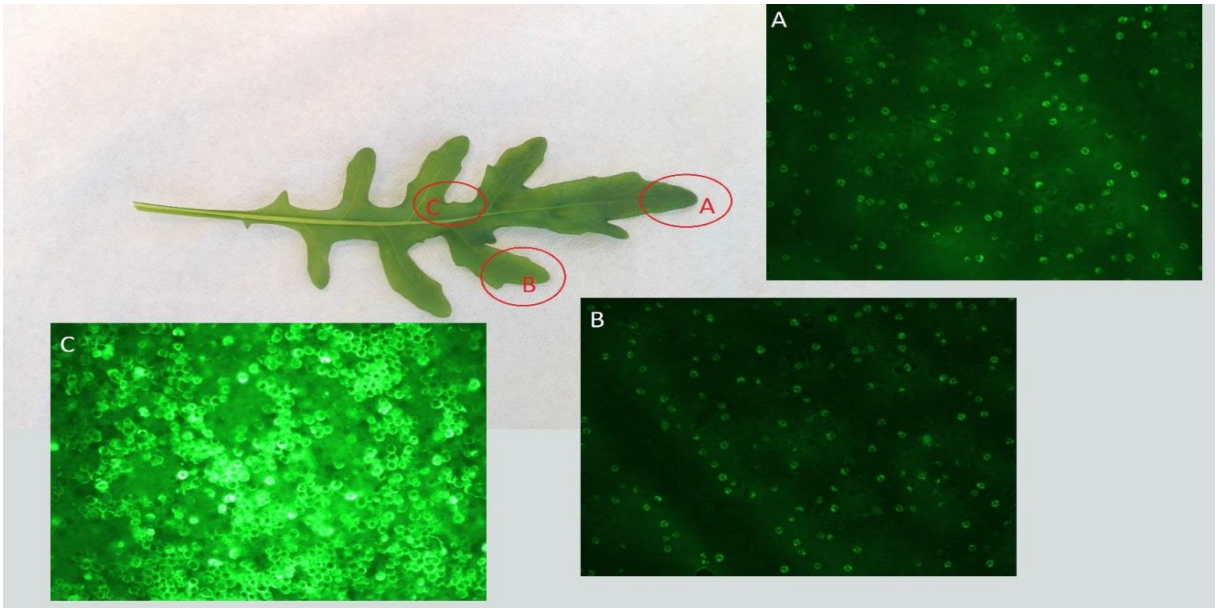


Figure 27. Typical Microscopic observation of a surviving leaf treated with PEF and VI. (A) area near the tip of the leaf, (B) the edge and (C) area near the main vein.

5.4. Influence of the morphology of the leaf on survival

The morphology of rucola leaves changed during growing in the green house. Figure 28A shows the typical rucola plants after 5 weeks of seedling. In one single plant could consists of different shape of leaves which depends on their life stages. The different shape of the leaves is shown by numbers 1 to 5 in the Figure 28B. The numbers do not represent a chronological order of changes on leaf morphology. However, as the plant becomes mature, the newer growth of the leaves become more and more uniform, looking more like leaf number 5 in Figure 28B. Unfortunately, the stages of morphology were not carefully observed during this study, which meant the time when the mature leaves (Figure 28B – number 5) started to appear was not recorded. During the experiment, different results were obtained when the mature leaves were compared to the younger leaves. The mature leaves had a better survival rate after thawing compared to the initial growth leaves (see Figure 28C). The main reason behind this remains unclear.

