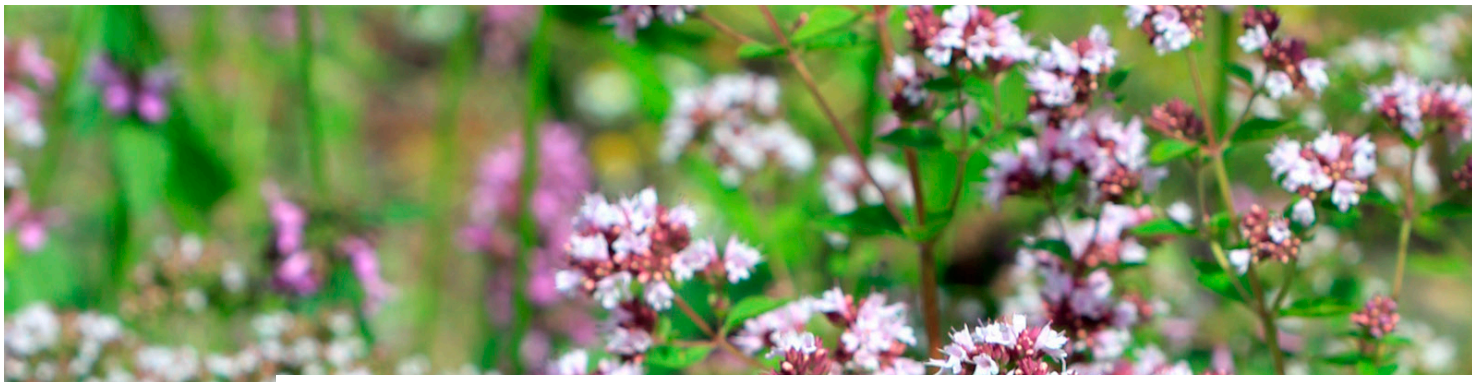

Application of pulsed electric fields and vacuum impregnation to improve the freezing tolerance of fresh *Origanum vulgare* leaves

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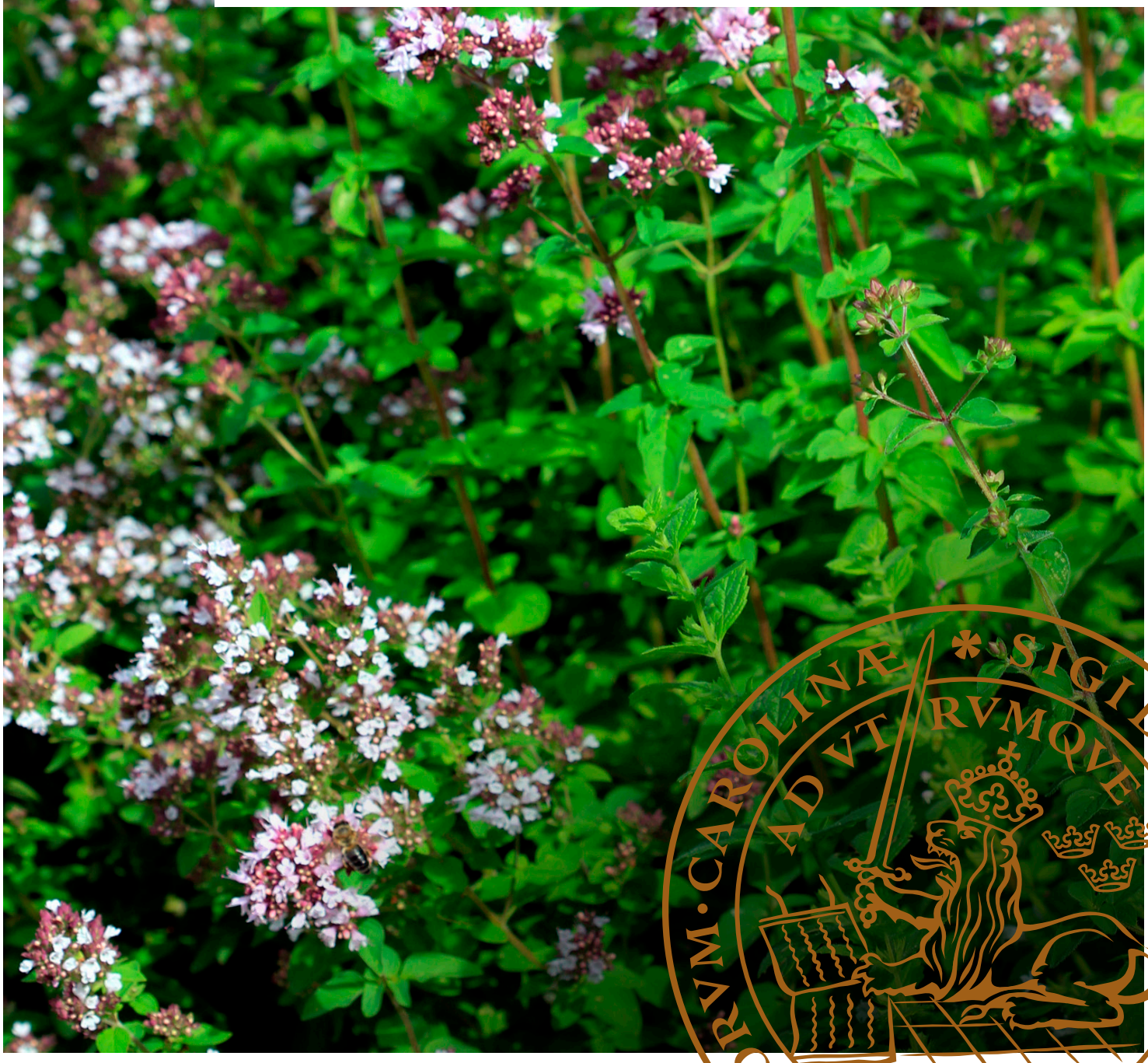

LUNDS UNIVERSITET

Department of Food Technology and Nutrition, Engineering and Nutrition

Master thesis

Application of pulsed electric fields and vacuum impregnation to improve the freezing tolerance of fresh *Origanum vulgare* leaves

"Oregano spice of life"

Master Student: Iliana Karasa
Supervisor: Federico Gomez Galindo
Examiner: Ingegerd Sjöholm
February 2018 Lund, Sweden

LUNDS UNIVERSITET

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Master examensarbete

Användning av PEF och vakuumimpregnering för att öka frystolerans för färska origanoblad

"Oregano spice of life"

Master Student: Iliana Karasa
Handledare: Federico Gomez Galindo
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Abstract

It is an undeniable fact that freezing is one of the oldest and widely used food preservation methods. Freezing reduces the chemical reactions, delays cellular metabolic reactions and does not allow microorganisms to grow. When freezing is applied on leafy vegetables such as spinach and fresh herbs such as fresh oregano, quality degradation is caused due to low freezing tolerance and thawing. Thawing damages leafy vegetables fresh herbs due to recrystallization. Recently, methods that can increase the freezing tolerance of fresh spinach leaves have been developed. It is cryoprotection and can be achieved by the application of two pretreatments. These pretreatments are vacuum impregnation (VI) of a cryoprotectant and pulsed electric fields (PEF).

During this project, increased freezing tolerance of fresh oregano leaves was tested by the combination of VI and PEF. The fresh oregano leaves were vacuum impregnated while they were immersed in isotonic trehalose solution 10 % (w/v). The ideal conditions for the vacuum impregnation on fresh oregano leaves were determined by applying low pressure and different duration of the different steps in the impregnation process after VI respectively PEF was applied. The applied PEF conditions were chosen in order to achieve homogenous and reversible electroporation of the leaves. After the combination of VI and PEF, the leaves were allowed to rest for 1, 2 and 3 days at 4^{o}C before freezing. The leaves were frozen and immediately thawed. The applied pretreatments of VI and PEF increased the freezing tolerance of fresh oregano leaves. However, leaves surviving the freezing and thawing cycle would rapidly loose viability few minutes after thawing.

Changing the time of resting after VI and PEF before the leaves were frozen influenced the viability after freezing and thawing. The longer the resting time, the slower was the rate in which the leaves lost their viability.

List of Abbreviations

FDA: Fluroscein Diacetate

PCD: Programmed Cell Death

PEF: Pulsed Electric Fields

PI: Propidium Iodine

VI: Vacuum Impregnation

Contents

1.0 Introduction

Origanum vulgare is an annual, shrubby and perennial herb that grows only in the eastern Mediterranean area. *O. vulgare* is not only used as added in food preparation such as meats, soups, salads and sausages but also to treat various diseases due to its stimulant and antiseptic properties (Hussain, 2001;Ozcan & Chalchat, 2009). The interests of food industry lead many researchers to focus on the antioxidant properties of *Origanum vulgare* (Hussain, 2011;Matsuura 2003).

