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Determination of Membrane Integrity in Onion Tissues Treated by Pulsed Electric Fields: Use of Microscopic Images and Ion Leakage Measurements

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Abstract

The influence of electrical field strength and number of pulses on cell rupture of onion tissues was investigated by two different methods to understand the changes in cell viability of plant tissues after pulsed electric field (PEF) treatment. The impact of pulsed electric field parameters on cell integrity of 20 mm diameter, 4 mm thick disks of Sabroso onions (*Allium cepa* L.) was determined by ion leakage measurements and microscopic method. The effect of treatments on cellular integrity was visualized by neutral red staining of the onion cells. Cell rupture is essential for optimal process design before extraction of desirable compounds and/or drying of plant tissues. Experimental results were obtained for onion disks treated with electrical pulses at field strengths of E≈ 167 V/cm and 333 V/cm, pulse width of ti=100 μs, frequency of f=1 Hz and the number of pulses, n=1 to 100. At 167 V/cm electric field strength treatment cell rupture was not observed however ion leakage increased and air spaces around cell walls disappeared, most likely due to changes in cell membrane permeability. Irreversible cell rupture occurred at 333 V/cm. Ion leakage values and ruptured cells count was increased with increasing pulse number. 92.2±5.9 % of the onion cells were ruptured after 333 V/cm and 100 pulse treatment. Small plant cells that are located near vascular bundles and upper epidermis showed higher resistance to pulsed electric field treatments.

Keywords: pulsed electric fields, plant tissue structure, onion, neural red staining, ion leakage

Industrial Relevance: The aim of this study was to determine the response of plant tissues with different cell type to the pulse electric field treatment. Two different methods, neutral red staining and ion leakage, were used to visualize and determine the cell rupture on onion tissues. Industry may choose one of these methods to evaluate treatment efficiency on the basis of cell rupture; especially ion leakage measurements which require lower investment cost and easy to apply or microscopic method for visualize the cells layer by layer. The paper stresses the importance of cell type, size and distribution of cells were found to the ability to resist cell rupture during pulsed electric field treatment. It is critical to explain overall changes caused by PEF on the structure of plant tissue at a cellular level in order to optimize the quality of PEF processed foods.

1. Introduction

Pulsed electric fields is an advanced technology that has been commercially applied for preservation of liquid food products such as juices (Barbosa-Canovas, Gongora-Nieto, Pothakamury & Swanson, 1999; Barbosa-Canovas & Zhang, 2001; Evrendilek & Zhang, 2005) as pre-step for solid food processes such as drying (Estiaghi & Knorr, 1999; Bazhal & Vorobiev, 2000; Vorobiev & Lebovka, 2006; Lebovka, Shynkaryk, El-Belghiti, Benjelloun & Vorobiev, 2007), and extraction (Bouzzara &Vorobiev, 2000; Bazhal, Lebovka, & Vorobiev, 2001; Eshtiaghi & Knorr, 2002). While there are a few commercial applications of PEF to whole plant tissues, the effects on the cellular structure of plant tissues is much less understood (Lebovka, Bazhal & Vorobiev, 2001). It has been established that pulsed electric fields are capable of

rupturing microbial cells, which destroys viability and results in extended shelf life (Zhang, Barbosa-Canovas & Swanson, 1995; Lebovka & Vorobiev, 2004).

Plant tissues are heterogenous materials, but the cells which compose them are generally made up of cells walls surrounding the plasma membrane, which contains the primarily liquid contents in the cytoplasm. The vacuole is an acidic storage comparment for sugars, acids and other solutes within the cytoplasm and oftentimes comprises a significant portion of each cell. Permeabilization of plant cell membranes by PEF can be reversible, in which case the cell membrane reseals following treatment, or irreversible, where the cell lyses or ruptures, as a function of the electrical protocols used (Zimmermann, Pilwat & Riemann, 1974). It is obvious that for optimization of electric field distribution during electroporation and cell rupture, the geometry of tissues, cell sizes and types present should be known. When an electrical current is forced to flow across different tissues, voltage drops at higher resistivity cell layers will be significant (Ivorra &Rubinsky, 2007). Therefore it is important to have sufficient electric field at the region of interest for cell permebialization or rupture to occur.

