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INCREASING THE RECOVERY RATE OF ARUGULA CELLS AFTER PULSED ELECTRIC FIELD TREATMENT

The effectivity of vacuum impregnation with glycerol and secondary metabolites on electroporated arugula leaves to improve freezing stability.

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Master thesis

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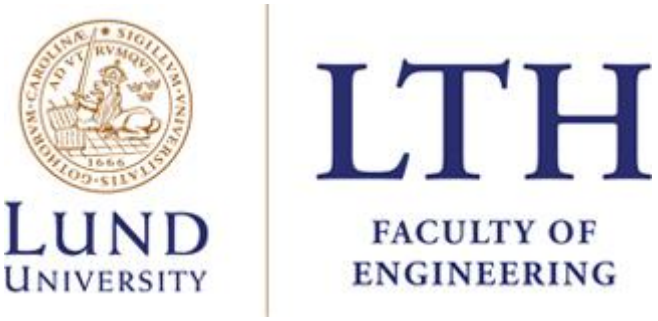

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Popular science Abstract

Increasing cell metabolism by vacuum impregnation of secondary metabolites in electroporated arugula.

This study will delve into the effects of vacuum impregnation of substances in combination with pulsed electric field on the cell behavior in arugula cells. This data will be used in order to develop freeze resistant arugula.

Arugula, commonly known as rucola or rocket, is a popular mustard green and is a staple food in Mediterranean areas. Which like many, does not like to be frozen. Freezing is one of the most well-known processes for food preservation. Unfortunately, it also has its drawbacks. When you freeze food products, freeze damage occurs. This often degrades product quality through dehydration of the cells. This is why it would be beneficial to invent a treatment after which arugula can be frozen. One theory for this is by using pulsed electric field and vacuum impregnation technology. Pulsed electric field is where small short pulses of electricity are fired through the arugula leaves which damages the plant tissue. This damage creates pores in the cell, which can be used to transport substances into the cell. The cell is then able to rebuild the cell over a period of time. Which can also be improved by adding secondary metabolites, which are substances that are naturally found in the plant cells, but in lower quantities. But how do we transport the substances? With vacuum impregnation. Vacuum impregnation is a method that uses pressure levels to introduce substances into products. The arugula is placed into a substance that helps improve the freezing resistance. When the pressure drops, gas in the cell expands. And when the pressure rises again, the gas will shrink and due to pressure differences inside the product, it will draw in the substance.

In this study, the combination of vacuum impregnation and pulsed electric field technology on the recovery after pulsed electric field technology was studied.

This was done by first vacuum impregnating the leaves with secondary metabolites and glycerol (glycerol improves the freezing resistance) and then treating the arugula with pulsed electric field technology. After this, the recovery of the arugula was studied. This was done by measuring the electrical resistance. The electrical resistance was measured because the damage of the pulsed electric field, causes substances to leech out of the cells, which reduces the electrical resistance. But as the cell recovers, the electrical resistance will increase again because the cell is taking up the substances again.

Results showed that vacuum impregnation with the secondary metabolites reduced the time the plant needed to recover after electroporation. It also showed to increase the survival rate after pulsed electric field treatment.

Abstract

Freeze damage is a common issue in many food items. The cellular dehydration caused by freezing can be prevented through addition of a cryoprotectant. Research has shown that it is possible to improve the freezing tolerance of plant materials by addition of cryoprotectants, which forms hydrogen bonds with water molecules. However, the cryoprotect needs to be in the cell to function. Which is where vacuum impregnation can be applied. Vacuum impregnation is a method of transfecting substances through pressure fluctuations. As the pressure drops, gas will be forced out of the intracellular space and solutes can be transfected into the intracellular space until an equilibrium is formed. However, the cryoprotectant needs to be within the cell membrane, where the vacuole is. Small pores can be formed on the cell membrane by using pulsed electric field technology (PEF). Hydrophilic pores are formed in the lipid bilayer of a cell membrane when exposed to electric pulses. As a result, hydrophobic pores are formed in the cell membrane by spontaneous thermal fluctuations of membrane lipids. If the voltage is too high or the number of pulses is too high, the cell dies. In some cases, with the right parameters, reversible electroporation can be achieved. This means that the cell is able to recover to its original state, with a closed cell membrane.

In this study, the effect of vacuum impregnation of arugula with glycerol solutions with secondary metabolites, in combination with PEF on the recovery rate, PEF survival rate, and freezing survival rate of arugula cells was studied.

The electroporation causes solutes to leech out of the cell, which can be measured by measuring the resistance. 100 Hz was used to measure the resistance of the arugula. At higher frequencies, the electrical current goes through the cell, whereas with lower frequencies, the electrical current goes around the cell, where the leeched out solutes will reduce the resistance. This reduction in resistance can be picked up by measuring the resistance over time, and as the cell recovers, the solutes are taken up by the cell and the resistance increases again.

Microscopy showed that bipolar electroporation with a pulse width of 1000μs, a pulse space of 20μs at 1000 V/cm with 1000 loops gave homogenous electroporation in arugula cells. Using these pulsed electric field and vacuum impregnation with different substances it has been shown that after only electroporation 40% of the leaves survived and recovery of the cells took 8 hours, no leaves survived the freezing experiment. Vacuum impregnation of different substances reduced the recovery time up to 3 hours and increased the freezing resistance from 0% to 10%.

Abbreviations & terminology

Below shows a list of abbreviations used throughout the document and their meaning.

Term	Definition
PEF	Pulsed electric field
VI	Vacuum impregnation
EP	Electroporation
Electroporation	Application of Pulsed Electric Field technology on material.
Recovered	The leaf has regained 95% of its initial resistance.
Dead leaf	The leaf shows has lost most of its turgor, thus the cells have not survived the treatment(s).

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1. Background

Arugula has been a staple ingredient in Mediterranean cuisine for many years. Where it is commonly consumed because of its nutty flavor. In Italy, arugula is commonly consumed as leafy vegetable, in salads and as topping for pizzas. Not only in Italy are pizzas a staple dish, in most western countries pizzas are eaten in large quantities. However, most of them, are not freshly made. They are prepared and then frozen, to be sold in the supermarkets for customers to bake them at home. This provides a challenge, since it would be wasteful to purchase a bag of arugula just to top several pizzas. But wouldn't it be possible to simply provide the arugula inside the pizza's package? The answer would be no. The arugula would wilt since the cells inside the arugula would die due to the freezing. Arugula cells, like many other plant cells, are not freeze resistant. During the freezing, ice crystals are formed which rupture the cells and the cell will stop functioning. This will cause the leaf to wilt and greatly lose its sensory attributes when thawed, which it is so famous for (Phoon et al., 2007). This slimy leaf would not be very pleasant to put on top of your delicious pizza.

Another reason why freezing would be interesting is due to the fact that in Scandinavia, amongst other northern European countries, arugula is often imported from Mediterranean countries due to the climate required for the growth. However, the logistic process of this is very time consuming, and is already impacting the shelf life. However, if it would be able to freeze the leaves, it would be possible to ship them frozen and thaw them when required. Which would make seasonal fluctuations in supply of lesser significance.

One theory is that if you can impregnate the cells with a cryoprotectant, the cell will be able to return to normal function after thawing (Gómez Galindo, 2017). This is due the fact that cryoprotectants are able to form hydrogen bonds with water molecules (Rouwkema et al., 2008). This cryoprotectant would need to be inside the plant material to function, due to the fact that when freezing damage occurs in the intracellular liquid, organelles can become damaged. This can be achieved through vacuum impregnation (VI) (Demir et al., 2018). However, since the cell membranes are not porous, the cryoprotectant will only enter the extracellular space of the plant tissue, which would limit its functionality.

This brings us to the next process, which is the application of a pulsed electric field (PEF) treatment. The PEF induces a large number of small electrical pulses through the plant tissue (Gómez Galindo, 2017). This will cause hydrophobic pores to be formed in the cell membrane by spontaneous thermal fluctuations of membrane lipids (Kandušer & Miklavčič, 2009). These pores could allow for infusion of the cell with solutes. Using the PEF at low voltages, allows the cells to recover afterwards, which means they will return to their normal cell function. Application of PEF technology in combination with vacuum impregnation would allow for a high effectivity of infusion with the cryoprotectant into the plant cells.