Nowadays, consumers are more demanding than ever. In the market, fresh food of high quality is preferred than processed food (ift.org). Frozen food is preserved through temperature reduction lower than the freezing point of water, in order to achieve low microbial, biochemical and enzymatic activity. In general, the application of fast freezing in food industries is preferred. This is explained by the fact that during fast freezing numerous of very small ice crystals are formed mainly intracellularly, thus better quality preservation is achieved. It is important to mention that the greater quality damage occurs during thawing of frozen foods because of recrystallization. As thawing takes place, there is loss of function in the cell membranes, due to conformational changes in the membrane proteins and the phase change of the lipids of the cell membrane (Mallet, 1993;Baust, 2002).

A decade ago, a group of Lund University scientists developed an innovative freezing method that improved the freezing tolerance of fresh spinach leaves. This method requires the pretreatment of food with pulsed electric fields combined with vacuum impregnation in trehalose solution (Phoon, et al., 2008). The method that was originally applied on fresh spinach leaves can be seen on Figure 1. In this thesis, this method was applied to fresh *Origanum vulgare* leaves.

Figure 1: Diagram with the method that was originally applied on fresh spinach leaves.

2.0 Objectives

The aim of this project was two-fold: first to evaluate the pretreatment of fresh *Origanum vulgare* leaves with vacuum impregnation in isotonic trehalose solution and pulsed electric fields on the freezing tolerance of the leaves. Second, to study the post-thawing survival of the leaves.

2.1 Specific objectives

- To determine the optimal parameters for vacuum impregnation (VI) and pulsed electric fields (PEF) as pre-treatments for improving the freezing tolerance of the leaves.
- To investigate the influence of different 'resting' times on leaf survival after freezing and thawing cycle.

3.0 Background

3.1 Leaf structure

A leaf consists of upper and lower epidermis (Hopkins & Huner, 2004). More specifically, the epidermis is a cell layer, which is found directly below the cuticle. The function of epidermis is not only to avoid water loss but also to provide an additional layer between the outside and inside of the leaf. Both upper and lower epidermis are thin and have stomata (Wikipedia.org). Guard cells, which are produced by the epidermis, control the closing and opening of stomata (Mamoucha & Rahul, 2016). The upper epidermis produces a waxy waterproof coating (Harvard University, 2011). Between the two epidermis, there is the mesophyll, which is the photosynthetic parenchyma tissue (Hopkins & Huner, 2004). Mesophyl is divided in palisade and spongy mesophyll. Palisade and spongy mesophyll are filled with chloroplasts in the cytoplasm (Hopkins & Huner, 2004). The leaf structure can be seen on Figure 2.

Palisade mesophyll absorbs more light whilst in the spongy mesophyll there are air spaces that allow CO_2 to diffuse through the leaf. Sugars are transferred to the plant through phloem tissue whilst the nutrients and the water are transferred through xylem tissue in the veins that are interconnected and closely parallel (Harvard University, 2011;Mamoucha &Rahul, 2016;Hopkins & Huner, 2004)**.** Those veins assure not only the efficient delivery of nutrients and water to every plant cell but also the removal of the product of photosynthesis (Hopkins & Huner, 2004). Stomata provide low resistance pathway for diffusional movement of gasses across the cuticle and epidermitis. When plants are photosynthesizing the stomata are opened due to the need of CO_2, whilst at night the stomata are closed because of the low demand of CO_2. The opening of the stomata depends also on the presence of water. When the water in the soil is not enough, then even when there is light stomata open less than normal (Zeiger , 2010).

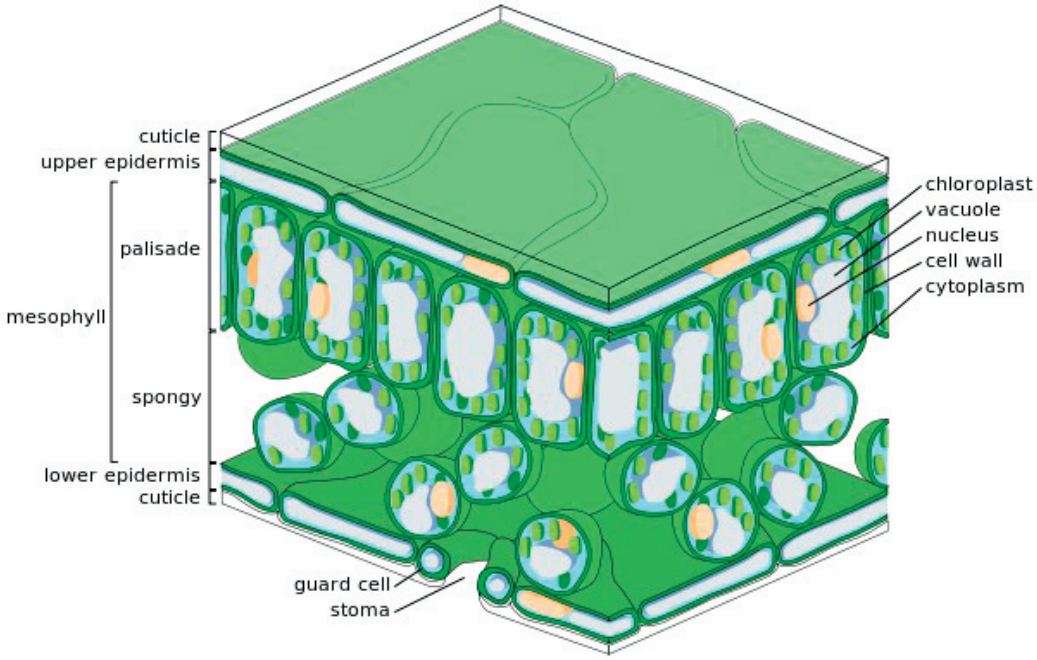

Figure 2: Schematic of leaf structure (from Wikipedia.org)

3.2 Origanum vulgare

It is an undeniable fact that oregano is one of the most used species in Mediterranean and Mexican cousin. Oregano in Greek means 'Joy of the mountains' (Denys, 2013). In Mediterranean countries, it can be found as *Origanum vulgare,* L, *Origanum viride*, and *Origanum virens* (Karamanos & Sotiropoulou, 2013). From a botanical aspect oregano is a herbaceous perennial plant with height up to 90 cm, bushy with branched woody stems, opposite leaves and creeping roots. Oregano has hairy ovate leaves of dark green color, erect flower bearing stalks and purple or white flowers that form terminal spikes. Every flower forms 4 small seed like structures (Denys, 2013). Specifically, *Origanum vulgare* is a perennial shrub that originates from the Cretan islands in southern Greece, and in coastal areas of the Greek mainland at altitudes up to 1500 m (Karamanos & Sotiropoulou, 2013).

Origanum vulgare has tenfold oil concentration (1.1 % - 8.2% v/w depending on its habitat) than its other relative species such as *O. vulgare subsp viridulum*, *O. vulgare subsp vulgare*, *O. vulgare subsp viride* and *O. vulgare subsp gracile* (Karamanos & Sotiropoulou, 2013). According to researches it has been shown that *Origanum vulgare* contains carvacrol, thymol, cymene and α-Pinene (Govaris, et. al., 2010;Gutierez, et. al., 2009;Rosato, 2009; Hussain, et. al., 2008;Pozzati, 2010). Origanum vulgare belongs to the family of *Lamiaceae* (Martinez-Rocha et al., 2008) and it is known that it has not only antimicrobial but also antioxidant activity (Ortega Ramirez, et al., 2016).

The flowering tops of *Origanum vulgare* are used for beer production whilst the dried leaves are usually used in sausages, olives, stew, soups, tomato based dishes and pesto. For some dishes, fresh oregano is used to obtain more intense flavor. *Origanum vulgare* is also used in pharmaceutical and cosmetic industries and perfumery. In folk medicine, oregano is used to treat various ailments such as dyspepsia, convulsive coughs, rheumatoid arthritis etc. Due to the antioxidant activity of oregano, it is used in foods with high lipid content because it delays the process of lipid peroxidation in fatty foods (Karamanos & Sotiropoulou, 2013).

3.3 Freezing and thawing

Freezing is defined as the transition phase in which a liquid becomes solid. This takes place when the temperature of the liquid is lower than the freezing point (less than 0^{o}C) (Wikipedia, 2017). Freezing is one of the oldest food preservation methods since it preserves better the original color and flavor (Kerry, et al., 2016). Ninety percent of fruits and vegetables consists of water. The rigid cell walls of fruit and vegetables contribute to the turgor pressure, thus to texture and structure. Freezing changes the mechanical properties of fruits and vegetables concentrate their solutes, increase osmotic pressure and increase the viscosity of the concentrated solution (Karel & Lund, 2003).

At around -5^{o}C both cells and their surrounding medium do not freeze due to supercooling and the depression of the freezing point by the protective solutes that are often present (Karel & Lund, 2003;Mazur, 1984). At temperatures between -5^{o}C to -15^{o}C, the surrounding medium of the cell partially freezes either as a result of seeding the solution with an ice crystal or in a spontaneous way. On the other hand, the cell contents remain supercooled and do not freeze because of the growth blockage of ice crystals into the cytoplasm by plasma membrane. This is explained by the fact that the water in the surrounding media of the cell that is partly frozen has lower chemical potential than the supercooled water in the cell thus, supercooled water flows out of the cell and freezes externally. Water retention of frozen cell depends on the cooling velocity (Mazur, 1984).