Living cells have a natural potential difference of approximately -150 mV (Coster, 1965). When the transmembrane potential difference exceeds a critical value, then the electrocompressive force exceeds the elastic forces and membrane defects, e.g. pore formation, occurs (Bouzrara & Vorobiev, 2003). Additional tension on the cell due to electrical stress may lead the further pore enlargements. When the pores can reseal after removing the external electric fields the effect is called reversible, whereas if the cells are permanently ruptured this is termed irreversible electroporation (Chang, Chassy & Saunders, 1992). Rupture occurs when the transmembrane potential of membranes reaches to 0.7-2.2 V at various field intensities (Angersbach, Heinz & Knorr, 2000). The electric field strength at which membrane breakdown occurs is called the

critical electric field strength or E_{cr}. In cellular systems such as potato, apple and fish tissues as well as in plant suspension cultures, the critical electric field strengths were found to be in the range of 150-200 V/cm (Angerbach, Heinz & Knorr, 2000).

Fincan and Dejmek (2002) studied in situ visualization of changes related to electropermeabilization of a single layer of onion epidermis during and after pulsed electrical field treatment with single pulse. However, the dynamics of cell disintegration in multi-layered tissues as a semi solid system composed of different types of cells has not been previously visualized. One method these authors used for determination and visualization cell disintegration is neutral red staining. The principle of this method is that nuetral red is an uncharged and non-ionized dye in alkaline solutions and it diffuses across plant membranes due to its lipophilic nature. The dye crosses the tonoplast membrane of the vacuole, where it ionizes and accumulates in the acidic vacuolar medium, appearing as dark red colored vacuoles in intact cells (Ehara, Noguchi & Ueda, 1996; Fincan & Dejmek, 2002). Neutral red staining has been widely used for the estimation of cell viability since penetration of the dye into the tissue depends on the integrity of the cell membranes and the capacity to maintain pH gradients (Repetto, del Peso & Zurita, 2008; Gonzalez, 2009).

Another method which has long been used as a measurement of the intactness and permeability of cell membranes (Vasquez-Tello, Zuily-Fodil, Pham Thi & Viera da Silva, 1990) is electrolyte leakage. An increase in ion leakage has been used to study chilling injury in a number of crops (Salveit, 1989). Since the leakage is from a high concentration inside the cell, to a low concentration outside it, the efflux may be considered to be passive diffusion, but the influx must be due to active transport. Increased injury indicated by the net leakage, may result from either

an increased efflux due to damage to the semipermeability of the plasmalemma, or a decreased influx due to damage to the active transport system (Palta et al. 1977a).

The disintegration of plant tissues treated with pulsed electric fields has been reported previously, using measurements of electrical conductivity by impedance meter where a model circuit of the cell membranes (tonoplast and plasmalemma) were assumed to be capacitors and the interior of both the vacuole and cytoplasm are resistors (Angerbach, Heinz & Knorr, 1999). Estimation of the disintegration of apple, carrot and potato tissues were also made using the kinetics of electrical conductivity in the course of PEF treatment and empirical dependencies of disintegration versus electric field were determined (Lebovka, Bazhal, & Vorobiev, 2002). The basic complexity of the optimum PEF treatment mode is obtained using correlations between the processing protocol and rupture (cell plasmolysis) or degree of damage to the biological tissue (Lebovka, Bazhal & Vorobiev, 2002). The influence of electromechanical stress or transmembrane potential on membrane discharge and rupture seems to be a function of various factors such as membrane properties, the external medium and the protocols of electroporators (Ho & Mittal, 1996).

In this study, the objective was to use neutral red staining and ion leakage to determine the effect of different electric field strengths applied for varying numbers of pulses on disintegration of the multiple cellular layers found in onion tissues. Another objective was to visualize the effect of PEF on different cell types and distribution.

2. Materials and Methods

2.1. Raw Material

Spanish yellow onions cv. Sabroso (approx. 8 cm in bulb diameter) were provided by Gills Onions (Oxnard, California) and used for pulsed electric field experiments. Whole onions were shipped to UC Davis and stored at 4 ºC until processing.

2.2. Sample preparation

The outer papery scales and the first fleshy scale and second layer of the onions were removed and then starting from the third scale, which is not damaged from the mechanical harvesting and postharvest conditions, samples were taken using a cork borer, which cut them into 20 mm diameter, 3 mm thick discs. Each single disc was processed in the PEF system and considered as one sample replicate.