This study will give an insight to cell behavior after electroporation and the effect of vacuum impregnation of secondary metabolites on cell recovery, cell membrane repair and cell metabolism. This knowledge will contribute to further development of freezer resistant plant leaves and improving cell metabolism using secondary metabolites.

1.1 Objectives

Within this study, several objectives are set which are to be studied.

This research will:

- Study the effect of vacuum impregnation of glycerol and secondary metabolites on the survival rate of arugula leaves.
- Study the effect of vacuum impregnation of glycerol and secondary metabolites on the recovery rate of arugula leaves.
- Study the effect of vacuum impregnation of glycerol and secondary metabolites on the freezing survivability of arugula leaves.

2. Theoretical background

Arugula is the collective name for several species of the Brassicaceae with pungent leaves. But most commonly it is the Eruca Sativa as shown on figure 1 (Morales et al., 2002). Which is the species used for this project. However, for simplicity's case, it will be referred to as arugula.

Figure 1 Eruca Sativa leaves (Amazon, 2020)

Arugula is consumed worldwide. It is consumed as a green, as part of a salad mix, as a cooked green and as pizza topping. The arugula is very popular due to its pungent properties (Morales et al., 2002). It is a fast growing crop which flowers under long days and high temperatures. It appears to be well adapted to the Midwest circumstances, which indicates that it can be available over a long period using season extension techniques, which can be made available in a greenhouse culture (Morales et al., 2002).

2.1 The leaf

Leaves are the organs of plants responsible for photosynthesis (Tsukaya, 2013). Arugula is autotrophic, meaning it does not obtain energy from other organic materials, rather it creates energy from photosynthesis. Energy from light is captured and used to make simple sugars, from carbon dioxide and water. These sugars can then be stored by in the shape of starch and further processed by chemical synthesis to form more complex organic molecules, like protein or cellulose. Cellulose is the structural material in plant cell walls (Feugier, 2006).

The leaves consist of three major tissue systems as shown in figure 2 (Coté, 2009).

- The epidermis covers the upper and lower surfaces. This layer is impermeable to water and forms the boundary which separates the plant's inner cells from the outside. The epidermis' most important function is to protect against water loss from transpiration
- Mesophilic tissue inside the leaves is where most of the photosynthesis in the leaf occurs.
- The stomata (or stoma), is a pore, found in the epidermis of leaves, stems, and other organs, that facilitates gas exchange.
- And the vascular tissue, which is an arrangement of veins that is responsible for transportation of water and minerals from the roots into the leaf.

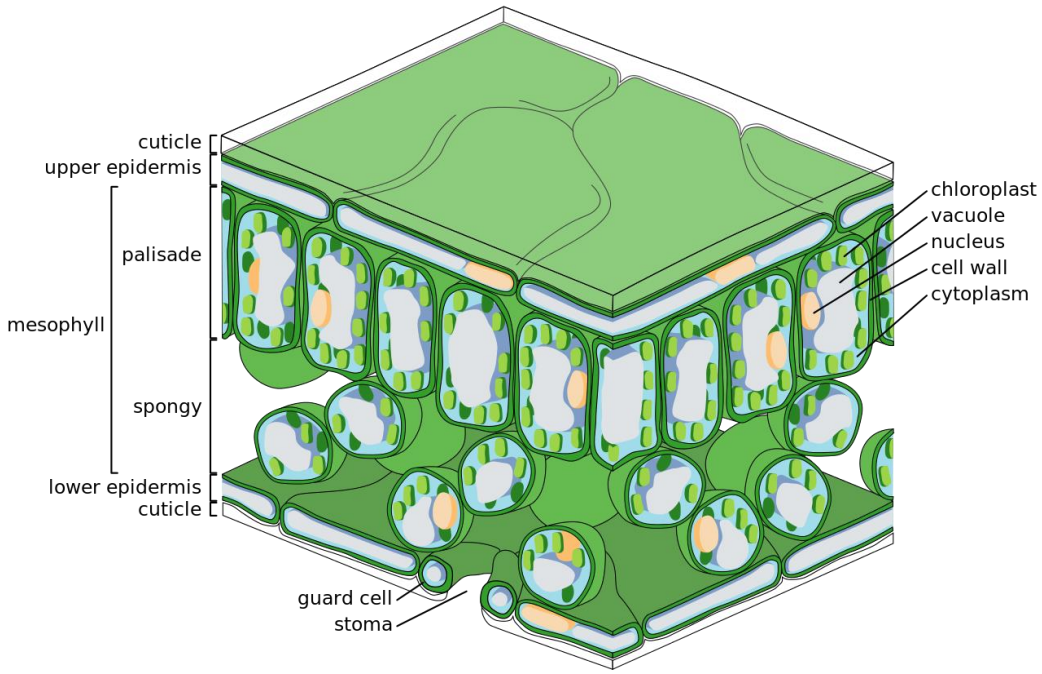


Figure 2 General structure of plant leaves (from Coté, 2009)

The cells inside the leaf are eukaryotic, which has distinctive features such as primary cell walls, which contain cellulose, hemicellulose and pectin. Many types of plant cells contain a central vacuole, which is filled with water. This vacuole controls the turgor inside the cell as well as the movement of molecules (Raven, 1997). A detailed schematic of a plant cell is shown in figure 3 below.

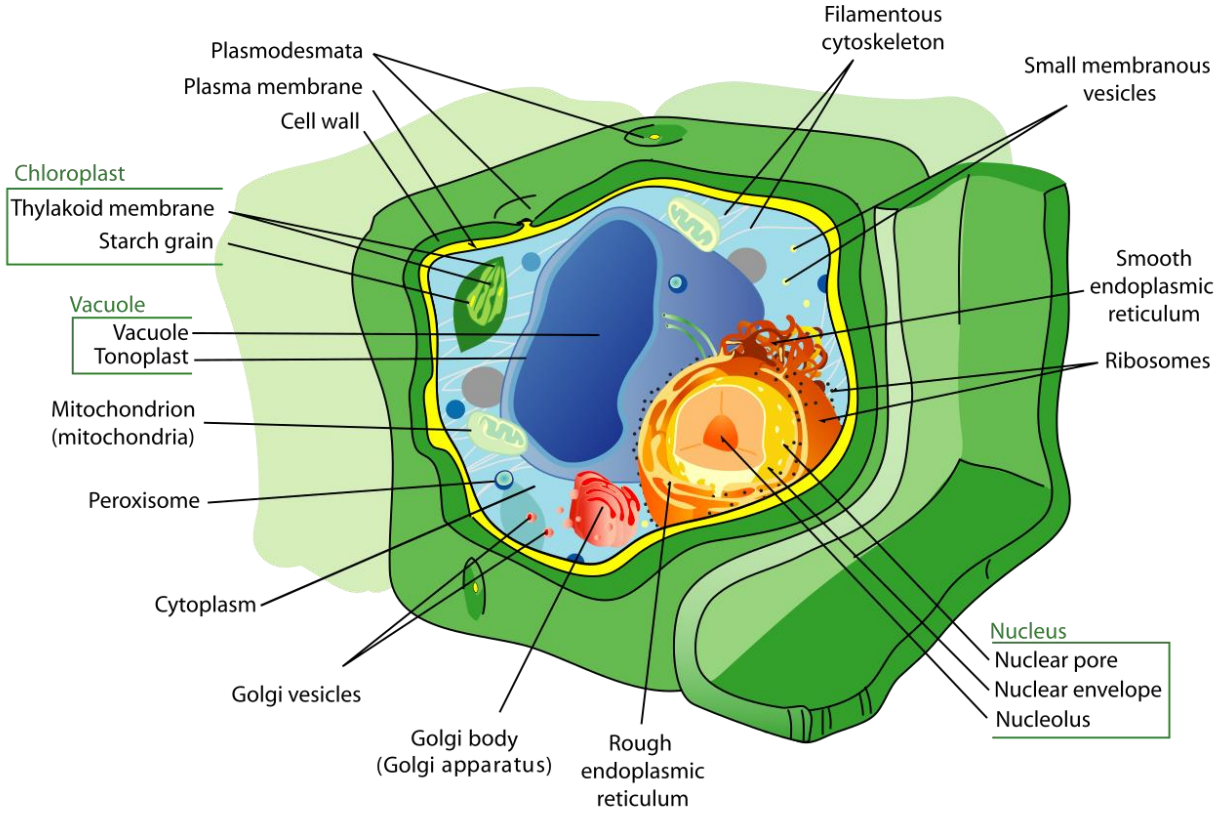


Figure 3 general structure of a plant cell (from Wikipedia, 2019)

2.2 Vacuum impregnation

For the cryoprotectant to function, it would have to be available to form hydrogen bonds within the cell. But for the cell take up the cryoprotectant, it would need to be present in the intracellular space. The availability of the cryoprotectant in this intracellular space will allow the cell to use its own transport mechanisms to take up the substance(s). This can be achieved using vacuum impregnation. This phenomenon occurs in vacuum impregnation due to the fact that the changing pressure levels expands the gas within the porous cells.