On Figure 3, there is the scheme of water retention of cells during slow, rapid and very rapid freezing. When the cooling velocity is sufficiently slow (Figure 3, upper right), dehydration of the cell occurs but not intracellular freezing. The explanation behind this, is that exosmosis allows rapid water lose from the cell, without supercooling. Consequently, the intracellular chemical potential will be stable in equilibrium with the chemical potential of extracellular water. In case that rapid cooling is applied, (Figure 3, center and bottom right) the cells are

supercooled and they freeze intracellularly. This is explained by the fact that cells do not have the capacity to lose water quick enough, so the equilibrium is achieved by intracellular freezing (Mazur, 1965).

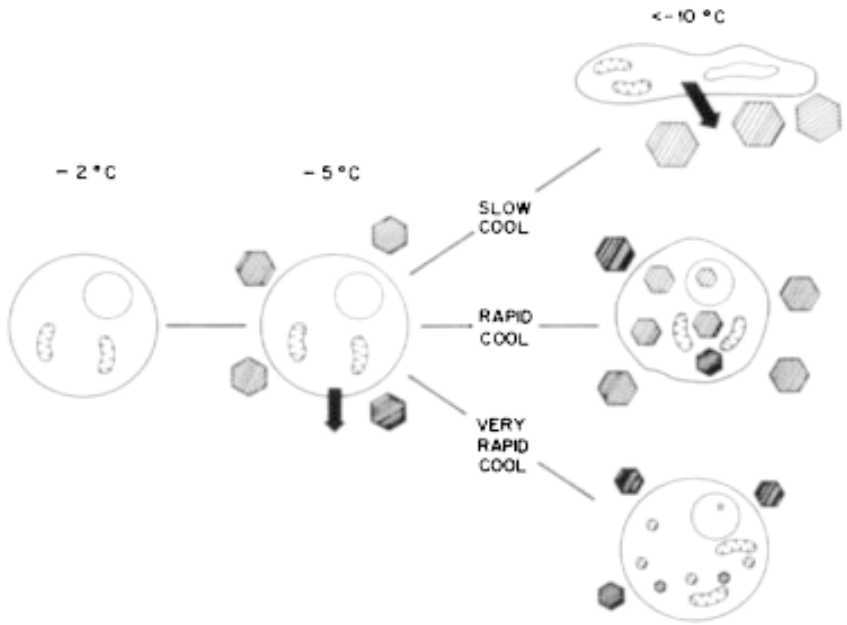

Figure 3: Scheme of the water retention of cells during slow, rapid and very rapid freezing (from Mazur, 1984)

During slow freezing, at rates lower than 1°C/min, occurs exosmosis. Intracellular water is rapidly lost, thus it the chemical potential of the intracellular water is maintained in equilibrium with that of the extracellular water. Thus, the cell does not freeze intracellularly but it is dehydrated. On the other hand, when cell freezes rapidly or very rapidly, it is incapable to lose water in a fast enough pace, so the equilibrium is not maintained. Thus, it is observed intracellular freezing to obtain the equilibrium (Mazur, 1984). As crystallization occurs, the solvent volume is reduced due to the development of ice crystals. During medium and fast freezing, cells are not able to lose water fast enough to maintain stable equilibrium. The water becomes more supercooled and finally obtains equilibrium by freezing intracellularly (Mazur, 1965). On Figure 3 can be seen the scheme of the physical events that take place in cells during slow, rapid and very rapid freezing. The majority of cells freeze at temperature between -5°C and -10°C. Ninety percent of cell water is converted to ice. At this temperature range, cytoplasm is supercooled and cell membrane loses the barrier properties for ice (Mazur, 1965).

The size, the location and the morphology of the ice crystals defines the textural and physical properties of the frozen food products (Petzold & Aguilera, 2009). The size and the location the ice crystals are governed by the freezing rate, the nature of the cell and the specimen temperature (Harnkarnsujarit, et. al., 2016). The factors that affect freezing rate are the composition, the volume of food, the initial food temperature and thermal conductivity, as well as the air velocity in the freezer (Karel & Lund, 2003). In general, food industries prefer fast freezing, because numerous small ice crystals are formed mostly intracellularly, which causes less structural changes on frozen foods during both freezing and thawing (Harnkarnsujarit, et al., 2016).

Cell membrane properties define the cell response to freezing. The ice nucleation temperature depends on the structure of the membrane. The factor that determines whether the cells will equilibrate by intracellular freezing or dehydration depends on the permeability of cell membrane to water and the nucleation temperature. When cells are frozen in subzero temperatures the cell membrane is harmed, the internal membranes and the cell shape are distorted (Mazur, 1984). Another aspect affecting the quality of frozen fruit and vegetables is recrystallization. During thawing, recrystallization occurs, which is defined as the phenomenon in which large ice crystals increase in size at the expense of smaller ones. Recrystallization, which is caused by temperature fluctuation, harms the cells because there is loss of function in the cell membranes, caused by phase changes of the lipids and conformational changes in the membrane proteins (Mallet, 1993;Baust, 2002).

The cause of recrystallization is the minimization of surface energy. Crystal's free energy is inversely proportional to its radius of curvature. Convex surfaces have higher energy than concave surfaces. Concave surfaces have negative curvature. Iso-mass recrystallization takes place because the ice crystals turn into spherical shape, accretion is driven by minimization of surface energy and migratory recrystallization takes place because small crystals have higher specific energy than the bigger ones. Moisture content over 80% leads to faster recrystallization (Erickson & Hung, 1997). The location and the size of ice crystals affect the quality of thawed products (Petzold & Aguilera, 2009). Five cycles of freezing thawing can cause loss of cell wall of papayas through expansion (Phothiset & Charoenrein, 2014). Vegetables with high starch content such as peas, corn, lima beans and shelled peas have better texture after thawing than non-starchy vegetables because of the support that starch grains offer to the cell wall (Lee, et al., 1946).

3.4 Pre-treatment strategy for improving freezing tolerance

The combination of VI and PEF as a pre-treatment of fresh spinach leaves has been successfully applied on spinach (Phoon, et al., 2008). The following sections describe the involved unit operations in more detail.

3.4.1 Vacuum Impregnation (VI)

Vacuum impregnation (VI) is defined as a food process that infuses a solution of specific composition into the porous matrices of fruit and vegetables through pressure difference. The inner air gaps of fruit and vegetables are filled with the used solution, which may provoke changes in the organoleptic characteristics and in the physio-chemical properties of food products. Vacuum impregnation can be used not only to modify taste, color, aroma, texture but also to change thermal properties, reduce pH and aw (Radziejewska-Kubzdela, et al., 2014).

The method of vacuum impregnation is based on diffusion (Radziejewska-Kubzdela, et al., 2014). Diffusion is characterized as the spontaneous movement of substances from regions of higher to lower concentration (Zeiger , 2010). Usually, vacuum impregnation is used as a pretreatment process. It is of great importance to determine the optimal parameters for achieving the desired result. The process of vacuum impregnation consists of two phases, the phase of reduced pressure and the phase of atmospheric pressure The filling of the leaves capillaries is achieved through hydrodynamic mechanism (HDM) and deformation–relaxation phenomena (DRP) (Radziejewska-Kubzdela, et al., 2014). The immersion of food in an external solution causes that a part of the solution penetrates the food matrix through opened pores. Thus, HDM is promoted by the deformation of cell walls, which is caused by the loss of gasses and native liquids (Derossi, et al., 2012). On Figure 4 HDM and DRP phenomena are shown in detail (Radziejewska-Kubzdela, et al., 2014).