2.3. Pulsed electric field (PEF) treatment

In preliminary experiments, onion discs were pulsed either 10 or 100 times at two levels of electrical field strength (E), e.g. 167 V/cm and 333 V/cm, which was derived from an applied potential of 50 to 100 V on a 3 mm thick disk. Initially at each electric filed strength 10 and 100 pulses were applied and tissues were visualized with light microscope. Then a subsequent set of experiment was designed to follow cell membrane rupture sequentially, by increasing the number of pulses applied at 333 V/cm from 2, 4, 6, 8 to a maximum of 10 pulses. Also, 50 pulses were applied at 333 V/cm. Monopolar positive pulses of rectangular shape with pulse width t_i = 100 μs and pulse frequency f = 1 Hz were used for each processing condition.

2.4. Pulsed electric field (PEF) equipment

PEF treatments were carried out using a system developed at the University of California, Davis. The PEF system consisted of a high voltage power supply (PowerPAC HV, BIO-RAD, USA), a

function generator (model 33220A, Agilent, USA), a PEF generator, sample holder and an oscilloscope (model TDS1012B, Tektronix, USA) for signal monitoring. The plexiglas cylindrical sample holder consists of a top and bottom chamber, and the bottom chamber has a well (gap) of a specific depth. The top chamber is assembled with a 2 cm diameter flat stainless steel electrode. The well of the bottom chamber, used in these studies, is 0.3 cm deep and 2 cm in diameter and has a flat stainless steel electrode fixed inside the bottom. The PEF generator regulated to provide monopolar positive pulses of rectangular shape with a pulse width t_i = 100 μs and pulse frequency f = 1 Hz.

2.5. Light microscopy

The light microscopy method which utilized neutral red for visualization of onion tissue integrity was used for one layer epidermis tissue of onions (Fincan & Dejmek, 2002) and the method was modified in our laboratory for 3 mm thick sample that has several layers of cells to observe the cells response to PEF (Gonzalez, 2009) and utilized in this study.

2.5.1. Microscope section preparation

From each onion disc, two 400 μm thick cross section specimens perpendicular to both epidermises were obtained for staining and microscopic analysis. 5 mm x 5 mm section specimens were cut and prepared using a Vibratome 1000 Plus (The Vibratome Company, St Louis, MO, USA.) (Gonzalez, 2009).

2.5.2. Neutral red (NR) staining

Freshly diluted stock dye solution was used during experiments. 0.5 % NR was dissolved for 30 min in acetone and then filtered twice using Whatman No.1 paper. The filtered stock solution

was diluted to 0.04 % in an isotonic solution of 0.2 M mannitol - 0.01 M HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethane-sulfonic acid]) buffer, pH 7.8, and used as dyeing solution.

Onion sections were cut and immediately rinsed in deionized water to remove cell debris and contents released by mechanical damage to the tissue during cutting process. Sections were dipped in 600 µl of diluted dye solution for a period of 2 hours, after which they were rinsed again for 30 min in the 0.2 M-0.01 M HEPES buffer solution. Specimens were mounted on a microscope slide with a drop of deionized water, covered with a cover slip and immediately observed with a light microscope (Olympus System Microscope, Model BHS, Shinjuku-Ku, Tokyo, Japan) at 2.5 or 4.0 X magnification. A digital color camera (Olympus MicroFire, Japan) was attached to the microscope to capture images (Olympus MicroFire software). Color photomicrographs (800 x 600 pixel resolution, white balance corrected) were captured from both parenchyma and vascular bundle cells in the outer epidermis of each specimen.

The entire range of processing treatments was replicated on separate days, with the pulsed electric field treatments carried out 4 times. Two onion discs were processed for each treatment. From each treated onion disc, two onion 400 µm specimens were obtained. At least two micrographs were taken from each specimen. The total number of images per PEF process treatment was as follows:

Pulsed electric field applied samples micrographs = 4 replicates/process treatment x 2 specimen/ onion discs x 2 micrographs/specimen = 16 micrographs per process treatment. In the figures, four images were selected to represent the visualization of treatments.