With vacuum impregnation, the pressure is lowered from atmospheric pressure. As the pressure lowers, the gasses inside the plant material expands due to the lowering pressure levels. This increase in volume will force the gas out of the intracellular medium. After an equilibrium is found, the pressure will slowly increase again. This increase will decrease the volume of the gas and will create an imbalance. This imbalance will cause for the plant material to take up the liquid and solutes it is placed in, until an equilibrium is found (Gras et al., 2003; Hui & Evranuz, 2015; Chiralt et al., 2001).

In Vacuum impregnation, there are two mechanisms that occur. The hydrodynamic mechanism (HDM) and the deformation-relaxation phenomenon (DRP). In HDM mechanism, the material is transported in and out of a porous material by vacuum diffusion and natural diffusion of the changing pressure levels. Whereas the DRP is a structural change which occurs in the product as a result of the hydrodynamic mechanism (Hui & Evranuz, 2015). The process of vacuum impregnation is described below.

Firstly the arugula is submerged in the solution. The vacuum impregnation is yet to start and the internal pressure in the pores is equal to the external pressure, which is at atmospheric levels.

At the initial stage of the vacuum impregnation, the volume of gas is increased due to the lowering of the pressure level, and due to the increase in volume, gas is leaving the cell until an equilibrium is reached.

At the second stage, the gas flow stops and the capillary pressure is induced, which initiates a mass transfer from the solution to the pore until another equilibrium is formed.

When the vacuum pressure returns to atmospheric pressure, the capillary pressure is increased due to the pressure difference. This induces an increase in transfer of the submerged solution into the pores. The solution will initially fill the porous space of the arugula, which is the extracellular space of the plant tissue. This solution will then enter the cell through its own transfer mechanisms and osmosis (Nyoto, 2019 & Hui et al., 2016).

2.2.1 Substances used in vacuum impregnation

There are several substances that can be beneficial to be vacuum impregnated, with various aims.

Glycerol

The damaging factor in the freezing process is the dehydration occurring during freezing. Cryoprotectants such as glycerol however, easily form hydrogen bonds with water molecules due to the chemical structure (figure 4). This reduces the formation of ice crystals and reduced dehydration that is occurring during freezing (Dashnau et al. 2006).

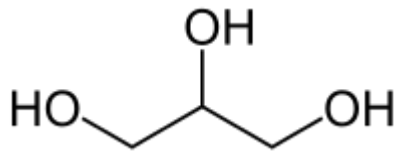


Figure 4 Glycerol structure

Vitamin B1

Vitamin B1 (thiamin) is essential for cell energy supply and plays an important role in metabolism. Vitamin B1 is synthesized predominantly in green tissues and is limited to plastids (Martinis et al., 2016). However, they do largely contribute do energy generating pathways in plant cells. It was shown that thiamin may act as a signaling molecule in response to biotic and abiotic stress (Goyer, 2010), allowing for faster stress response in plant cells. Furthermore, vitamin B1 has been found to respond to DNA damage in plant cells ranging from concentrations of 1.5um to 100um (Raschke et al., 2007) Vitamin B1 has the potential to play a key role in the recovery of cell damage caused by electroporation.

Folic Acid

Folic acid a key contributor in the methyl cycle which provides one-carbon groups for methylation reactions. Furthermore, folic acid is involved in the synthesis of many biomolecules such as amino acids, nucleic acids and vitamin B5. These functions make folic acids essential in plant cells. Folic acid is key in DNA synthesis and a significant part of the folic acid in the cell is located in the vacuole. Lower folic acid levels are associated with reduced nucleotide production in plant cells (Gorelova et al., 2017). Thus, impregnation with Folic Acid could significantly benefit the plant cell in its restorative capabilities.

2.3 Pulsed electric field

The main ideology behind the invention of the pulsed electric field technology leads back to exploring alternatives to heat induced microbial inactivation (Donsì et al., 2010). However, it quickly showed that the application of pulsed electric field (electroporation) was not just limited to microbial inactivation. The application of short and intense electric pulses can also be used for the introduction of different molecules into the cell, electrofusion, water treatment and enhancing the efficiency of pressing, extraction and drying processes in food production (Donsì et al., 2010).

However, there are two methods of electroporation with vastly different applications. When a higher voltage is applied, the cells are unable to recover their normal functionality due to the fact that the cell membrane is too damaged to recover due to the strength of the electrical pulses is larger than the target tissue is able to recover from (Garcia et al., 2010) (Donsì et al., 2010). However, when the electricity applied with the electrodes is lower than the target's tissue is able to recover from, the electroporation is reversible. The cells are able to repair their phospholipid bilayer and continue to their normal cell functions (Garcia et al., 2010).

The exact mechanisms of electroporation are yet to be fully understood. However, the most widely accepted is the transient aqueous pore model, which will be discussed in chapter 2.3. One thing most theories have in common though, is that the cell membrane plays an important role in amplifying the applied electrical field. Since the conductivity of the intact membrane is significantly lower than the conductivity in the extracellular medium and cytoplasm (Donsì et al., 2010).

Reversible permeabilization can be created when the electric field applied reaches values close to the critical value (E_c). The critical value indicates the point in between reversible and irreversible electroporation (Donsì et al., 2010). The reversible electroporation allows for the introduction of different molecules into the cell. Which in medicine, is commonly used for a more effective drug delivery (El-Andaloussi et al., 2012). However, it is not just limited to biomedical applications. A similar process can be used to introduce molecules into plant cells, for food and botanical purposes (Donsì et al., 2010). Reversible electroporation can be achieved when the cell is able to recover from the stress that is applied from the electrical field. If the stress becomes too much, the cell is unable to recover and dies. The amount of stress the cell can withstand is among other things, based on the cells biology. Depending on the growing conditions and the maturity and variety of the plant.

Research has shown that for arugula, the optimal treatment for survivability and porosity is achieved by electroporating with 500 pulses, a pulse width of 20μs, a pulse space 1000μs with an voltage of 1100V/cm (Nyoto, 2018). There is however, an area in between reversible and irreversible. By tweaking the settings, the stress can be increased, but the plant cells still survive, but not all of them. Some cells will survive and some won't. This makes it excellent for studying the effects of secondary metabolites. Using this mixture of reversible and irreversible, the effect of the secondary metabolites can be highlighted.

Resistance

To categorize the behavior of the leaf, the resistance over time will be determined. This will show how the cell membrane behaves and how solutes flow out of the cell as the membrane is electroporated. A leeching out of solutes, in combination with a damaged cell membrane will increase the conductivity, which results in a decrease in resistance.

The value of the impedance is strongly correlated to the frequency. At higher frequencies, the electrical current will go through the cell. At lower frequencies however, the current will go around the cell. In this area, you are able to measure the leeching out of the solutes after electroporation (Zimmerman & Williams, 1982). Which are shown by a decrease in resistance, due to an increasing conductivity by the solutes. This means that external stress factors, such as PEF can be determined using electrical resistance measurements in low frequency ranges. At high frequency, no difference can be determined between the impedance of intact cells and compromised cells (Donsì et al., 2010).

2.3.1 Influence of electroporation on plant cells

The transient aqueous pore model describes the formation of hydrophilic pores in the lipid bilayer of a cell membrane when it is exposed to electric pulses. As a result, hydrophobic pores are formed in the cell membrane by spontaneous thermal fluctuations of membrane lipids. After electroporation, the presence of an induced transmembrane potential provides the free energy necessary for structural rearrangements of membrane phospholipids and thus enabling hydrophilic pore formation (Barbosa-Cánovas et al., 1999) (Kandušer & Miklavčič, 2009). The induced transmembrane voltage that is subjected to an electric field should reach a certain value to trigger the formation of pores in the cell membrane (Kandušer & Miklavčič, 2009).