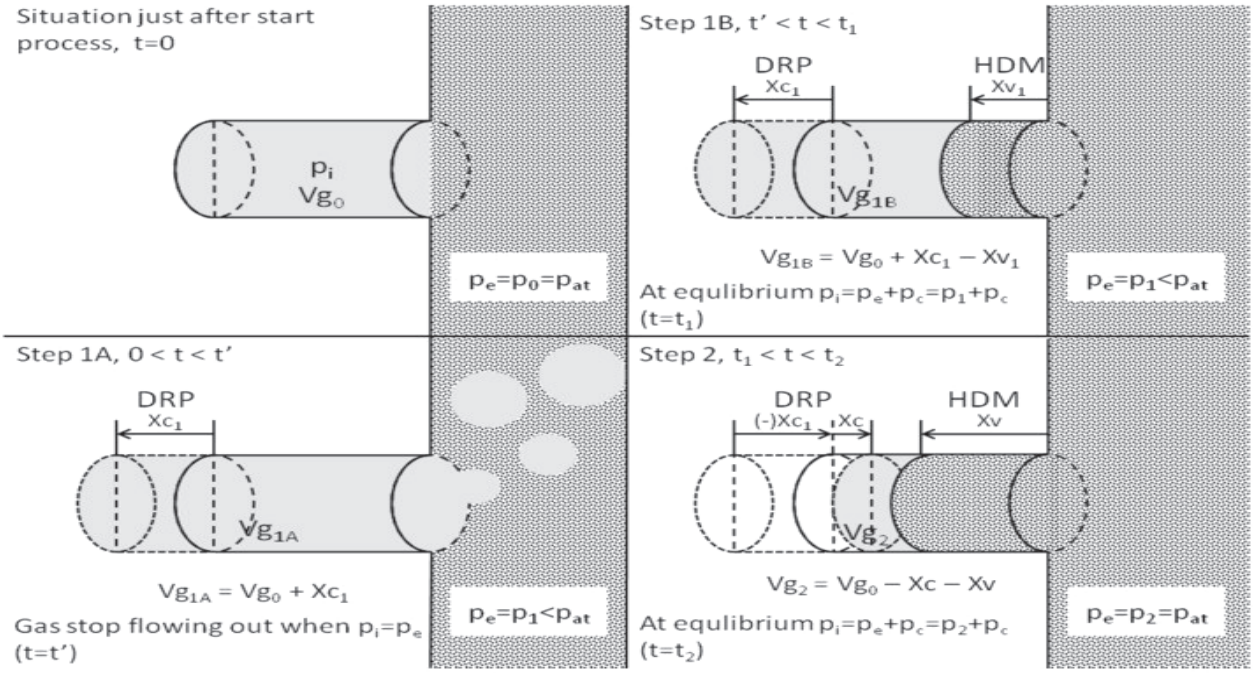

Figure 4: Illustration of hydrodynamic mechanism (HDM) and deformation relaxation phenomena (DPR) (from Radziejewska-Kubzdela 2014)

At the time (t_0) the food is immersed in the solution, the pressure of the outside (p_e) and inside (p_i) the capillary is equal to 1 Pa. The initial volume of capillaries is filled with gas. The 1st phase of the process starts with the reduction of the pressure (p1 < pat). The gas is removed from the capillaries due to the difference of pressures. During the first part of deformation relaxation phenomena (DPR), deformation and expansion of the capillaries is noticed. The capillary volume rises ($Vg1A= Vg_0+X_{c1}$). This stage of the process lasts until the pressure inside and outside the capillaries becomes equal (Radziejewska-Kubzdela, et al., 2014).

On step 1B (see Figure 4), the hydrodynamic mechanism causes the gradual filling of the capillary with liquid, *p*i slightly increases, at the same time the volume of gas trapped in the capillary as result of DRP, is reduced. In the second stage of vacuum impregnation method, the pressure becomes equal to the atmospheric pressure, and deformation relaxation phase (DRP) changes to the relaxation time. Just before the process start, the capillaries get even smaller than they were before the beginning of the vacuum impregnation process. High liquid inflow is observed from the outside to the inside of the capillaries caused by the pressure and decompression of the capillaries. The volume of the final gas decreases. The impregnation of food occurs in the stage of relaxation phase. An important thing that should be taken in consideration while designing vacuum impregnation is the speed of vacuum. The air should not be removed very fast because this will cause not only inhibition of the hydrodynamic

mechanism but also closure of the capillary vessels (Radziejewska-Kubzdela, et. al.,2014;Fito, et. al., 1996).

Successfully vacuum impregnated leaf is defined as the leaf where its extracellular air is replaced by the impregnating solution. The percent of leaf that is impregnated depends on different factors such as the reduced pressured applied, the molecular weight of the solute and the tonicity of the solution used (Andreas, et al., 2011). The factors that influence the process of vacuum impregnation are: sample porosity, sample size and shape, temperature of process, capillaries size and shape, level of applied pressure, duration of vacuum process, needed time to reach vacuum, sample mechanical properties, composition and concentration of the solution (Gras, 2013;Fito, et. al., 1996;Zhao & Xie, 2004).

3.4.1.1 Impregnated solution- Cryoprotection

A cryoprotectant is defined as a substance, which protects organic tissue from freezing (Wikipedia, 2017). Natural cryoprotectants are alcohols, sugars (e.g. glucose, sucrose, and trehalose), diols or amides. The sugars with cryoprotectant activity are sucrose, glucose and trehalose (Fuller, 2004). In this current research, the cryoprotectant that is used is trehalose. Trehalose (CAS Number 99-20-7, anhydrous, CAS Number 6138-23-4, dehydrate) is a non-reducing (FAO, 2017) disaccharide which consists of two glucose molecules with α, α 1→1 glycosidic bond (Kahl, 2000). It has a relative sweetness of 40-45 % of sucrose (Novel Foods, 2005).

Since trehalose is not a reducing sugar, it does not react with amino acids to initiate the Maillard reaction. It has white or almost white crystals; it is soluble in water but slightly soluble in ethanol (WHO, 2015). Trehalose can be found in a variety of organisms in nature. It is safe for human consumption; thus, it is used in a quite wide range of applications in food industry for example taste enhancer, humectant, nutritive sweeteners or stabilizers and thickeners. Trehalose can be produced with microbial fermentation or extraction from natural sources or enzymatic conversion (Kahl, 2000). Trehalose, which is used in food industries as additive, is produced enzymatically with isoamylase from *Pseudomonas amyloderamosa* or alpha-amylase from *Bacillus licheniformis*, the carbon source used is cornstarch (WHO, 2015). Most people can digest trehalose but lower consumption is suggested (less than 10-50 grams) for celiac individuals because trehalose may cause diarrhea or abdominal bloating (Abbot & Chen, 2004;Food standards Australia, 2003).

3.4.2 Pulsed electric fields (PEF)

Pulsed electric fields or Electroporation is defined as a low temperature, non-chemical, non-thermal, low impact process (Kempkes, 2010) which impacts on the permeability of cell membranes by applying high voltage and short time pulses (Barba, 2015). Pulsed electric fields process causes pore formation. These pores formed affect the mass transfer of the cells. (Tylewicz, 2017). The advantages of Pulsed Electric Fields are that it is instant, energy efficient, flexible and non-thermal (Pereira et. al., 2016). . PEF has the capacity to increase the processing yield of various extraction procedures such as red juice beet, carrot juice and sugar beet juice. Higher yield is achieved through the mass transfer increment that is caused by increased cell permeability. Additional applications of PEF are preservation of semi solid and solid foods such as soup with peas, rice pounding and enhancement of drying (Raso & Heinz, 2006). Through PEF, microorganisms may be reduced by 5-9 logs, which is similar to pasteurization (Kempkes, 2010).

The mechanism of permanent electroporation and electrical membrane breakdown is explained by the theory called ‘electromechanical instability theory’. On Figure 5, the theory is shown. According to this theory, the cell membrane is a capacitor, which is filled with dielectric material that has very low dielectric constant compared to the environment and the inner cell. Thus, this causes accumulation of free charge on the outer and inner side of the cell membrane so a transmembrane potential of 10 mV is generated. The cell needs to maintain the transmembrane potential stable to survive. The cell turgor and inner cellular pH are maintained through the entrance of certain substances, H^+ and OH^- in the cell. When electric field is applied, the cell membrane is compressed and its thickness is reduced due to the increment of transmembrane potential. Electrical membrane breakdown occurs in case the transmembrane potential exceeds the viscoelastic limits of the cell membrane. The electric field strength and time of process determines the number and the size of formed pores. Short treatment time or low electric field forms small number of pores of small size. Irreversible permeabilization or mechanical disruption of cells takes place with the application of intense PEF treatment (Raso & Heinz, 2006).

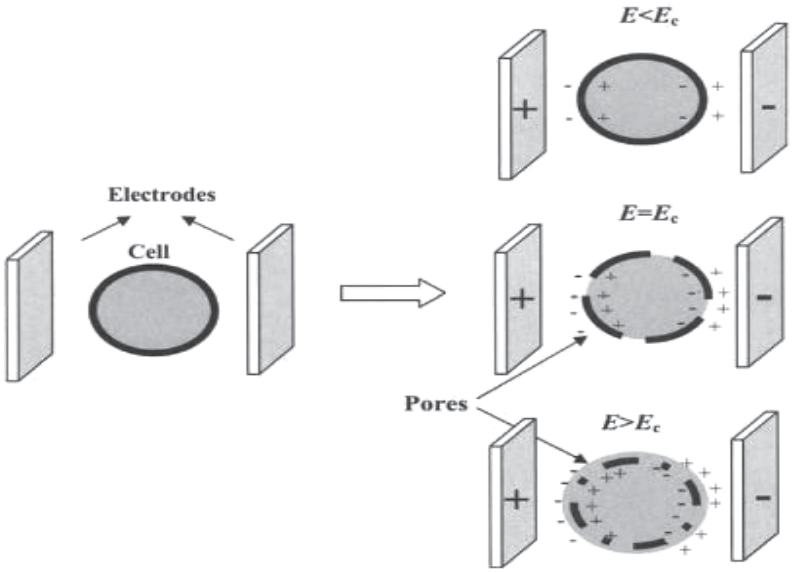

Figure 5: Illustration of cell membrane during PEF treatment (From Raso, 2006)

Plant leaves have heterogeneous structure and are very anisotropic thus, it is challenging to achieve homogeneous electropermeabilization within the leaf tissue. This can be explained by the different cell size distribution and shapes throughout the leaf structure and that there are differences of cell orientation. Additionally, there are differences in the electrical properties and extracellular space configurations among the same specimen (Dymek, et al., 2014). Pulsed electric fields with application of less severe parameters can cause reversible electropermeabilization of cells (Benz & Zimmermann, 1981). Reversible permeabilized cells have the capacity to recover, since the formed pores on the cell membrane close again and its permeability properties are restored, irreversible permeabilized cells cannot recover and die (Kwao, et. al., 2016).