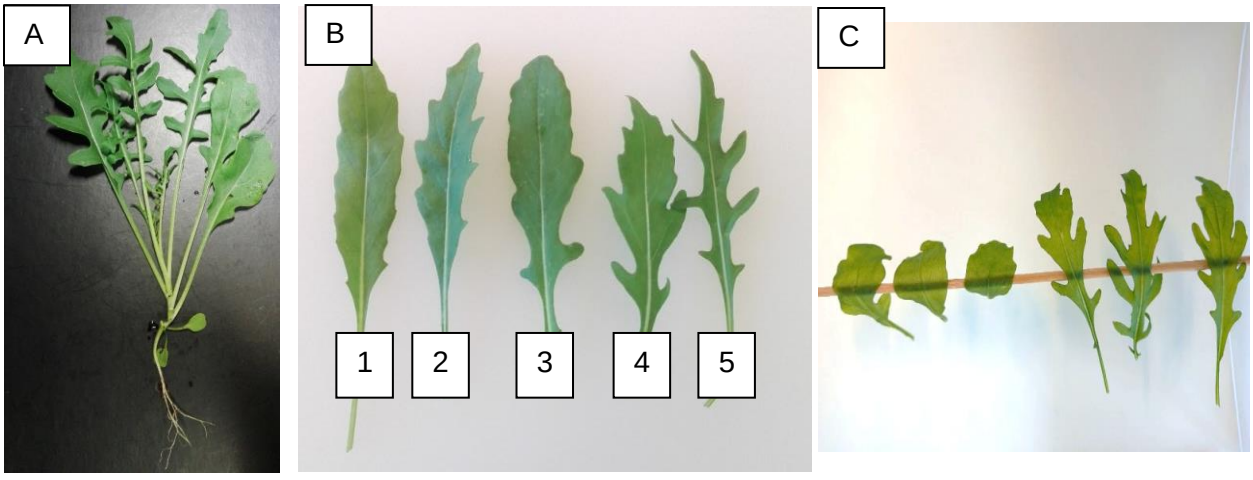


Figure 28. A typical 5 weeks old rucola plant (A). Different morphology of harvested GH rucola leaves (B). Wilting test of frozen and thawed GH leaves with different morphology (C).

6. Discussion

6.1. Effect of PEF treatment on conductivity

The PEF treatment in this experiment was applied to produce electroporation with reversible permeabilization of the cell membranes. Phoon et al. (2008) stated that cells were able to recover over time after the application of PEF and this recovery was dependent on the initial impedance (Z, Ohm) of spinach leaves. According to Figure 17 the value of conductivity for both treatments (PEF A and B) was increased after the PEF treatment. This indicated some leakage after PEF, (a phenomenon categorized as normal), as PEF treatments produced electroporation that allowed liquid content to move from inside the cell to the outside of the cell (Donsi et al., 2010). Phoon et al. (2008) stated that differences in value of impedance before and after treatments might provide information regarding the condition of the cell membrane. In this thesis, the value of conductivity before and directly after PEF was not significantly different. However, it did differ compared to the conductivity of negative control (dead-untreated sample). It was therefore concluded that the treatment had not completely damaged the cell membrane.

6.2. Effect of treatment on weight loss and recovery

From Figure 21 showed that both treatments with commercial and GH leaves suffered from weight loss. This is relevant as the glycerol solution used for the experiment was hypertonic. However, there were different results for treatments using a combination of PEF and VI compared to treatment only using VI. Weight loss for commercial leaves only subjected to VI was greater compared to those treat with both PEF and VI. However, using glycerol solutions 1 and 2, the weight loss for GH leaves treated only with VI (SC 5 and SC6) was lower than those treated with both PEF and VI (SC1 and SC2). The weight loss obtained for only VI treatment (SC7 and SC8) using glycerol solution 3 and 4 was similar than that obtained using the combination of PEF + VI (SC3 and SC4) . According to Hui et al. (2016), using a combination of high pulse electric field and vacuum impregnation improves the liquid infusion as the permeability of cell membrane will be disturbed. This might be relevant for the results that showed that a treatment combining PEF and VI has lower weight loss since some liquid could penetrate the cell while treatment with only VI would just allow the liquid to occupy porous spaces in the tissue. However, the reaction of plants to osmotic stress is complicated and widely varied, depending on many factors such as cultivar, plant parts and maturity (Hui et al., 2016).