The hydrophilic pores that are formed can then be used to transfect new molecules into the cell. Transfection is the process of delivering organic compounds into eukaryotic cells, which are in this case; glycerol & secondary metabolites (Bio, 2020).

2.4 Freezing and thawing

Freezing damage

Freezing occurs when the cells cannot prevent nucleation. When the temperature drops below the freezing point of water (can vary 1-2°C in presence of solutes), the water molecules come together to form a stable ice nucleus. The lower the temperature, the lower the amount of water required to form a nucleus. Homogenous nucleation can be observed under temperatures of -38,5°C. However, cells with highly viscous contents are likely to form a glass, instead of freezing (Pearce, 2001). During the freezing, the cell matrixes influence tissue responses at freezing temperatures. One of the most disastrous effects of freezing is the propagation of ice which occurs between bordering cells (Acker et al., 2001). After an ice crystal is formed, ice propagation is initiated, causing a spreading of ice crystals, which leads to the tissue being frozen (Karel et al., 1975).

The reason freezing is an important aspect of this project is the damage caused by freezing. First of all, freezing causes cellular dehydration. The extent of the cellular dehydration is largely dependent on the freezing parameters. Secondly, cells die when the limit for supercooling is exceeded. And lastly the effect of mechanical stress from large ice crystals on the tissues or structures of the plant material (Pearce, 2001; Xin & Browse, 2000).

The size distribution of ice crystals is not necessarily stable. This distribution might change over time in storage, where the system is minimizing its free energy by redistributing the ice crystals. The free energy is minimized if a crystal form with a lower free surface energy is obtained or if the total surface area of the crystals is minimized (Karel et al., 1975). The way that freezing induces cellular dehydration is due to the fact that the water potential of ice is smaller than the water potential of liquid water. As a result, the extracellular ice crystals grow by drawing water from the cytoplasm through the plasma membrane by osmosis, until the potential of both the ice and the cell are equal. which causes dehydration in the cell. The extracellular ice is formed because the intercellular fluid's freezing point is higher than the cytoplasm's. The membrane will be damaged when the dehydration caused by freezing exceeds the tolerance of the cell(s). This dehydration results in a reduction of cell volume, loss of structural water and the structural integrity of the cell membrane (Pearce, 2001; Bhandal et al., 1985).

During storage of frozen products, recrystallization might occur. Which is initiated due to fluctuations of storage temperature. These temperature fluctuations induce the growth of larger ice crystals and causes the smaller ice crystals to disappear, resulting in a reduction of the total ice crystals, which leads to increased cell damage and thus further increasing dehydration. This phenomenon is initiated due to the fact that all thermodynamic systems will over time grow into a state of minimum energy, with the aim to decrease surface energy in the crystalline faze by growing large ice crystals at the cost of smaller ones (Arora & Palta, 1991).

The freezing itself is often less damaging than thawing. During thawing, the cells spent much more time in the most damaging temperature, which is just below the freezing point of water (Karel et al., 1975).

3. Materials & methods

In this chapter, all methods and materials used in this study are described and explained.

3.1 Raw material handling

For most experiments, arugula grown in the greenhouse were used. However, due to a thrip infestation, the development of the method was done using arugula from Svensk Cater Malmö AB.

3.1.1 Commercial leaves

The leaves from Svensk Cater Malmö AB were classified by size and turgor. The leaves used in experiments were 7.0 cm (±1.0 cm) in length, and the width was 3.5 cm (±0.5 cm).

The arugula from Cater Grönt originate from Italy and the logistics cover 4 days before it reaches the warehouse in Malmö. The leaves are stored at 2-6°C, washed and packed within 4 days of arrival, the total transit time being 8 days after harvest before usage in experiments. The arugula then has a shelf life of 9 days (Cater grönt, 2020). After arrival at Lund University, the leaves were sorted by size and stored in the fridge until experiments commence.

3.1.2 Greenhouse arugula

The arugula was grown in a greenhouse in the biology department of Lund University. Seeds were planted weekly to sustain the demands for the experiments. The growing medium was prepared using Weibulls potting soil and perlite (90:10). The arugula seeds were bought from Enza Zaden under the brand name Prudenzia F1. Three trays (56 x 25 x 6 cm) were filled with 2.5kg of the soil mixture. Within the soil, three rows of 7 holes that were 2 cm deep were made. Each hole was then filled with 5-7 seeds and sealed off with soil. The arugula was grown under 200W HPI-T (High Wattage Quartz Metal Halide) lamps, operating 16 hours a day. Trays were rotated every other day to evenly distribute the light the arugula was receiving through the glass panels in the greenhouse. The plants were watered daily and fertilized one and three weeks after planting. 14g of YaraTera KRISTALON YELLOW (13-40-13 + B, Cu, Fe, Mn, Mo and Zn) fertilizer was added to two liters of tap water and distributed to the arugula. The trays were rotated clockwise every other day to evenly distribute incoming light from the windows of the greenhouse. The greenhouse was maintained at 16-18°C and 45-55 % RH and the leaves were harvested every Monday after 7 weeks. And experiments were initiated the same day as the plants were harvested, limited to one sample per week. The harvested leaves were kept in a Tupperware box with wet paper towels on the bottom and a plastic cover as described in 3.1.3. This prevented dehydration of the arugula leaves.

3.1.3 Storage

To prevent any (de)hydration, the leaves were kept in a Tupperware container (24 x 17 x 5,5 cm) with moist paper towels. 5 layers of paper towels were placed on the bottom of the container and soaked in 10 grams of tap water (17°C) the paper towels were covered with the lid of a smaller Tupperware container (21 x 15 cm). Leaves were numbered and placed on top as shown on Figure 5.

Figure 5 Ten leaves were placed in a plastic container with moist tissues on the bottom.

The Tupperware container was stored in the fridge at 4°C ± 2°C and leaves were used the same day as they were harvested.

3.2 Solutions

The following solutions were prepared for the experiments.

Propidium iodine solution for nuclei staining

125µM PI solution (78 µS) was made by dissolving 0.0835g of propidium iodine to 1L of distilled water from the Milli-Q IQ 7000.

250 µS conductive solution

20mM sodium chloride solution was made by adding 1.25g of sodium chloride to 1L of distilled water from the Milli-Q IQ 7000. Distilled water was then added until a conductivity of 250 µS was reached.

Vacuum impregnation solutions

Isotonic glycerol solutions were made using the concentrations stated in table 1.

Table 1 Vacuum impregnation solutions

Name	Glycerol % (V/V)	Vitamin B1 % (W/V)	Folic Acid % (W/V)
Pure glycerol	11	0	0
Glycerol & Vitamin B1	11	50 µmol/L	0
Glycerol & Folic Acid	11	0	400 µmol /L

3.3 Treatments

The treatments of VI, PEF, freezing & thawing were performed on ten leaves. Ten leaves were harvested the same day of the experiments from seven week old arugula plants and twenty leaves were harvested to assess the impact on freezing resistance.

The order of the experiments & measurements is as follows;

Measure resistance→ VI → Measure resistance→ PEF → Measure hourly (8 hours) or until recovered.

3.3.1 Vacuum impregnation

Lukhava, 2020 determined that a isotonic 11% glycerol solution could be used to improve freezing resistance without any noticeable off-flavors. The three different solutions from table 1 were used for vacuum impregnation. Vacuum impregnation started at an atmospheric pressure of 1000 mbar, from which the pressure was reduced to a pressure of 90mbar over a time period of 15 minutes. Once it reached 90mbar the pressure was maintained for one minute, after which the vacuum was slowly released over a ten minute period until atmospheric pressure was reached.

3.3.2 Pulsed electric field

The electroporation protocol developed by Nyoto (2019) was applied with adjustments of some parameters, as described in table 2. Electroporation was bipolar with the settings in table 2.

Table 2 PEF settings

Name	Pulse Width (us)	Electric field strength, V/cm	Pulses	Pulse space (us)
Nyoto, 2019	20	1100	500	1000
De Kuijer, 2020	20	1000	1000	1000

Electroporation was performed directly after vacuum impregnation.