There are many applications of PEF in food processing. PEF applications are used both in the irreversible and reversible forms. Reversible form of PEF has been applied for cryoprtotection of fresh baby spinach leaves (Phoon, et. al., 2008) and drying of basil (Kwao, et. al., 2016). Regarding the improvement of the tolerance of spinach to freezing, reversible PEF increases the mass transfer of trehalose solution in the cells of the leaf, thus there is cryoprotectant both intracellularly and extracellularly. Regarding the improvement of drying of basil, when low electric field is applied, the process of drying is improved since the leave's stomata open irreversibly, resulting in shorter drying time, improved the retention of volatile aroma compounds, the color of the final product and the rehydration capacity compared to the untreated basil leaves (Kwao, et. al., 2016).

3.4.2.1 Consequences of the application of reversible PEF

The mechanism in which that plant tissues respond to reversible PEF conditions has not been investigated and understood very well. Processing plant tissues with reversible PEF conditions affects the metabolism of plant cells. The metabolic responses depend on the intensity and width of the pulse, the size and the persistence of the pores that were created. When the plasma membrane pores open, polar molecules move out and in the cell. When the pores are closed again then oxidative stress is caused, which produces reactive oxygen species (ROS). ROS consists mainly of H_2O_2. As the ionic species move, energy release may occur. Additionally, ATP hydrolysis occurs when the pores are resealed. Additionally, cell wall organization changes due to the stress that PEF causes. The cell wall permeability decreases when PEF treatment is applied, as the cell wall porosity decreases (Gomez Galindo, 2008b).

3.4.3 Programmed cell death

Programmed cell death is defined as a sequence of events that guide to the organized and controlled destruction of the cell (Lockshin & Zakeri, 2004). The utility of PCD includes the appropriate development of the plant body and the defense to stop the spreading of unwanted pathogenic microorganisms (Lam, 2004). There are two forms of cell death: apoptosis and necrosis (Kerr, et al., 1972). During apoptosis cells shrink, the integrity of mitochondria is lost (Adrain & Martin, 2001;Green & Reed,1998), the cytoplasm becomes denser, the nucleus condensates and fragmentizes and finally the cells break into apoptic bodies (Adrain & Martin, 2001). On the other hand, during necrosis the cells swell, and die in an uncontrolled way. The swelling of the cells is caused by its incapability to osmoregulate, thus there is a flow of ions and water into the cell (Lennon, et al., 1991). Programmed cell death in plants is a process, which occurs from xylogenesis till senescence (Beers, 1997;Rogers, 2005).

Distinction if there is apoptosis or necrosis in plant cells depends on the duration and severity of the insult (Lennon, et al., 1991). Usually, plant cells are not broken into apoptotic bodies (McCabe & Pennell, 1996). The morphological descriptions of dying and dead plant cells indicate apoptotic-like programmed cell death (AL-PCD). Apoptotic-like programmed cell death responds to gene regulation and specific molecules signaling. Weather the plant cells will die due to AL-PCD or necrosis would also depend of temperature. If the temperature is moderate, the cells die via AL-PCD whilst heat stress in higher temperatures causes necrosis (Reape, et al., 2008).The heating stress affects the availability of intracellular ATP and caspases (Leist, et. al., 1997;Denecker, et. al., 2001).

Pulsed electric field process applied in nanoseconds has shown to induce apoptosis in certain types of mammalian cells, mainly cancer cells. According to scientists, cells that actively grow are triggered intracellularly by external nanosecond of pulsed electric fields and nanoelectopulse trains repeated in a frequency of 2-2000 Hz (Vernier, et. al., 2004). Healthy, normal eukaryotic cells contain phosphatidyserine (PS) in the plasma membrane lipid bilayer on the inner leaflet (Verhoven, et al., 1995). Externalization of PS is a signal of phagocytosis and disposal. Apoptotic cells have externalized PS (Fadok, et al., 2001). Less than 100kJ/mol is needed to facilitate the cell membrane restructuring and PS translocation from the cell membrane (Vernier, et. al., 2004). PS externalization can occur in erythrocyte membranes when electroporation is applied in microseconds (20ms, 0.3-0.9MV/m). This can be explained by the rearrangement of the membrane bilayer and the enhancement of external portative effects. Additionally, PS translocation can be achieved when ultrashort high field pulses 7-300ns of 2.5-30MV/m are applied.

3.5 Methods to assess reversible cell permeabilization

3.5.1 Propidium Iodine

Propidium Iodine ($C_{27}H_{34}I_2N_4$, Cash No 25535-16-4) is a substance, which is used for the fluorescent staining of nucleic acids. It has molecular weight equal to 668.45, the solubility in deionized water is 1 mg/mL (1.5mM). Propidium Iodine does not bind only with DNA but also with RNA, thus for discrimination is needed treatment with nucleases. Propidium Iodine is used in flow cytometry, fluorescence microscopy, fluometry and confocal laser scanning microscopy to evaluate DNA content or to evaluate cell viability. The viability is examined by the monitoring in microscopy by the presence of green dots (Wikipedia, 2017), (Ibidi, 2015). Propidium iodine cannot go through living cell membrane, so alive and dead cell can be distinguished (Ibidi, 2015). Additionally, propidium iodine has the ability to bind to RNA, so DNA and RNA can be distinguished (Wikipedia, 2017). PI is used to monitor the electroporation efficiency because it cannot enter intact viable cells. It can only enter the cells upon electroporation. Propidium Iodine can determine the effect of electric pulses parameters on the level of cell membrane permeabilization (Kanduser & Usaj, 2015).

3.5.2 Fluroscein Diacetate (FDA)

Fluorescein diacetate (FDA) ($C_{24}H_{16}O_7$,CAS Number 596-09-08) is a substance which is used as an indicator of cell viability (Ibidi, 2015), (SigmaAldrich, 2017). It has molecular weight of 416.38 (SigmaAldrich, 2017). Fluorescein diacetate is converted by the cells to fluorescein, which has green fluorescent. The conversion is dependent to esterase (Ibidi, 2015). It was used FDA as it was described by Dymek et. al. 2014.

4.0 Experimental summary

The experimental steps that were followed can be seen of Figure

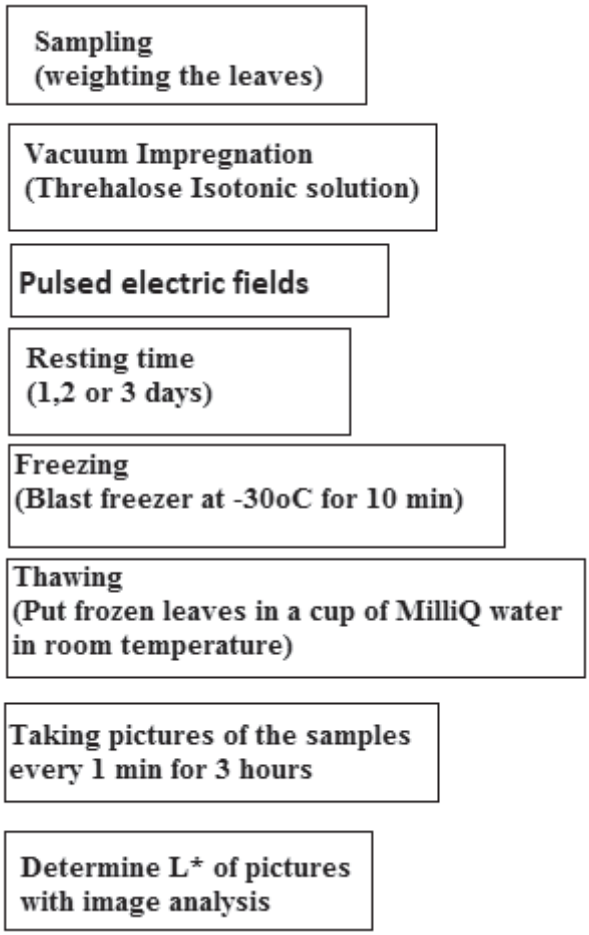

Figure 6. Diagram of the experimental procedure

5.0 Materials and Methods

5.1 Raw material preparation

Ecologic potted oregano (*Origanum vulgare*) was bought from a local farm (Kabbarps Trädgård AB, Åkarp, Sweden) and brought into the laboratory. The side handles and the top of the carton box were covered with plastic bags for extra protection from cold and rain. The plants were maintained in stable room temperature, moisture and light exposure in the lab for maximum 1 week. The bases of the pots were filled with water once they were bought and the lights remained on during day and night. For the experiments, fresh oregano leaves were used. The dimensions of the leaves used were 2.9±0.2cm long, 2.04±0.2cm width and 0.05±0.02g weight.