2.6. Ion leakage (%) determination

Two onion discs, either control or PEF treated, were placed into 50-ml plastic tubes containing 20 ml of an isotonic solution, 0.2 M mannitol. Ion leakage was measured as electrical conductivity (σ) in 0.2 M mannitol solution using a conductivity meter (Accument portable AP65 Fisher Scientific) over a 4 h time period (t_i) at 25 °C (Salveit, 1989-2002). Ion leakage was calculated as a percentage of total electrical conductivity of the sample. Total conductivity was measured from samples that were frozen (-18 °C) and thawed twice, resulting in 100 % ruptured cells. The ion leakage experiments were replicated 4 times per PEF process treatment.

$$Ion\ leakage\ (\%) = \frac{Conductivity\ (t_i)}{Total\ Conductivity} \times 100 \qquad \text{Eq. 1}$$

3. Results and Discussion

3.1. Vital staining after exposure to 167 V/cm PEF treatments

Neutral red (NR) staining of onion tissues is used for visualizing the viability of plant cells. Viable cells are identified by NR dye uptake into the vacuole, where the red color is concentrated, and also by the smooth appearance of the intact cells as visualized in the light microscope (Gonzalez, 2009). White cells are considered to be ruptured (inviable) cells which are not able to accumulate the neutral red dye in the vacuole. Micrographs of untreated control onion tissue had both stained and unstained parenchyma cells (Fig. 1). The unstained, inviable cells are assumed to result from mechanical damage that occurs during tissue sectioning of cells larger than 400 μm in diameter in the longitudinal plane, causing rupture of membranes and loss of viability. The black edges near the cell walls surrounding many of the stained cells are air spaces in the apoplast, or extracellular spaces between cell walls of adjacent cells (Gonzalez, 2009). Samples treated with 10 pulses of electric field strength of 167 V/cm showed similar

results as control samples, except that in some sections the black edges in the extracellular spaces had disappeared. When the number of the pulses at the same electrical field strength was increased to 100, it was evident that black edges at the extracellular spaces disappeared entirely. The similarity of micrographs for control and 167 V/cm treated samples shows that there was no PEF discernible effect on the cell viability at this electrical field strength. But the presence or absence of air in the extracellular spaces between the cell walls was affected by the number of pulses applied.

These observations are similar to micrographs previously obtained from onions which have been exposed to vacuum treatment, e.g. when the air is physically removed from extracellular locations, the black edges were lost (Gonzalez, 2009). Interstitial air spaces were quantified in control specimens of parenchyma onion tissue by image analysis (Dražeta, Lang, Hall, Volz & Jameson, 2004; Gonzalez, 2009).

The disappearance of intercellular air spaces following PEF treatment at 167 V/cm treatments could be the result of fluids leaking from the cell into the intercellular spaces. Because no cell rupture occurred at this field strength (Fig. 1), reversible electroporation may have occurred. This has been suggested to occur at a lower level than the critical electric field strength, which is necessary to rupture the cell membranes. Fincan & Dejmek (2002) used an in situ image visualization method to quantify cell permeabilization in onion epidermal tissue during and after pulsed electric field treatments at 170, 350 and 520 V/cm, using single pulse treatment. They recorded micrograph images at different time intervals over 4 min 55 s period and found that permeabilization occurred in the epidermal cells after PEF treatments at 350 and 520 V/cm. These authors also found that significant changes occurred in the extracellular air space and in conductivity (mS/cm) values.

3.2. Ion leakage after exposure to 167 V/cm PEF treatments

In Figure 2, changes in percent ion leakage are illustrated for the control and samples treated at 167 V/cm electric field strength. Ion leakage in control samples was 11.5±1.3 %. Ions in the cell wall and extracellular spaces, in particular at the cut surface of the tissue, rapidly diffuse from the tissue after it is cut and immersed in an aqueous solution, and this causes a transient elevated rate of ion leakage. Washing the excised tissue to remove cellular contents released by wounding would lessen this transient increase in leakage and reduce the background level of conductivity (Salveit, 2002). In this study, however, it was decided not to wash the cut tissues because this may have influenced diffusion of ions from the remaining tissue. Ion leakage after the 10 and 100 pulse treatments at 167 V/cm was 21.6±1.7 % and 41.4±0.4 %, respectively. Ion leakage rates increased gradually due to transfer of ionic species from intracellular spaces through extracellular spaces and into the mannitol. This occurred through PEF induced permeabilization of membrane. Pore openings in membranes caused a free flow of intracellular liquid through the cell wall.