3.3.4 Combination of vacuum impregnation and pulsed electric field.

To more effectively deliver solutes into the cell, a combination of VI & PEF was used. Four different samples were set up to study the effect of VI, PEF and a combination thereof, as shown in table 3.

Table 3 Experiment setup

Name	VI	PEF	Glycerol	Vitamin B1	Folic Acid
PEF	No	Yes	No	No	No
VI G	Yes	Yes	Yes	No	No
VI B1	Yes	Yes	Yes	Yes	No
VI FA	Yes	Yes	Yes	No	Yes

3.3.5 Freezing & resting

20 arugula leaves per sample were first vacuum impregnated and then electroporated using the settings mentioned in table 2 and 3. After these treatments, the leaves are allowed to recover for 72 hours. This was done using the method described in 3.1.4.

After 72 hours, the arugula was blotted to remove any excess liquids. After which, they are transferred to Tupperware box without moist paper towels and were placed in the Opti Freeze freezer at Opti Freeze AB and stored at -22°C for 24 hours.

3.3.6 Thawing

After being in the freezer for 24 hours, the leaves are removed from the box and thawed at room temperature on paper towels for 5 minutes. After 5 minutes, the turgor was determined by placing the leaves on a ruler. Wilted leaves were classified as dead, leaves that showed any turgor were classified as viable.

3.4 Analysis

To determine the effect of different treatments and samples, several analyses were conducted.

3.4.1 Microscopy

To verify homogenous electroporation, leaves were submerged in a 125µM propidium iodine solution in the PEF chamber and analyzed under a microscope (Nikon ECLIPSE Ti – u) to verify homogenous staining of the nuclei.

3.4.2 Resistance

The electrical resistance was measured using a Hewlett Packard 4192A Impedance Analyzer 5Hz – 13MHz. The samples were measured before any of the beforementioned treatments, after each treatment, and hourly for 8 hours after PEF & VI + PEF.

Resistance of 10 arugula leaves, harvested on the same day was measured. Experiments were done over a period of 2 weeks with the age of the harvested plants being between 49 & 51 days (7 weeks). The resistance was measured by placing covering two stainless steel electrodes (1.0 x 0.5 cm) with filter paper (1.0 x 0.5 cm) (MN 713 MACHEREY NAGEL, Germany) and wetting them by pipetting 10 µL of NaCl conductive solution (250µS) onto them. The electrodes are then placed into a Styrofoam box with 5 sheets of moist paper towel (10 grams of water) to prevent dehydration. The arugula was placed in the electrode in a sandwich-like fashion, securing them using a washing pin on leaf, as seen in figure 6 below. This was repeated for each sample. After each treatment (electroporation, vacuum impregnation), resistance was directly measured. Leaves were then put in the Tupperware box and stored in the fridge until the next measurement. Samples were measured for 8 hours or until recovered. The recovery threshold was set to 0.95 of the initial value due to the fact that the resistance stabilizes once it reaches that threshold. All measurements within one series were conducted the same day, directly after harvesting.

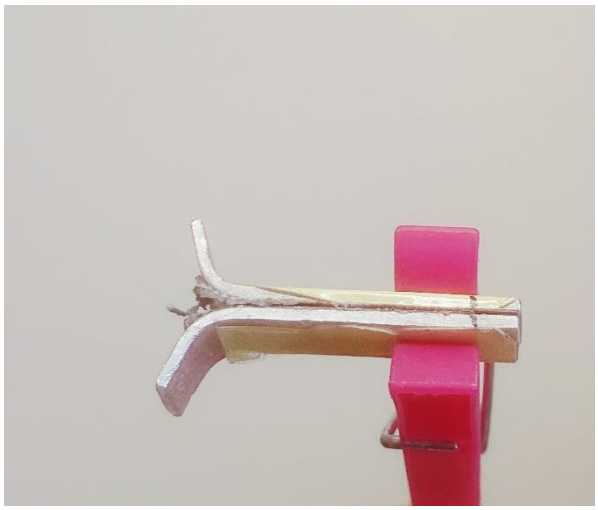


Figure 6a Laundry pin placement

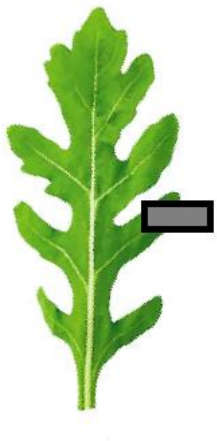


Figure 6b Electrode attachment on arugula leaf

3.4.3 Method development

The best method to measure leaves has been developed overtime, adjusting several parameters and even switching equipment in order to get the most reliable measurements.

Electrode size

0.5 x 1.0 cm electrodes were compared with 1.0 x 1.0 cm electrodes. 0.5 x 1.0 cm gave reproducible results as opposed to the 1.0 x 1.0 cm electrodes.

Volume of conductive solution

As the filter paper needed to be moist enough to support an electrical current throughout but not too moist so that the leaf could absorb moisture. Experiments were run with 15, 20 and 25 µL per cm^2. 20uL gave the most consistent results.

Impedance meter

The initial idea was to conduct the measurements on the ADITUS Medical CythorLab AMP02 – 001. However, due to the nature of the measurement the Aditus deemed to be too inconsistent for this purpose. Thus the switch was made to the Hewlett Packard 4192A Impedance Analyzer 5Hz – 13MHz.

Frequency

100 Hz showed to be more reliable than 1 kHz and 10 kHz to study the effect of PEF on the cell membrane recovery over time after electroporation.

Storage conditions

Initially, the leaves were separated from the moist paper towels by a plastic gauze. The small holes in the gauze however were still too large and contact between the leaf and paper towels was possible. The switch was then made to cover the paper towels with a smaller Tupperware lid. This also made numbering and tracking the leaves far easier.

Laundry pin placement

As the resistance is also dependent on the pressure applied by the electrodes and filter paper onto the leaf, the pressure needed to be identical with each measurement. Best results were achieved by placing the laundry right before the contact point of the electrodes

3.4.4 Control

To exclude any influence on the resistance from storage conditions, a control was set. This was done by measuring the resistance over time of 5 freshly harvested leaves from the greenhouse. The leaves were stored using method 3.1.4.

3.4.5 Wilting test

To determine whether or not the arugula leaf has survived the treatments and freezing, a wilting test was performed. In this test the turgor of the leaf will be assessed. The thawed leaves were placed on a ruler and turgor was assessed using the force of gravity as seen in figure 7. Wilted leaves were classified as ‘dead’ and whereas leaves with turgor were classified as ‘alive’.

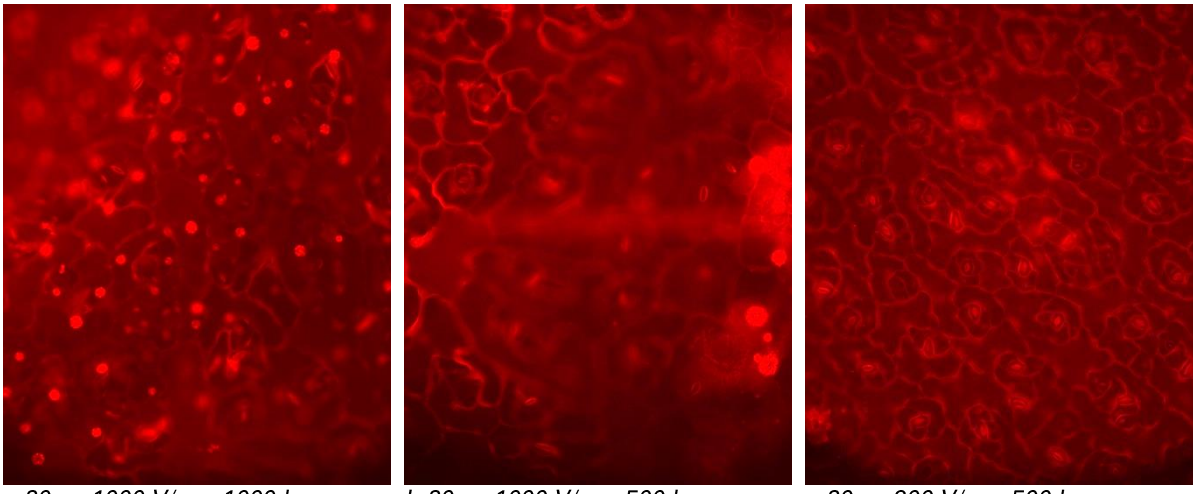
Figure 7 wilting test

4. Results & discussion

4.1 Microscopy

Microscopy was used to determine whether or not the electroporation was successful. Below shows an effective electroporation with homogenous staining of nuclei by the PI solution (Figure 8**Error! Reference source not found.**a), non-homogenous e lectroporation of the leaf (Figure 8b) and lastly, an unsuccessful electroporation (Figure 8c), in which the PI is seen in the vacuole of the cell.