5.2 Determination of trehalose isotonic solution for impregnation

For the determination of trehalose isotonic solution, 20 oregano leaves were immersed in 400 ml of 5% (w/v), 7% (w/v), 10% (w/v), and 12% (w/v) trehalose solution for 0, 2, 4 and 6 hours. Every two hours the leaves were removed from the solution, their surface dried with a tissue and weighted. The experiment was done three times.

5.3 Vacuum Impregnation (VI)

Five oregano leaves were weighted and immersed in 100ml of 10 % (w/v) isotonic trehalose solution in a beaker. Plastic nets were placed on top to avoid floating of the leaves. For additional security, the nets were fixed with tape on the walls of the beaker. After the completion of the process, the leaves were taken out of the solution, taped with a tissue to remove excess solution on the surface and they were weighted for second time. The percent of weight gain was calculated according to the formula GW% = ((Wf-Wi)/Wi)*100. The vacuum impregnation system that was used was the S.I.A Supervisions System Automation from Bologna (Panarese, et al., 2014). Few VI conditions were tested in order to determine the ones with the highest percent of weight gain. Because of the small size of the fresh oregano leaves, it was decided to apply the lowest pressure that the VI system could achieve, which was 126 mBar. The VI experiments were focused on the time combination of reduced pressure phase and atmospheric pressure phase. Every experiment was repeated twice. On Table 1 the applied VI conditions are reported. Table 1 is a result of many trials.

Table 1: Duration of pressure phase (sec) and atmospheric pressure phase for different applied protocols A, B, C and D.

VI Protocol	Reduced pressure phase	Atmospheric pressure phase	Weight gain %
A	9.07 min	17min	18.36
B	13.07 min	25min	24.00
C	21.07min	25min	19.50
D	25.07min	17min	19.50

5.4 Pulsed electric fields (PEF)

The applied PEF parameters were: biopolar pulses, electric field strength = 600V/cm, pulse width =150µs, pulse space=760µs, number of pulses=65, pulse train space=10, number pulse train=1 as described by Phoon et. al (2008). The vacuum impregnated oregano leaves (using VI protocol B), were immediately transferred into the electroporation chamber. The electroporation chamber was filled with 200ml of 160µs NaCl solution. Three leaves were placed carefully, so they would not cover each other. The NaCl solution was prepared by adding small amount of NaCl into MilliQ water, while measuring the conductivity. The NaCl volume was decided based on trials to cover the electrodes once the chamber was shield. The electroporation chamber that was used had gap size between electrodes equal to 0.8 cm. Both electrodes were connected to the pulse generator (ARC Aroma Pure AB CEPT, Lund, Sweden), and the electric pulses were observed by an Oscilloscope Fluke 123 (Washington, USA).

5.5 Resting, Freezing and Thawing

Six VI and PEF pretreated oregano leaves were rested for 1, 2 and 3 days in a plastic container with saturated moisture at 4^{o}C. In order to create a saturated atmosphere in the plastic container, wet paper tissues with MilliQ water were placed inside for 24 h at 4^{o}C. The leaves were frozen in a blast freezer at -30^{o}C. It was assured that the core of the leaves reached -30^{o}C by placing a thermocouple as a sandwich between two leaves. It was estimated that the freezing time was 10 minutes. When the freezing process was completed, the leaves were thawed by placing them in a bicker full of MilliQ water at room temperature for 5 seconds.

5.6 Viability verification of leaves

It was very important to prove the viability of leaves before and after each process step. More specifically VI treated leaves, VI and PEF treated leaves, VI and PEF treated leaf after 1, 2 and 3 days of resting. This was done with fluorescence microscopy using Nikon Fluorescence Microscope Ti-U (Japan). The dye that was used was Fluorescein diacetate (FDA). The positive control was a freshly cut leaf and the negative control was a dead leaf which was frozen at -20^{o}C and then thawed in room temperature in MilliQ water.

5.7 Post thawing evaluation of viability-image acquisition

The VI and PEF pretreated oregano leaves that were rested, frozen and thawed were photographed using a camera. The camera that was used was Nikon D5100 with 18-55 kit, notation 4. The camera was stabilized on a tripod. The processed leaf was tapped with a tissue to remove excess water from its surface. Next, the processed leaf was placed in a 100ml glass beaker. The beaker that contained the processed leaf was placed on a black table. The height of the tripod was adjusted so that the camera's lense was 20 cm across from the inner center of the beaker. On each side of the camera were putted led lights of stable intensity and on stable position. Once a leaf was placed in the center of a beaker, the photography setup, which included the led lamps, the camera on the tripod, the black photography stand, and the sample in the bicker were covered with a black sheet. Additionally the lab ceiling lights were switched off. Thus, the camera started taking a picture of an already thawed leaf every minute for 3 hours. In order to keep the humidity of the sample stable (because of the exposure in intense lighting for long time), wet tissue with MilliQ water was used in the bottom around the sample and on the top of the beaker.

5.8 Post thawing evaluation-image analysis and measure lightness (L*)

Every picture was analyzed with ImageJ to define lightness, L*. When a picture was uploaded on Image J, it was divided in three individual channels, each of them represented L*(lightness), a* (redness/greenness) or b* (yellowness/ blueness) of the initial picture. Only the L* picture was saved. The L* picture was uploaded on ImageJ. Then using the mouse, only the area of the picture that illustrated the leaf surface was carefully selected. Thus, the program produced a lightness histogram of the leaf surface. The lightness histogram provided the average L* value of the selected leaf area. The same was done for all the pictures of each resting day.

6.0 Results and Discussion

6.1 Trehalose isotonic solution for vacuum impregnation

On Figure 7, the diagram obtained by immersing fresh oregano leaves in trehalose solution with different concentrations is reported. The weight of the leaves is stable when immersed in 10% (w/v) trehalose solution, thus it is implied that the isotonic solution for fresh oregano leaves is 10% (w/v). The trehalose solutions with concentration 5% (w/v) and 7% (w/v) are hypotonic whilst 12% (w/v) is considered hypertonic.

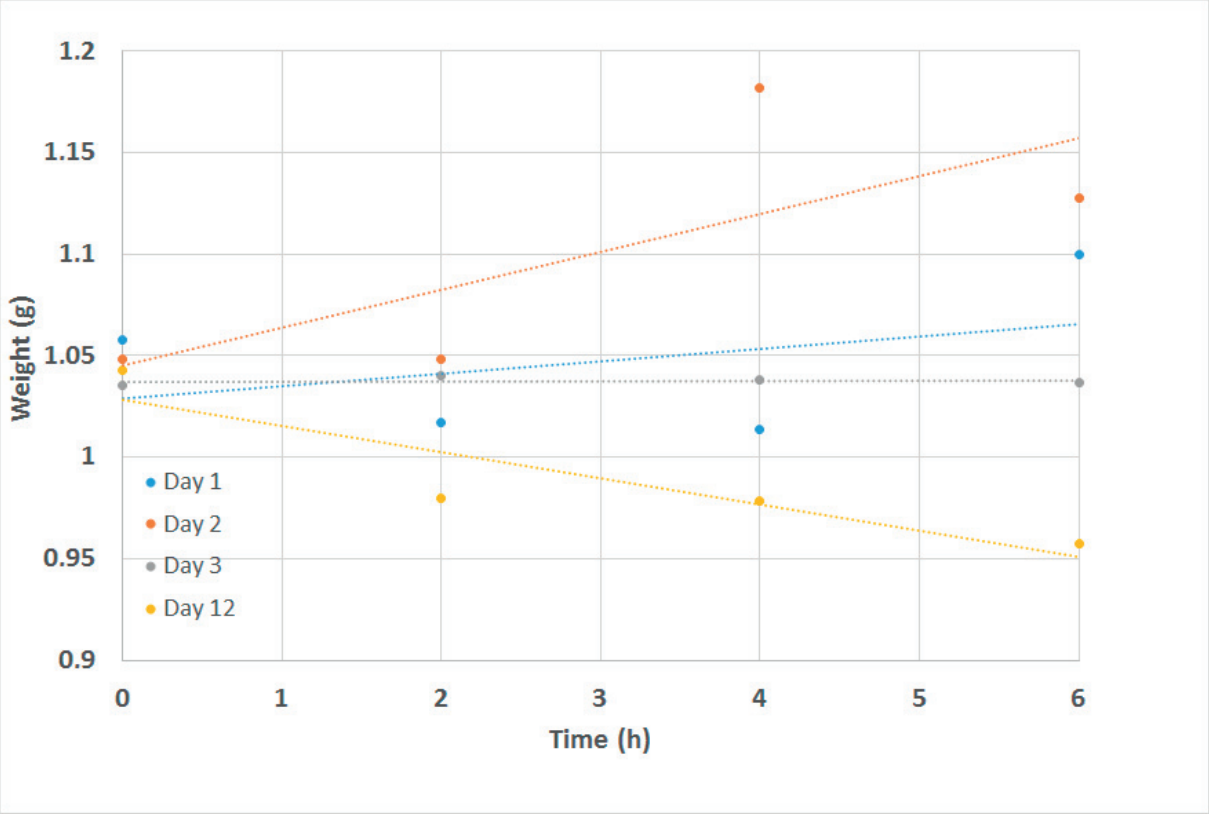

Figure 7: Weight of oregano leaves immersed in 5% (w/v), 7% (w/v), 10% (w/v), 12% (w/v) trehalose solution over time. Standard deviation was less than 5%.