Fincan & Dejmek (2003) reported similar results following more intensive PEF treatments. These authors stated that PEF treatment causes larger pores to be made in the cell membrane of onion epidermis which allow faster escape of liquid cell contents and thereby faster relaxation. In an earlier study, Fincan & Dejmek (2002) reported the change in conductivity as a function of applied electrical field strength, using only a single pulse application to one cell layer of onion epidermis. At 350 and 520 V/cm field strengths, conductivity increased in 60 s, whereas the treatment at 170 V/cm did not result in an increase in conductivity which was similar like control. Their result showed that ionic species from the permeabilized intercellular space spread through the extracellular spaces to the electrodes and/or the outside path gradually and

causedconductivity increase. Contact resistances between electrodes and the internal extracellular spaces of the tissue were found higher than the parallel resistance in the moisture path along the outside of the sample so no sudden change was observed in conductivity.

3.3. Vital staining after exposure to 333 V/cm PEF treatments

In Fig.3, images from parenchyma, vascular bundle and outer epidermal cells are shown for control and samples treated at 333 V/cm for different number of pulses. The 10, 50 and 100 pulse treatments at 333 V/cm resulted in less concentrated NR staining, therefore indicating tonoplast membrane rupture. Almost 100 % cell rupture occurred after 100 pulse treatments at 333 V/cm, whereas following 50 pulse treatments some cells still appeared to be viable. In 10 pulse treated samples only a few viable parenchyma cells were seen, but most of the cells around the vascular bundles were viable. Treatment with 2 to 10 pulses at this constant electric field strength caused less rupture of the parenchyma cells.

Background color differences occurred in images where the cells are ruptured, due to interaction of the protonated dye with negative charges in the cell walls (Stadelmann & Kinzel, 1972). NR dye interaction with DNA (Wang, Zhang, Liu & Dong, 2003) has also been reported and may account for the retention of stain in inviable cells.

Onion parenchyma tissue is composed of very large cells, with some cells close to 400 μm in diameter in the longitudinal plane (Gonzalez, 2009). Cells around the vascular bundles are smaller than the parenchyma cells and these cells are more resistant to the pulsed electric field treatments. Cell size and shape effects on electroporation have been studied with mammalian cells and protoplasts (O`Hare, Ormerod, Imrie, Peacock & Asche 1989; Rouan, Montané, Alibert & Teissié, 1991; Kanduser & Miklavcic, 2008). Studies with protoplasts determined a linear

relationship between protoplast size and critical field strength needed for permeation (Montane, Alibert & Teissi, 1990; Rouan, Montané, Alibert & Teissié, 1991). Our results are the first published illustration of the effect of field strength and number of pulses on heterogeneous cell types in multiple cell layer plant tissues. The cells around vascular bundles are smaller than the parenchyma cells and were more resistant to the rupture effect of PEF applications.

In electroporation, when a current is forced to flow across different tissue layers, those with higher resistivity will be subjected to higher electric fields. This implies that some tissue layers will be more prone to electroporation than others (Davalos, Rubinsky & Mir, 2003). Plate electrodes do not produce homogeneous electric fields when the tissue to be treated has an irregular shape. Moreover in heterogeneous electric fields, there is also a voltage drop at those higher resistivity layers which will be significant and in most cases, uncontrollable (Ivorra & Rubinsky, 2007). Impedance at the electrode–tissue interface also makes the uniform application of pulsed electric fields a challenge. Hence, factors such as cell size, geometric distribution of cells, thickness of tissue layers and heterogeneity of electric fields caused the non-uniform cell rupture in the onion tissue illustrated in Fig. 3. It is also reported, for a spheroidal cell, the maximum induced transmembrane potential strongly depends on its orientation with respect to the electric field. It is maximum when spheroidal cell is parallel to the applied electric fields (Valic et al. 2003).

3.4. Ion leakage after exposure to 333 V/cm PEF treatments

In Figure 4, percent change in ion leakage is illustrated for the control and samples treated with different numbers of pulses at electric field strength of 333 V/cm. Following application of 2, 10 and 100 pulses, ion leakage of the samples went from 24.5±9.0 % to 67.5±10.0 % to 92.2±5.9 %.