Figure 8 PI staining after homogenous (a), semi-homogenous (b) and unsuccessful (c) electroporation



a 20µs, 1000 V/cm, 1000 loops *b 20µs, 1000 V/cm, 500 loops* *c 20µs, 900 V/cm, 500 loops*

This shows the importance of having the right settings for electroporation. Even though a reduction in resistance might be shown, this does not necessarily mean the electroporation has been homogenous.

4.2 Resistance measurements

Solutes leeching out into the cell will show a strong decrease in resistance due to the increase of conductivity around the cell from the solutes. As the solutes are slowly being recovered by the cell, resistance will increase over time Arora & Palta (1991) have shown that partially damaged cells will reabsorb leaked ions. Meaning that if the cell is able to fully reabsorb the leaked ions, it can recover its functions as well. Thus, as the resistance is closing in on the initial resistance before PEF, the leaked ions are nearly fully recovered, it can be concluded that the cell is recovered as the solutes are no longer present in the extracellular space. Due to the nature of arugula leaves, large variations might occur in absolute resistance values. Which is why it has been chosen to assess data in relative values. The initial resistance will be marked as 1.00 and following measurements will be relative to the initial 1.00. This will visualize the effect of VI and PEF on the recovery time of each individual leaf.

4.2.1 Recovery rate after PEF

Recovery rate of the surviving leaves was measured and plotted after PEF and is shown in figure 9. Please note that PEF takes place after 15 minutes (0.25 on the x axis).

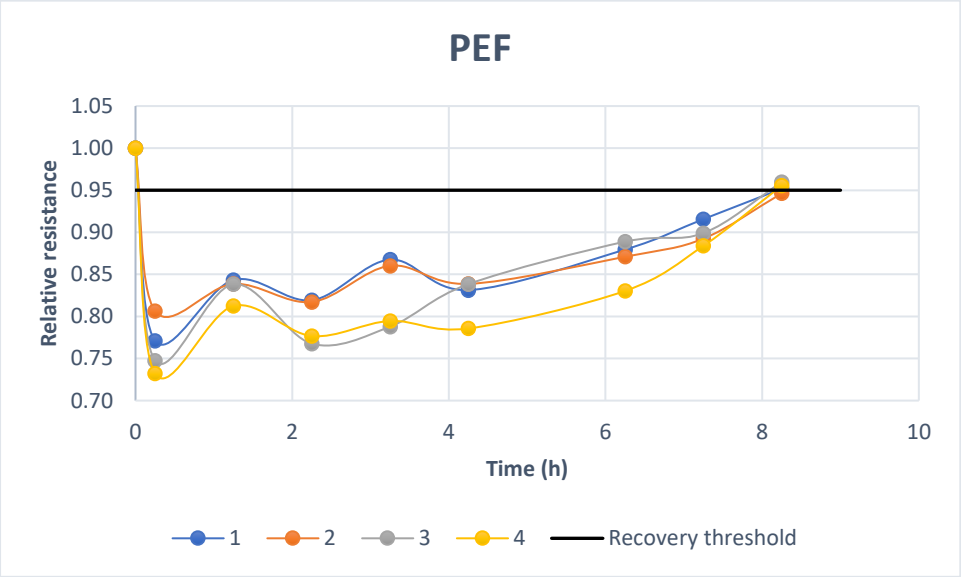


Figure 9 Recovery rate of arugula treated with pulsed electric field

The samples treated with PEF all show a similar recovery trend with recovery taking up to eight hours after PEF.

4.2.2 recovery after the application of VI with different substances and PEF

Samples were treated with VI with solutions described in table 1, followed by PEF treatment were measured. The resistance of these samples was monitored over a time period of 8 hours.

Figure 10 shows the recovery time of the recovering samples which were vacuum impregnated with glycerol and electroporated.

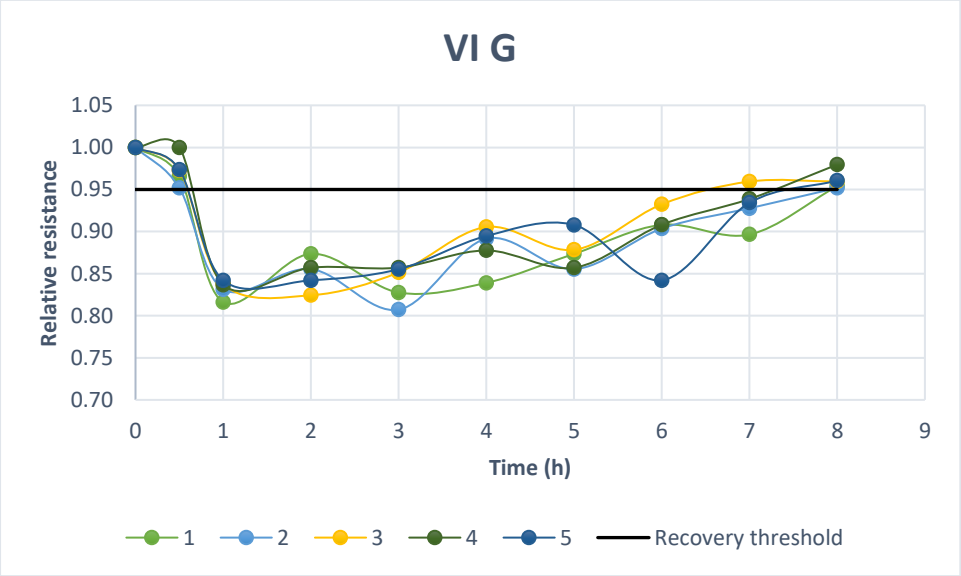


Figure 10 Recovery rate of arugula leaves treated with PEF & VI (11% glycerol)

Figure 11 shows the recovery time of the recovering samples which were vacuum impregnated with folic acid and electroporated.

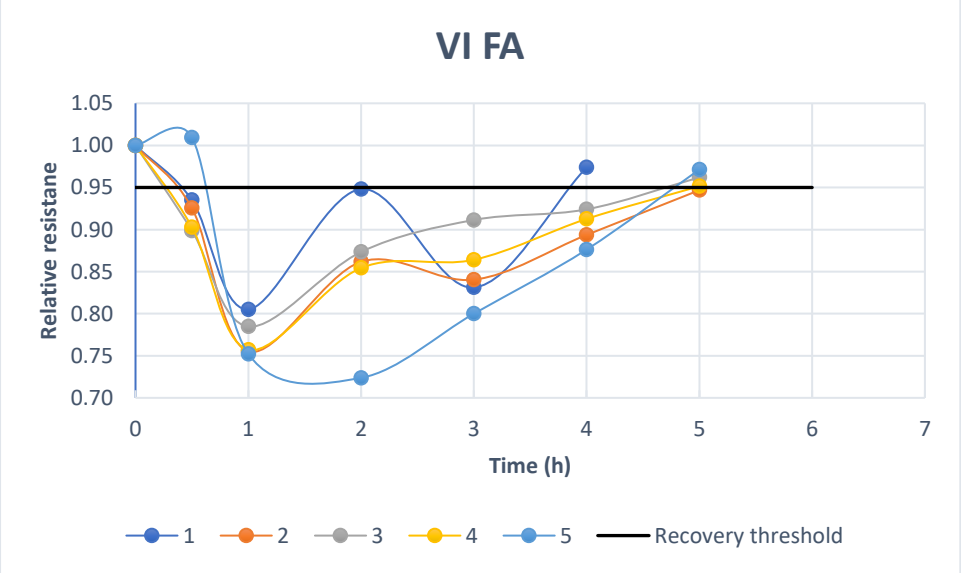


Figure 11 Recovery rate of arugula leaves treated with PEF & VI (11% glycerol, 400umol folic acid)

Figure 12 shows the recovery time of the recovering samples which were vacuum impregnated with folic acid and electroporated.