6.2 Optimal VI conditions for fresh oregano leaves

The weight gain for the different VI protocols tested in this study is reported in Table 2. Thus it was decided to apply the VI conditions of protocol B as the highest percent of weight gain was obtained.

VI protocol	**Weight gain %**
A	18.36
B	24.00
C	19.50
D	19.40

Table 2: Weight gain (%) applying 4 different VI protocols

6.3 Permeabilization of fresh oregano

Figure 8 shows that the permealization of the leaf surface is homogenous. It can be easily seen that there are clusters of cells that have been permeabilized successfully. The red dots that fluorescence are the result of propidium iodine (PI) that has entered the cell through the pores that have been formed when PEF was applied. These clusters are distinct all over the leaf surface, thus the applied permeabilization process permeabilized the leaf surface homogenously.

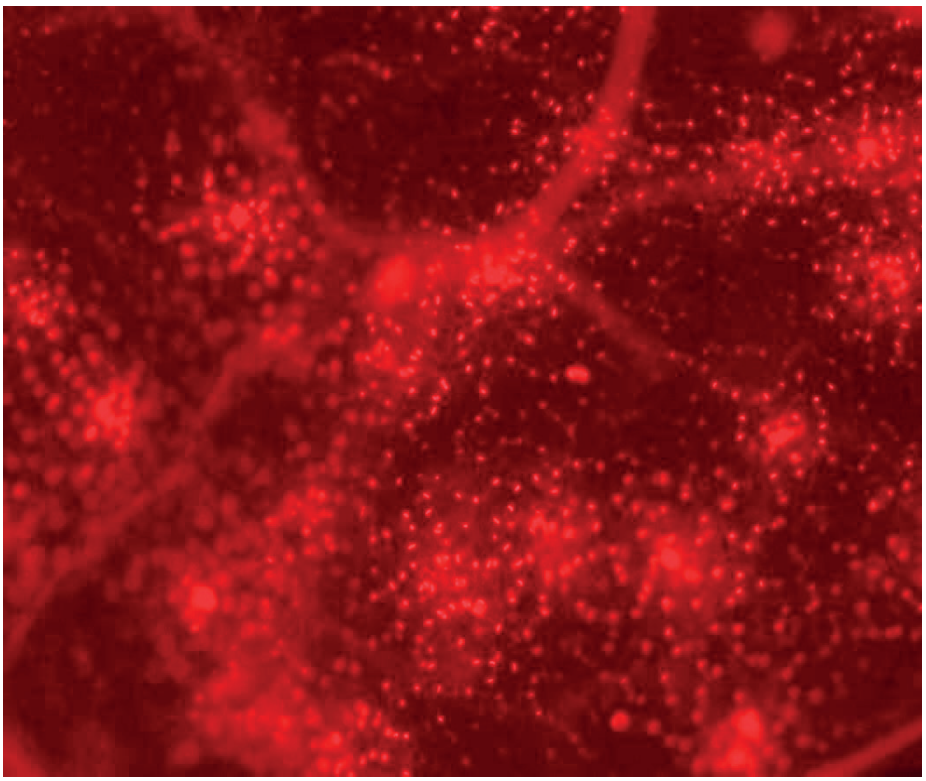

Figure 8: Sample treated with PEF under propidium iodide (PI). PEF was applied as specified in the Materials and methods section. The magnification used was x10.

6.4 Viability verification of the leaves

The pictures obtained from the microscope when testing viability after the different processing steps are reported in Figure 9. On the pictures A, C, D, E, D, G and I it is clear that the cells are alive since they show green florescence. On picture B, there is no fluorescence since the leaf was killed. The fluorescence is explained by the fact that fluorescein diacetate has converted by the cells to fluorescein, which has green fluorescent.

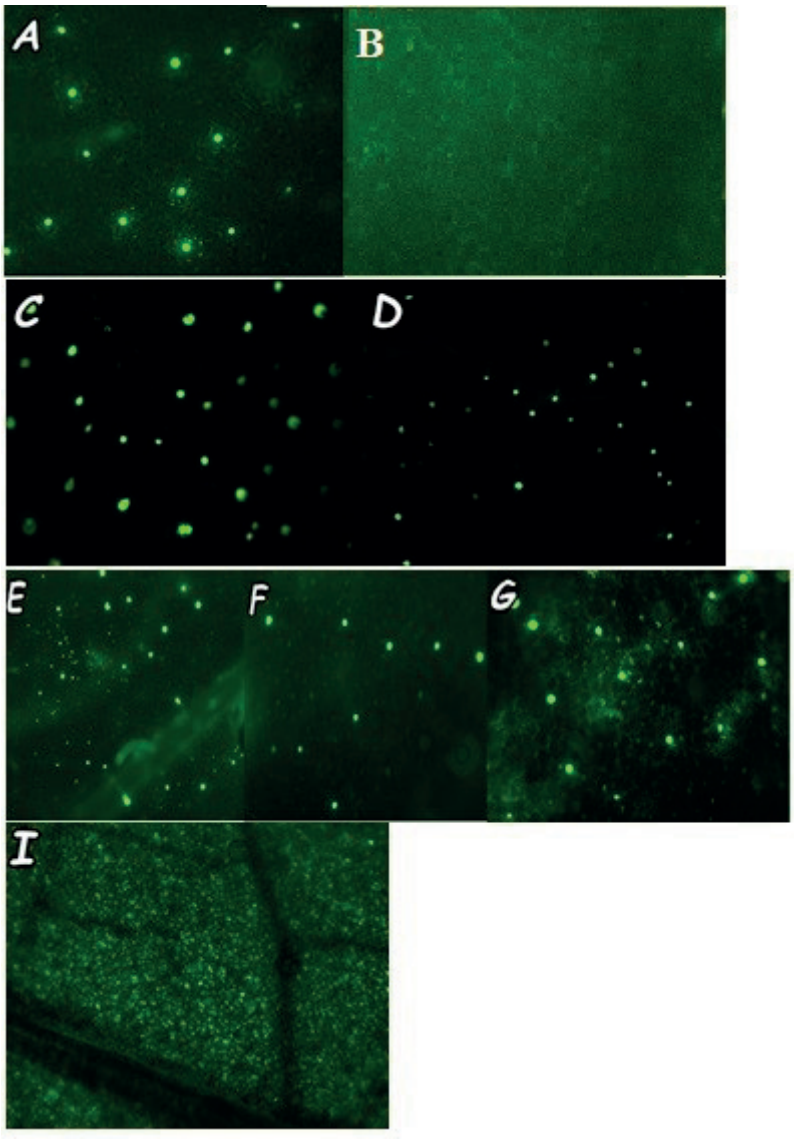

Figure 9: Fluorescence microscope pictures showing viability of cells. Control freshly cut leaf (A), control dead leaf (B), VI treated leaf (C), VI and PEF treated leaf (D), VI and PEF treated leaf rested at 4°C for 1, 2 and 3 days (E), (F), (G), VI and PEF treated leaf rested for 3 days, frozen and immediately thawed (I).

Cryoprotection of fresh oregano leaves was achieved when the combination of vacuum impregnation (VI) and pulsed electric fields (PEF) was applied before resting and freezing. Cryoprotection was accomplished by protecting the leaf cells both extracellularly and intracellularly with the presence of trehalose as cryoprotectant. PEF induces metabolic effects on plant cells that depend on the intensity, width of the pulse, the size and the persistence of the pores that were created (Gomez Galindo, 2008b). Those metabolic changes such as ATP hydrolysis and alteration of cell wall organization (Gomez Galindo, 2008b), followed by VI may assist the cryoprotection of leaves' cells (Phoon, et. al., 2008).

6.5 Post-thawing evaluation of viability

Since fresh oregano leaves had slightly different green color, it was decided to use relative values to achieve easier comparison. Two replicates were done for each resting time. On Figure 10A the diagram for the relative L* values (%) is reported for the first 60 minutes after thawing for leaves that were rested for 1, 2 and 3 days. On Figure 10B the diagram for the relative L* values (%) for 61-180 minutes after thawing is reported for leaves that were rested for 1, 2 and 3 days.