Application of field strengths of 333V/cm resulted in cell membrane rupture, as illustrated in Figure 3. Electrolyte leakage has long been used as a measurement of the intactness and permeability of cell membranes (Vasquez-Tello, Zuily-Fodil, Pham Thi & Viera da Silva, 1990; Gonzalez, 2009).

The most common method for determination of the extent of plant and animal tissue disintegration after electrical treatments is based on measurement of conductivity using an impedance meter (Knorr & Angerbach, 1998). Other researchers have previously determined disintegration of tissues by measuring electrical conductivity at low frequencies (1-5 kHz) (Lebovka, Bazhal & Vorobiev, 2002) using an LCR Meter HP 4284A (Hewlett–Packard) at the frequency of 1000 Hz (Lebovka, Praporscic & Vorobiev, 2004). A more complex method measures the electrical conductivity of treated and intact materials in the range of 3-50 MHz using impedance meter (Angerbach, Heinz & Knorr, 1999). Measurements with impedance meter require investment cost but the results delivery is very quick. Our method is easy to apply and economical for plant tissue experiments according to impedance meter measurements which is cheaper in instrumentation investment if the aim is finding the degree of cell rupture while the determination of ion leakage requires approximately 4 h measurement time.

4. Conclusions

Electric fields applied to irregularly shaped plant tissues resulted in a range of effects. With constant electrical field strengths, an increase in the number of pulses induced cell rupture. Cell size and distribution of cells were found to be very important to the ability to resist cell rupture. Small plant cells showed higher resistance to pulsed electric field treatments. Parenchyma cells were distributed horizontally in the onion tissue, and these ruptured easily. However cells in the

vascular bundles and the outer epidermis were often found to exist longitudinally between the upper and lower electrodes and these had greater resistance to the electrical field applications. Tissues treated with 100 pulses at 167 V/cm were determined to maintain viability although ion leakage rates were as high as 40%. Treatments at 333 V/cm for only 4 pulses, on the other hand, had the same level of ion leakage but were inviable. This indicates that low electric field applications result in reversible electroporation while higher electric field applications were irreversible.

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Figures Captions

Figure 1. Micrographs of parenchyma cells processed at 167 V/cm for variable pulse numbers at 1 Hz and 100 µs pulse width.

Figure 2. Ion leakage (%) of PEF treated samples at 167 V/cm for variable pulse numbers at 1 Hz and 100 µs pulse width.

Figure 3. Micrographs of parenchyma, vascular bundles and outer epidermis cells processed at 333 V/cm for variable pulse numbers at 1 Hz and 100 µs pulse width.

Figure 4. Ion leakage (%) of PEF treated samples at 333 V/cm for variable pulse numbers at 1 Hz and 100 µs pulse width.

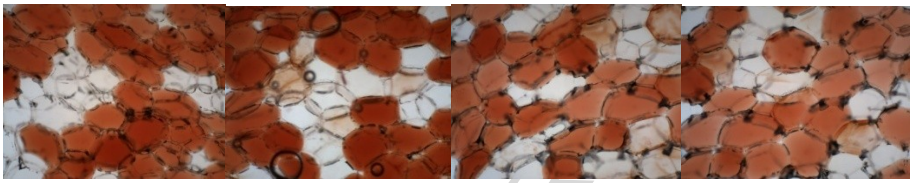
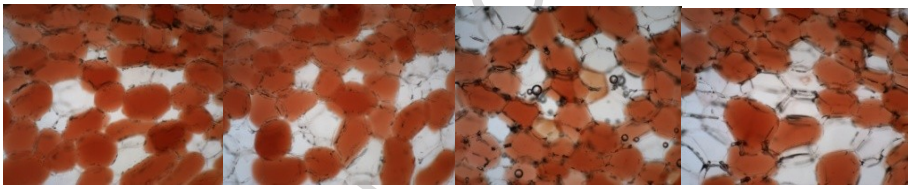
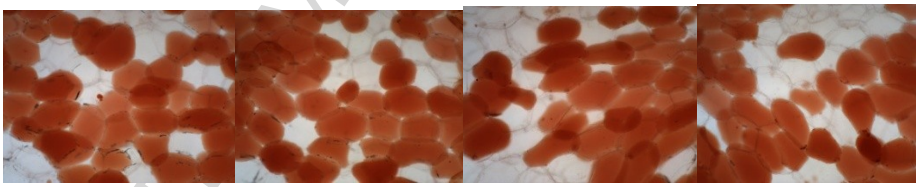
Treatment	Parenchyma Cells
Control	
167V/cm-10 pulse	
167V/cm-100 pulse	

Figure 1.