The recovery among commercial and GH leaves were quite different. The commercial leaves were only treated using the lowest glycerol concentration because the percentage of recovered leaves was very low (less than 50%). The main reason for this low recovery rate remains unknown. Correlating the weight loss with the percentage of recovery, the commercial leaves had quite a large weight loss. For example, commercial leaves lost over 36% weight when treated with only VI, and only 18.67 % of them regain its structure. All treated GH leaves suffered between 13.46 – 22.77 % weight loss, but all of them were recovered, almost 100%. This may be related to the resting time, as in this experiment, the same resting time was applied for both commercial and GH leaves. It is also convinced by Gómez Galindo (2016), that the resting time is essential for the tissue to recover from stress before the next process operation is initiated.

6.3. Frozen and thawed survival

The result of conductivity (resistance) measurements was not describing the actual percentage survival of frozen and thawed sample. In this project, the samples for resistance measurements were chosen randomly before they were frozen. This was the case because the resistance had to be measured before freezing to match the values with their resistance after freezing and thawing. Which means there were possibility that all measured samples were not survived (such as shown in Figure 25, SC2). There was also high variation of the un-survived leaves conductivity. This could be the fact that the resistance measurement was dependent on the specific area of the tissue that was used in the measurements. Some of the leaves showed higher electrolyte leakage, even when compared with dead leaves. The variation might also be a result of the calculation, as the calculation was based on average value of the leaf thickness while the thickness of the leaves varied.

In the wilting test using GH leaves, SC4 performed the best in terms of survival during the first hour. This might be due to the fact that SC4 had the highest cryoprotectant concentration of all the treatments. However, if the results for treatments with both PEF and VI and for treatments only using VI (Figure 24) are compared, it showed that PEF gave highest contribution when it was combined with VI using glycerol solution 3. PEF gave higher contribution when the samples were measured during the first hour after being thawed. The reasons behind these results are not known.

6.4. FDA observation of viable cells

As reported in Figure 27, the viable cells after thawing are mainly situated near the main vein. Differences in the porous structure between the centre and the edge of the leaf might have contributed to this result. Differences in the porous structure between edge and centre of spinach leaves have been reported by Panarese et al. (2016). A similar result was also reported by Demir (2012), regarding the survival of baby spinach leaves.

7. Conclusions

The lowest possible pulse width that could be applied for obtaining uniform electroporation was 20 µs. At this pulse width, both commercial and green house leaves showed some percentage of surviving leaves after freezing and thawing.

The optimal treatment for minimalizing freezing injury for the GH leaves was to apply PEF using 20 µs pulse width at 1100 V/cm field strength, 500 pulses, 1000 µs pulse space and 1 train for the GH leaves. The treatment with a combination of PEF + VI using glycerol hypertonic solution 4 (highest tested concentration) yielded the highest survival rates. At this concentration, the survival rate was quite high: Up to 88.41%, 72.46% and 57.97% for 5 minutes, 30 minutes and 1hour storage time after thawing, respectively. However, at 2 hours storage time after thawing, the contribution of the application of PEF was no longer significant in comparison to the leaves treated only with VI. PEF treatment gave the highest contribution when it was used in combination with VI using glycerol hypertonic solution 3. Cells near the main vein were less damaged compared to cells at the edge of the leaves among the cells that survived.

The rucola leaves have different morphology at initial growth stages. It is better to avoid using the leaves from initial growth stages (see Figure 28, left, number 1 – 4) so that only leaves with similar morphology (Figure 28, left, number 5) are studied.

8. Recommendations and future work

Bug attacks (aphid) occurred repeatedly when growing the rucola in the greenhouse. These bug attacks started approximately three weeks after planting. This could and should be prevented by biological treatment in future research.

An optimal concentration of glycerol needs to be established based on sensorial evaluation of the leaves, as sweetness and certain after taste can be caused by the high concentrations used in this study. Additional additives such as GABA and antifreeze protein may help to reduce the amount of glycerol, avoiding possible negative influences on the taste.

Analyzing the conductivity of the whole leaf can be an improvement of the method used in this study. Other analytical methods, such as calorimetric analysis, might provide information regarding aspects neglected in this study.