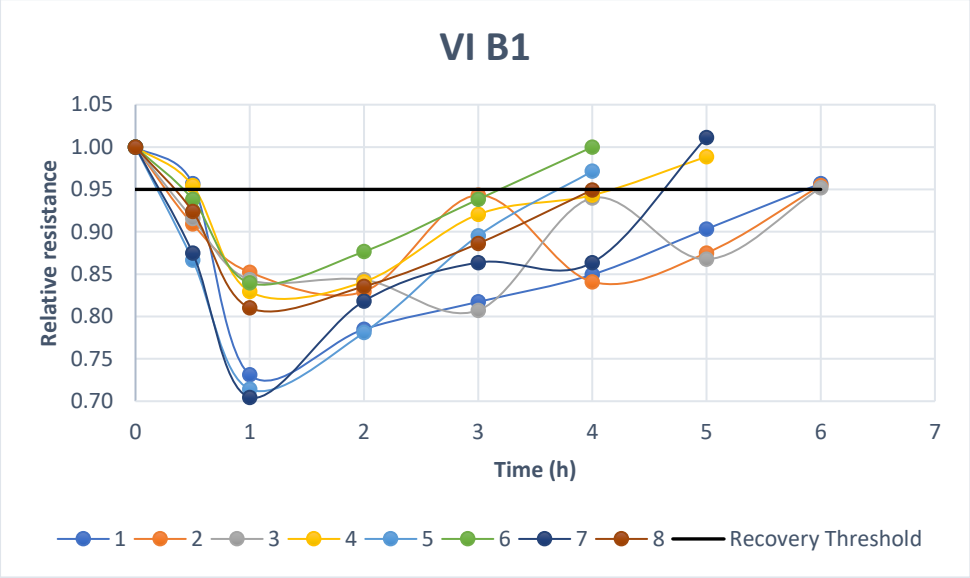


Figure 12 Recovery rate of arugula leaves treated with PEF & VI (11% glycerol, 50umol vitamin B1)

Comparing these results, it can be shown that there is a significant increase in the recovery rate ($P<0.05$) of the samples vacuum impregnated with secondary metabolites which is shown in table 4 below. The only samples that showed no significant difference ($P<0.05$) between the recovery time were the samples VI FA and VI B1.

The data also shows an indication of an increased survival rate after impregnation with secondary metabolites. To claim this however, replications should be conducted.

Table 4, survival rate and recovery time.

Sample	Survival rate	Slowest recovery (h)	Fastest recovery (h)	Avg. recovery (h)
PEF	4/10	8	8	$8.0 \pm 0_{ab}$
VI G	5/10	7	6	$6.8 \pm 0.4_{ab}$
VI FA	5/10	4	3	$3.8 \pm 0.4_{a}$
VI B1	8/10	5	3	$4.0 \pm 0.8_{b}$

4.2.3 Control

Due to the nature of arugula leaves, large variations might occur in resistance values. Which is why it has been chosen to asses data in relative values. The initial resistance will be marked as 1.00 and following measurements will be relative to the initial 1.00. This will visualize the effect of VI and PEF on the recovery time of each individual leaf.

To exclude any influence on the resistance from storage conditions, a control was set.

Greenhouse arugula

A graph of the average resistance in the control, with a slope of -0.0002, a mean of 0.99 and a standard deviation of 0.026 was obtained by plotting the data from the resistance measurements (Figure 13). This shows that there is no weight gain or loss of the leaves. This ensures that the setup is appropriate to measure resistance over time to characterize the recovery of the arugula over time.

The measurements show small fluctuations (±2,6%) in resistance in the control and should be considered when measuring the resistance over time.

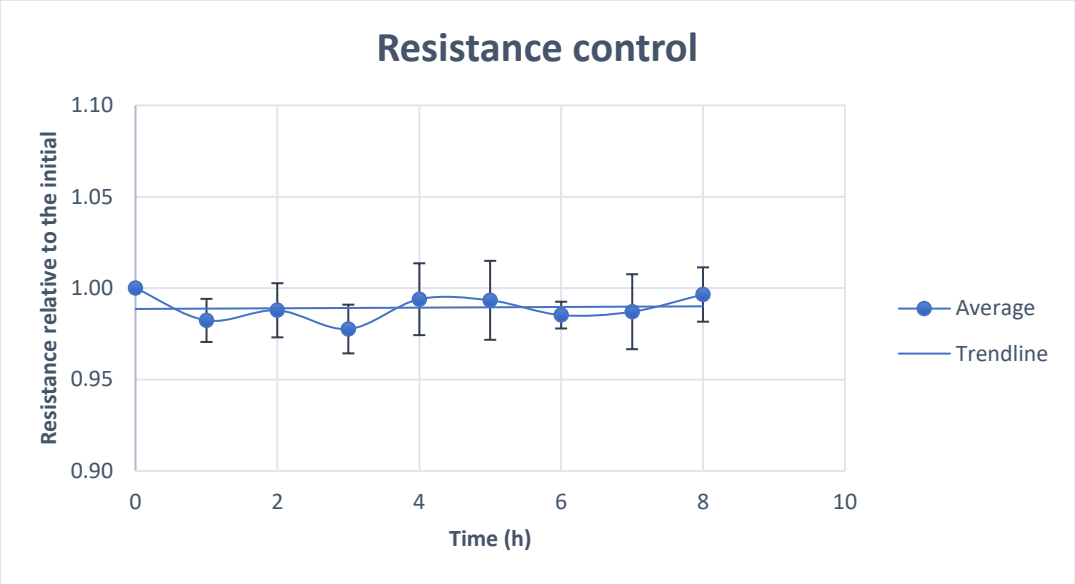


Figure 13 Average relative resistance curve of stored greenhouse arugula samples.

Cater grönt arugula

The same was done for arugula from cater grönt, which were used to optimize the methodology. However, using the arugula from grönt resulted in a reduction of resistance, indicating hydration (shown in figure 14). This was solved by hydrating the arugula in the storage Tupperware using the storage method in 3.1.4 for 16 hours.

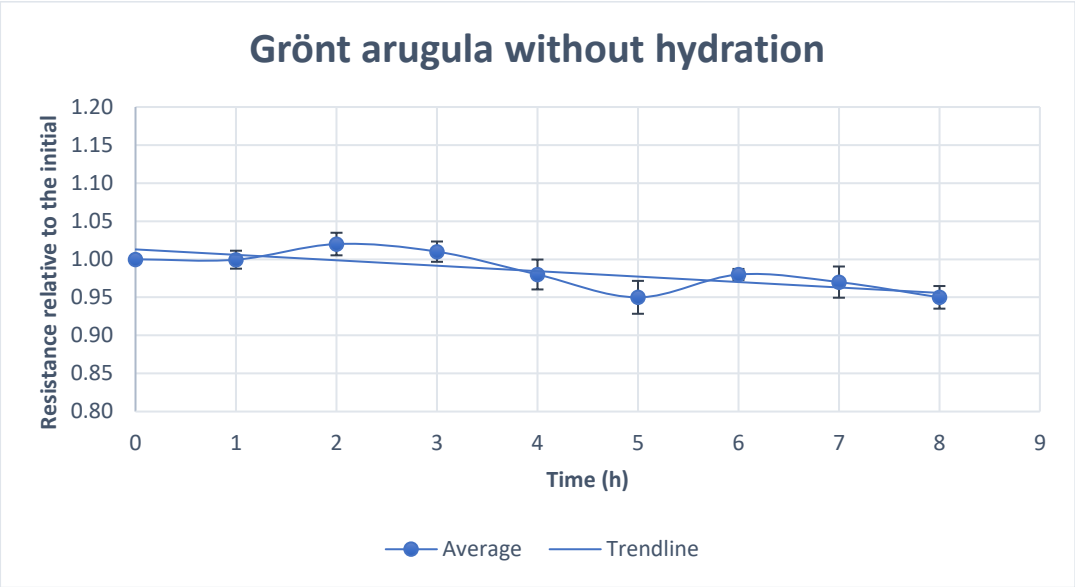


Figure 14 Average relative resistance curve of catered arugula, without hydration period.