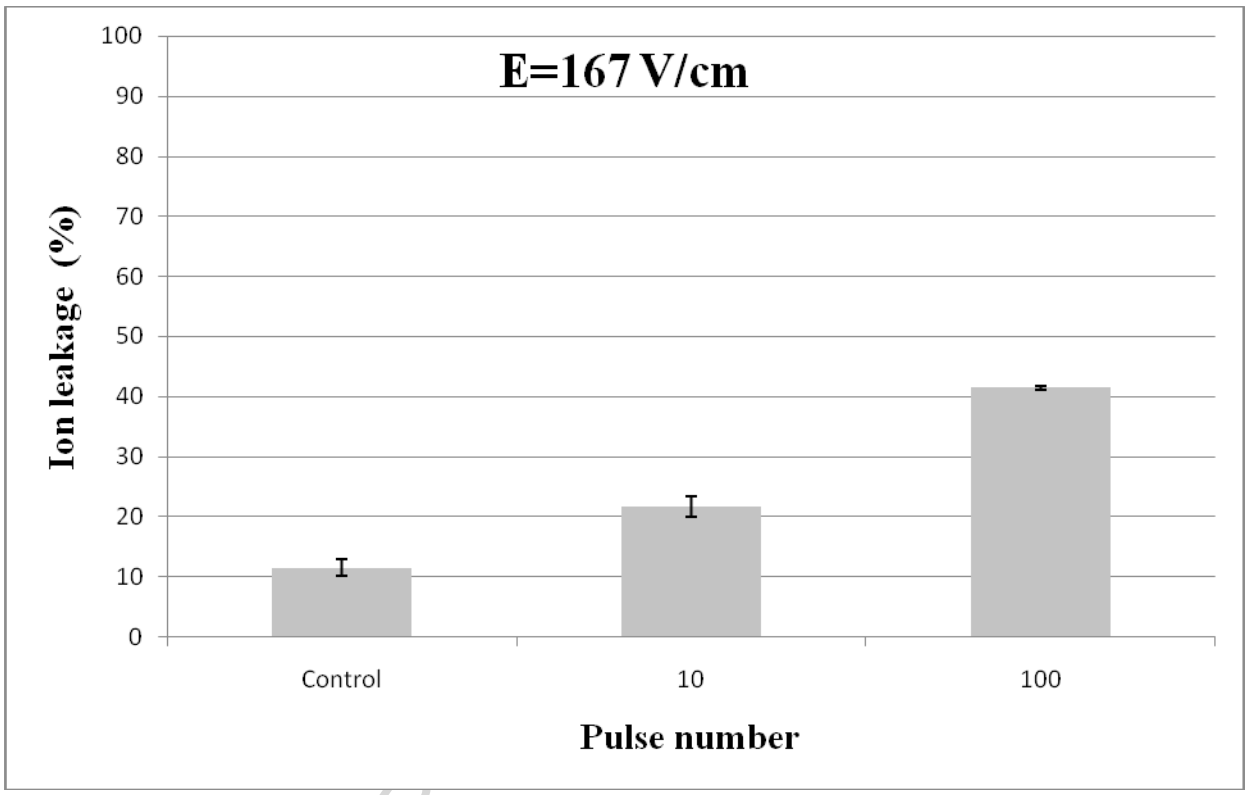


Figure 2.