References

Ahmed, J. and Rahman, S. (2012). Handbook of food process design. 1st ed. Chichester, West Sussex, U.K.: Wiley-Blackwell, 2-3,10-11.

Anon, (2016). Reversible Freezing in Frogs and Toads The.... [online] Available at: http://23pairsofchromosomes.com/post/138204765728/reversible-freezing-in-frogs-and-toads-the [Accessed 25 Jun. 2018].

Betoret, E., Calabuig-Jiménez, L., Betoret, N., Barrera, C., Segui, L. and Fito, P. (2016). Sustainable Innovation in Food Science and Engineering. Innovation Strategies in the Food Industry, 149-165.

Cater Grönt. (2018). Information by email on 3rd September 2018.

Day, J. and Stacey, G. (2007). Cryopreservation and freeze-drying protocols. Totowa, N.J.: Humana Press.

De Vito, F., Ferrari, G., I. Lebovka, N., V. Shynkaryk, N. and Vorobiev, E. (2007). Pulse Duration and Efficiency of Soft Cellular Tissue Disintegration by Pulsed Electric Fields. Food and Bioprocess Technology, 1(4), 307-313.

Demir, E. (2012). Optimization of vacuum impregnation and pulsed electric field parameters for improving the cryoprotection of spinach leaves. Master of science thesis. Lund University.

Demir, E., Dymek, K. and Galindo, F. (2018). Technology Allowing Baby Spinach Leaves to Acquire Freezing Tolerance. Food and Bioprocess Technology, 11(4), 809-817.

Dickison, W. (2000a). Integrative plant anatomy. San Diego, CA [etc.]: Academic Press, 132-133.

Dickison, W. (2000b). Integrative plant anatomy. San Diego, CA [etc.]: Academic Press, chapter 1.

Doležalová, I., Duchoslav, M. and Dušek K., (2013). Biology and Yield of Rocket (Eruca sativa Mill.) under Field Conditions of the Czech Republic (Central Europe). Notulaebotanicae, 41(2), 530-537.

Donsì, F., Ferrari, G. and Pataro, G. (2010). Applications of Pulsed Electric Field Treatments for the Enhancement of Mass Transfer from Vegetable Tissue. Food Engineering Reviews, 2(2), 109-130.

Dymek, K., Rems, L., Zorec, B., Dejmek, P., Galindo, F. and Miklavčič, D. (2015). Modeling electroporation of the non-treated and vacuum impregnated heterogeneous tissue of spinach leaves. Innovative Food Science & Emerging Technologies, 29, 55-64.

En.wikipedia.org. (2018). Electrical resistivity and conductivity. [online] Available at: https://en.wikipedia.org/wiki/Electrical_resistivity_and_conductivity [Accessed 1 Jul. 2018].

FAO (2012). Cryoconservation of animal genetic resources. Rome: Food and Agriculture Organization of the United Nations., 85-94.

Fito, P., Chiralt, A., Barat, J., Andrés, A., Martínez-Monzó, J. and Martínez-Navarrete, N. (2001). Vacuum impregnation for development of new dehydrated products. Journal of Food Engineering, 49(4), 297-302.

Food and Agriculture Organization of the United Nations. (2018). Food Loss and Food Waste. [online] Available at: http://www.fao.org/food-loss-and-food-waste/en/ [Accessed 5 Jul. 2018].

Gómez Galindo, F. (2016). Responses of Plant Cells and Tissues to Pulsed Electric Field Treatments. Handbook of Electroporation, 1-15.

Galindo, F. and Dymek, K. (2017). Pulsed Electric Fields in Combination with Vacuum Impregnation for Improving Freezing Tolerance of Vegetables. Handbook of Electroporation, 2135-2151.

Hall, M., Jobling, J. and Rogers, G. (2012). Some Perspectives on Rocket as a Vegetable Crop: A Review. Vegetable Crops Research Bulletin, 76(-1), 21-41.

Hincha, D., Zuther, E. and Thalhammer, A. (2014). Plant cold acclimation. New York: Humana Press, 15-24.