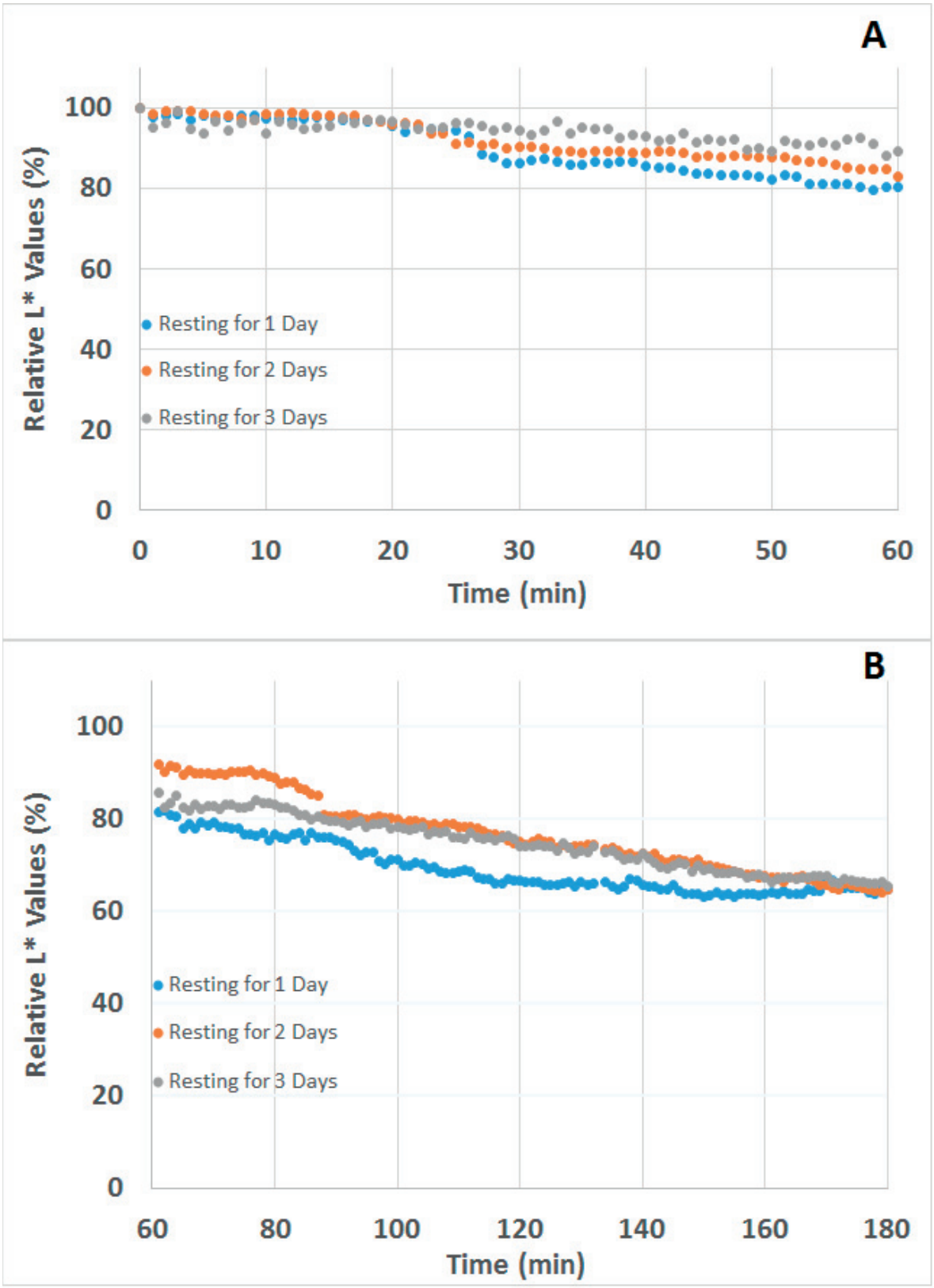

Figure 10: (A) Illustrates the relative L* values (%) obtained from the average of two replicates for the first 60 minutes after thawing. The leaves were rested for 1, 2 or 3 days. (B) Illustrates the relative L* values (%), obtained from the average of two replicates for 61-180 minutes after thawing. The leaves were rested for 1, 2 or 3 days.

Figure 10A shows the relative lightness values (%) of oregano leaves that were vacuum impregnated in isotonic trehalose solution, treated with pulsed electric fields, rested for 1,2 or 3 days, frozen and thawed. The relative lightness values (%) correspond to the first 60 minutes of thawing. For resting day 1, from 0 to 18 minutes the lightness values did not change. However, from 18 to 60 minutes there is a progressive decline of the leaves lightness. The leaves that were rested for 2 days, show lightness values that are steady between 0 to 25 minutes. In the time range from 25 to 60 minutes the relative lightness shows a continuous decline. When leaves were rested for 3 days before freezing, from 0 to 49 minutes the lightness values were stable. But, at 49 minutes the lightness values start to drop progressively. Clearly, it can be seen that from 49 to 60 minutes there is an uninterrupted decline. The slopes that were estimated from Figure 10A, are reported in Table 3. It is noticed that as the resting days increases, the slope of the decreasing lightness over time gets closer to value zero. A slope closer to zero, indicates smaller changes of relative lightness values (%). Resting time of three days provides more stable lightness values, compared to 1 day and 2 days of resting.

Figure 10B, shows the relative lightness values (%) corresponding to the 61-180 minutes after thawing. The slopes representing the changes of lightness over time after thawing are reported in Table 3. By observing Figure 10B and the calculated slopes, it can be concluded that the slope reduces faster when the leaf rested for 2 days, next follows the leaf that was rested for 3 days and lastly the leaf that was rested for 1 day. It would be interesting to compare the slopes from 0-60 minutes with the slopes from 61-180 minutes. By looking at Table 3, it is easy to observe that the greatest slope difference from 0-60minutes to 61-180 minutes is noticed when the leaf was rested for 1 day. On the contrary, the slopes from 0-60 minutes and 61-180 minutes for resting day 2 and resting day 3 are similar. This indicates that the biggest lightness changes occur the first 60 minutes, when the leaf was rested for 1 day. The lightness changes for resting day 2 and 3 were more constant. Thus, loss of tissue viability is faster in the first 60 minutes after thawing when the leaf was rested for 1 day than after resting for 2 and 3 days.

Table 3: Slopes estimated from Figure 10A and 10B, and inflection point calculated from Figure 10A. The slopes and inflection points refer to the changes in lightness of leaves that were rested for 1, 2 or 3 days.

Resting time (Day)	Slope from 0 to 60 minutes (Figure 10A)	Inflection point (min) of lightness curves (Figure 10A)	Slope from 61 to 180 minutes (Figure 10B)
1	-0.3653	18	-0.1447
2	-0.2737	25	-0.2333
3	-0.1183	49	-0.1672

The survival of cells to freezing and thawing is influenced by the physiological conditions prior freezing (Park, et al., 1997). Despite the achievement of successful cryoprotection, its duration was limited. It is considered that the reduction in relative lightness values is caused by cell death. Probably the reduction of relative lightness is progressive over time, due to the fact that cells do not die at the same time, but progressively. Cells start losing viability on different spots of the leaf surface. The number of cells that die or have died increases and starts to become obvious on the surface by small dark brown spots. Gradually, these dark brown spots agglomerate, become larger until all leaf surface turns from green to dark brown. There are two types of cell death apoptosis and necrosis (Zimmermann, KC.,et., al., 2001). The environmental temperature affects the occurrence of necrosis or apoptosis. High environmental temperatures lead to necrosis whilst moderate environmental temperatures lead to apoptosis (Reape, et al., 2008).

The resting time obviously affects the leaves' viability over time. Specifically, the VI and PEF treated leaves that were rested for 3 days took longer time to start decreasing their viability, reflected in the lightness measurements; however, they did not survive longer than 49 minutes. This might be an effect of stress that leaves go through during the pretreatments, before freezing. Clearly, PEF pretreatment induces oxidative stress (Gomez Galindo, 2008b). Additionally, during freezing, mature leaves are also induced to oxidative stress (Li, et al., 2018). It is known that oxidative stress causes programmed cell death (Vernier, et. al., 2004). Investigations on PEF treatment on cells showed that it may cause apoptosis due to relocation of phosphatidyserine (PS) from the plasma membrane lipid bilayer on the inner leaflet (Fadok, et. al., 2001;Vernier, et. al., 2004). It seems that the resting time allows plant cells to begin programmed cell death, apoptosis or necrosis later than would start without the pretreatment of VI and PEF. Maybe it would be a good idea to repeat the same process using milder PEF conditions or other cryoprotectants such as proline or betain.

7.0 Conclusions

- Through VI and PEF pretreatments, cryoprotection was achieved.
- The achieved cryoprotection only lasted few minutes and was affected by the resting time between PEF treatment and freezing.
- The resting time affects the rate at which the processed oregano leaves loose viability after thawing.

8.0 Future Work

- Use of another cryoprotectant instead of trehalose for example proline or betain.
- Apply milder PEF conditions.
- Apply PEF first and secondly VI.
- Rest the leaves in room temperature instead of 4^{o}C.

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