The data shows a slope of -0,0071 with a standard deviation of 0.025, which could indicate a 5% ± 2,5% hydration on average over a period of 8 hours.

After the hydration period, the resistance was measured over a time period of 8 hours as shown in Figure 15.

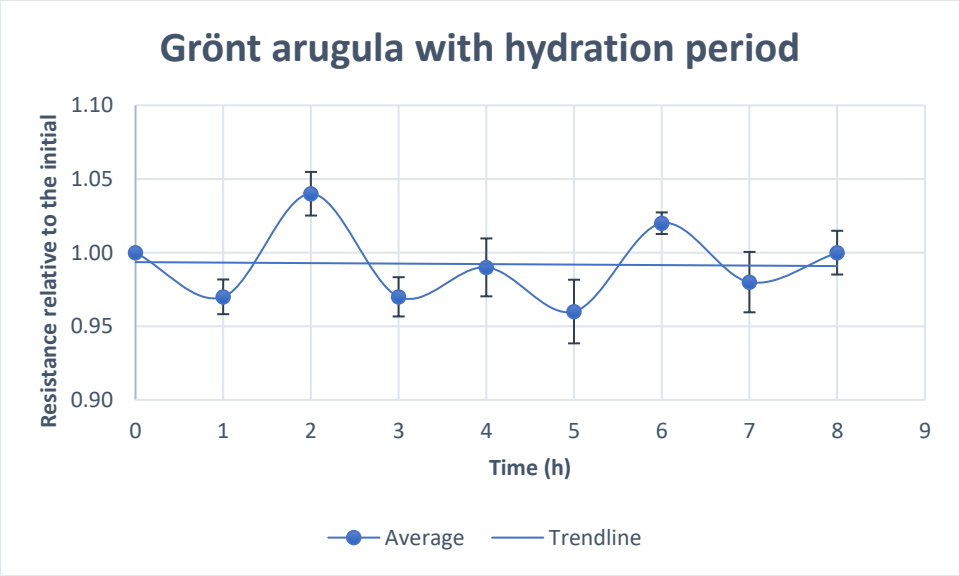


Figure 15 Average relative resistance curve of catered arugula, with hydration period.

This shows that there is a loss of moisture caused by either the transportation of the arugula or the storage of the arugula at the caterer. This can be solved by a hydration period before using the leaves for experiments. Which showed a slope of -0,0003 and a standard deviation of 0.027. Which shows that there is no effect of external factors on hydration or dehydration on the measurements after the hydration period.

4.3 Freezing resistance

Freezing resistance was determined using the wilting method. The results are shown in table 5 below.

Table 5 Survival rate of frozen arugula

Name	Survival rate	Survival rate (%)
PEF	0/20	0%
VI G	0/20	0%
VI FA	1/20	5%
VI B1	2/20	10%

Data shows that without vitamin B1 and folic acid, none of the arugula leaves in this study survived. The samples vacuum impregnated with FA & B1 indicate an increase in survival. Though replications should be conducted to determine the significance of these results.

4.4 Discussion

A significant reduction of recovery time was in samples treated with vitamin B1 and folic acid when compared with samples treated with only PEF & samples vacuum impregnated with VI and PEF. This shows the importance of vitamin B1 and Folic acid in cell metabolism and repair. There was no significant difference between the recovery time of B1 and the recovery time of samples impregnated with folic acid.

This study also shows that the combination of VI & PEF have been successful in impregnating the plant cells with the substances. The fast recovery rate in VI B1 can be attributed to role of vitamin B1 in metabolic pathways and response to DNA damage (Raschke et al., 2007). Which might not be limited to just DNA damage but also to damage to other parts of the cell. Folic acid has also been effective in reducing the recovery time in arugula cells. This phenomenon could be attributed to the fact that folic acid is responsible for the synthesis of DNA as well as nucleotide production (Gorelova et al., 2017). Surprisingly, vacuum impregnation with solely glycerol also significantly reduced the recovery time of the arugula after electroporation. A study by Aubert et al (1994) shows that glycerol can play a role in plant cell metabolism and thus could explain this reduction of recovery time. The large spread in the recovery time between individual samples could be attributed to the effectivity of electroporation and vacuum impregnation on different leaves.

The strong indication that vitamin B1 increase the survival rate after electroporation can be explained by its biological function in plants cells. Goyer, 2010 states that its serves as cofactor in for 2-oxoglutarate dehydrogenase, which is an essential enzyme in the krebs cycle of energy production. Goyer, 2010 also states that thiamin (vitamin B1) plays a role in metabolism of amino acids and acts as signaling molecule for biotic and abiotic stress.

This study indicates that vitamin B1 and folic acid can also play a role in increasing the freezing resistance in arugula leaves. However, to determine any statistical significance of the freezing experiment, replications should be performed.

Furthermore, the leaves that were put to the freezing test were not assessed beforehand for viability. Following the trends from chapter 4.3, this could well mean that 40-50% of the leaves in PEF, VI G & VI FA were already deceased before subjected and higher survival rate of 80% in VI B1 might be responsible for the higher survival rate in the freezing test.

Additionally, the PEF settings showed that they were sub-optimal, 60% of the leaves deceased after electroporation without VI. Fortunately, these settings highlighted and importance of vitamin B1 and folic acid in cell metabolism. With optimized PEF settings, the effect of vitamin B1 and folic acid on the survival rate might be less significant, but might be more important for survival after freezing.

It is safe to say that folic acid and vitamin B1 do improve the recovery of arugula cells in this study. Which can be attributed to their effects on the plant's metabolism which corresponds with the studies conducted by Raschke et al.(2007) and Gorelova et al. (2017). Additionally, to determine the significance of the increased freezing resistance in samples vacuum impregnated with folic acid and vitamin B1, replications would need to be conducted and with a population of leaves with 100% survival after treatment and before freezing.

4. Conclusion

The results presented herein show that impregnation of glycerol, vitamin B1 and folic acid with the aim of increasing cell metabolism to improve the survival rate after freezing is feasible. Samples in this study vacuum impregnated with vitamin B1 and folic acid showed an significant increase in recovery rate. Samples without these vitamins showed a significantly slower recovery rate.

There was a significant ($P<0.05$) reduction of recovery in all vacuum impregnated samples. With a strong reduction in samples impregnated with vitamin B1 and in samples impregnated with folic acid. These samples required roughly only half of the time to recover to its initial resistance value.

Freezing resistance of samples in this study could only be achieved through a combination of vacuum impregnation with glycerol, folic acid or vitamin B1 and electroporation. Samples impregnated with solely glycerol were unable to survive the freezing experiments. Addition of Vitamin B1 and Folic Acid seemed to be essential to increase freezing resistance of these samples although replications are required to determine the significance of this result.

Microscopy of arugula leaves used in this study, that were electroporated with 1000 V/cm at 1000 µs pulse width and a pulse space of 20µs at 1000 pulses showed a homogenous electroporation of the leaf. The survival rate of arugula leaves after electroporation with these settings was 50% and a recovery time of 7 hours.

Lastly, it can be concluded that the order of VI → PEF → freezing was effective in the transfection of substances into cells of arugula leaves used in this study. Shown by a decreased recovery time and increased freezing resistance.

5. Recommendations

Further recommendations for future research of this study would first of all be to optimize the PEF parameters for the Aditus Medical. 50% survival rate after only PEF is on the low side and could be further increased by tweaking the electroporation settings further.

Secondly, the vitamin B1 and folic acid concentrations should be optimized. It is clear that they have an effect on cell recovery and survival rate. The optimal concentrations however, are still unknown. It is possible that even faster recovery or a higher survival rate is possible with alternative concentrations. Another interesting could be study of the synergetic effects of vitamin B1 and folic acid. They both play separate roles in the repair and metabolism of the cell, and it would be interesting to see how the combination of both would affect the recovery rate and survival rate.

Replications of the freezing experiment should be conducted to determine the significance and reproducibility of the effects of vitamin B1 and folic acid on the survival rate. Additionally, arugula leave should be assessed after the resting period before freezing. It is likely that electroporation already induced cell death in the arugula before freezing. Automatically resulting in favorable results for vitamin B1 impregnation.

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"It is in fact 'rocket' science."

Oscar de Kuijer

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