Hui, Y. and Evranuz, E. (2016). Handbook of vegetable preservation and processing. Florida: CRC Press, 251-257.

Livsmedelsverket. (2018). Nutrition content - Arugula rocket salad. [online] Available at: http://www7.slv.se/SokNaringsinnehall/Home/FoodDetails/2561 [Accessed 23 Apr. 2018].

Morales, M. and Janick, J. (2002). Arugula: A Promising Specialty Leaf Vegetable. Trends in new crops and new uses. ASHS Press, Alexandria, VA. 418-423.

Muldrew, K. and McGann, L. (1990). Mechanism of Intracelular Ice Formation. Biophysical society, 57, 525-532.

Padulosi, S. and Pignone, D. (1997). Rocket: a Mediterranean crop for the world. International Plant Genetic Resources Institute, Rome, Italy. 2-3.

Paes, S., Stringari, G. and Laurindo, J. (2006). Effect of Vacuum Impregnation-Dehydration on the Mechanical Properties of Apples. Drying Technology, 24(12), 1649-1656.

Panarese, V., Herremans, E., Cantre, D., Demir, E., Vicente, A., Gómez Galindo, F., Nicolai, B. and Verboven, P. (2016). X-ray microtomography provides new insights into vacuum impregnation of spinach leaves. Journal of Food Engineering, 188, 50-57.

Pearce, R. (2001). Plant Freezing and Damage. Annals of Botany, 87(4), 417-424.

Petzold, G. and Aguilera, J. (2009). Ice Morphology: Fundamentals and Technological Applications in Foods. Food Biophysics, 4(4), 378-396.

Phoon, P., Galindo, F., Vicente, A. and Dejmek, P. (2008). Pulsed electric field in combination with vacuum impregnation with trehalose improves the freezing tolerance of spinach leaves. Journal of Food Engineering, 88(1), 144-148.

Sigma-Aldrich. (2018). Fluorescein diacetate F7378. [online] Available at: https://www.sigmaaldrich.com/catalog/product/sigma/f7378?lang=en&region=SE [Accessed 24 Jul. 2018].

Taiz, L. and Zeiger, E. (2010a). Plant physiology. Sunderland: Sinauer Associates, 2-5.

Taiz, L. and Zeiger, E. (2010b). Plant physiology. Sunderland: Sinauer Associates, 57

Taiz, L. and Zeiger, E. (2010c). Plant physiology. Sunderland: Sinauer Associates, 174

Taiz, L. and Zeiger, E. (2010d). Plant physiology. Sunderland: Sinauer Associates, 607-608

United Nations Sustainable Development. (2018). Sustainable consumption and production. [online] Available at: https://www.un.org/sustainabledevelopment/sustainable-consumption-production/ [Accessed 5 Jul. 2018].

Thermo Fisher Scientific. (2018). Propidium Iodide. [online] Available at: https://www.thermofisher.com/order/catalog/product/P3566 [Accessed 24 Jul. 2018].

Velickova, E., Tylewicz, U., Dalla Rosa, M., Winkelhausen, E., Kuzmanova, S. and Gómez Galindo, F. (2013). Effect of vacuum infused cryoprotectants on the freezing tolerance of strawberry tissues. LWT - Food Science and Technology, 52(2), 146-150.

Wu, X., Zhang, M., Adhikari, B. and Sun, J. (2016). Recent developments in novel freezing and thawing technologies applied to foods. Critical Reviews in Food Science and Nutrition, 57(17), 3620-3631.

Xin, Z. and Browse, J. (2000). Cold comfort farm: the acclimation of plants to freezing temperatures. Plant, Cell and Environment, 23(9), 893-902.

Yusof, N., Rasmusson, A. and Gómez Galindo, F. (2016). Reduction of the Nitrate Content in Baby Spinach Leaves by Vacuum Impregnation with Sucrose. Food and Bioprocess Technology, 9(8), 1358-1366.

Zimmermann, U. (1982). Electric field-mediated fusion and related electrical phenomena. Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes, 694(3), 227-277.