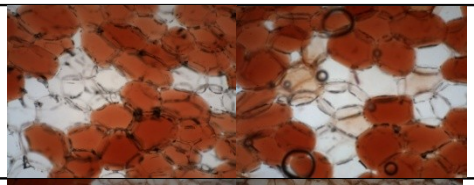
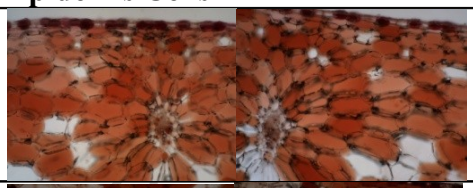
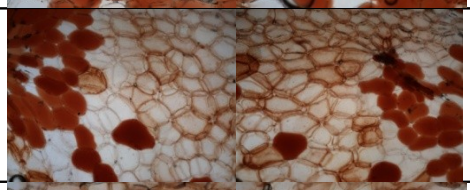
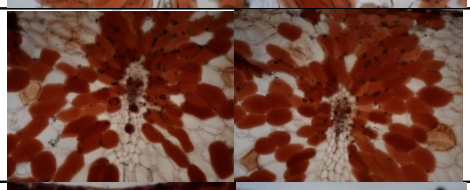
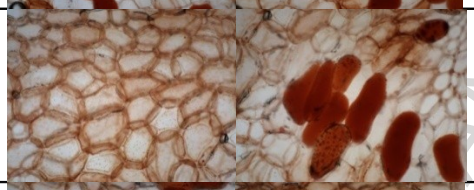
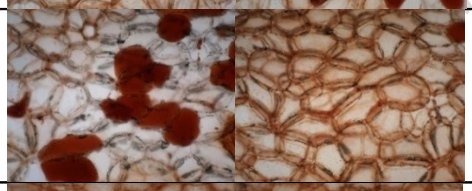
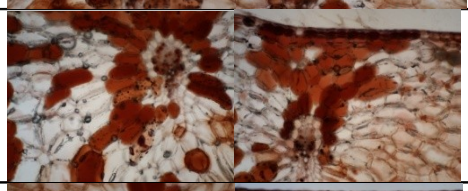
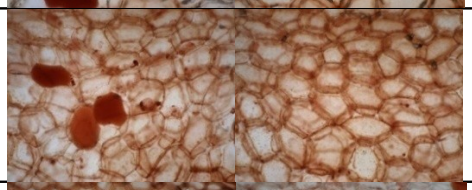
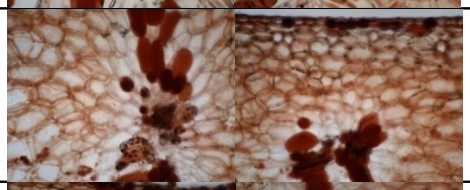
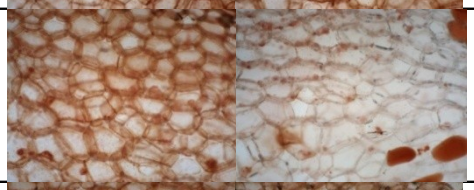
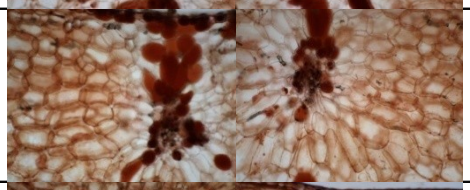
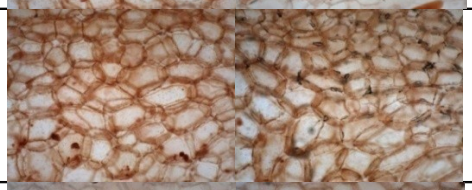
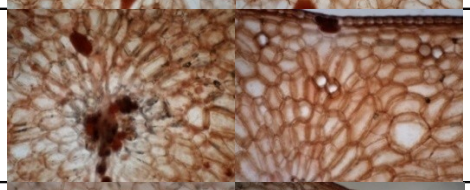
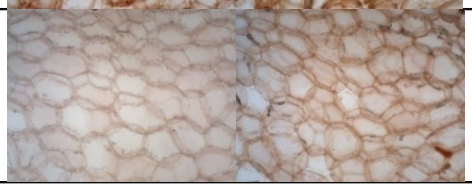
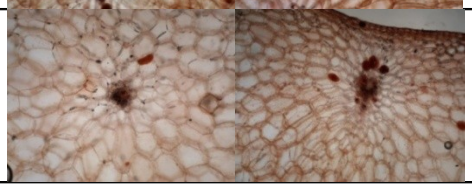
Treatment	Parenchyma Cells	Vascular Bundles and Outer Epidemis Cells
Control		
333 V/cm-2 pulse		
333 V/cm-4 pulse		
333 V/cm-6 pulse		
333 V/cm-8 pulse		
333V/cm-10 pulse		
333V/cm-50 pulse		
333V/cm-100 pulse		

Figure 3.

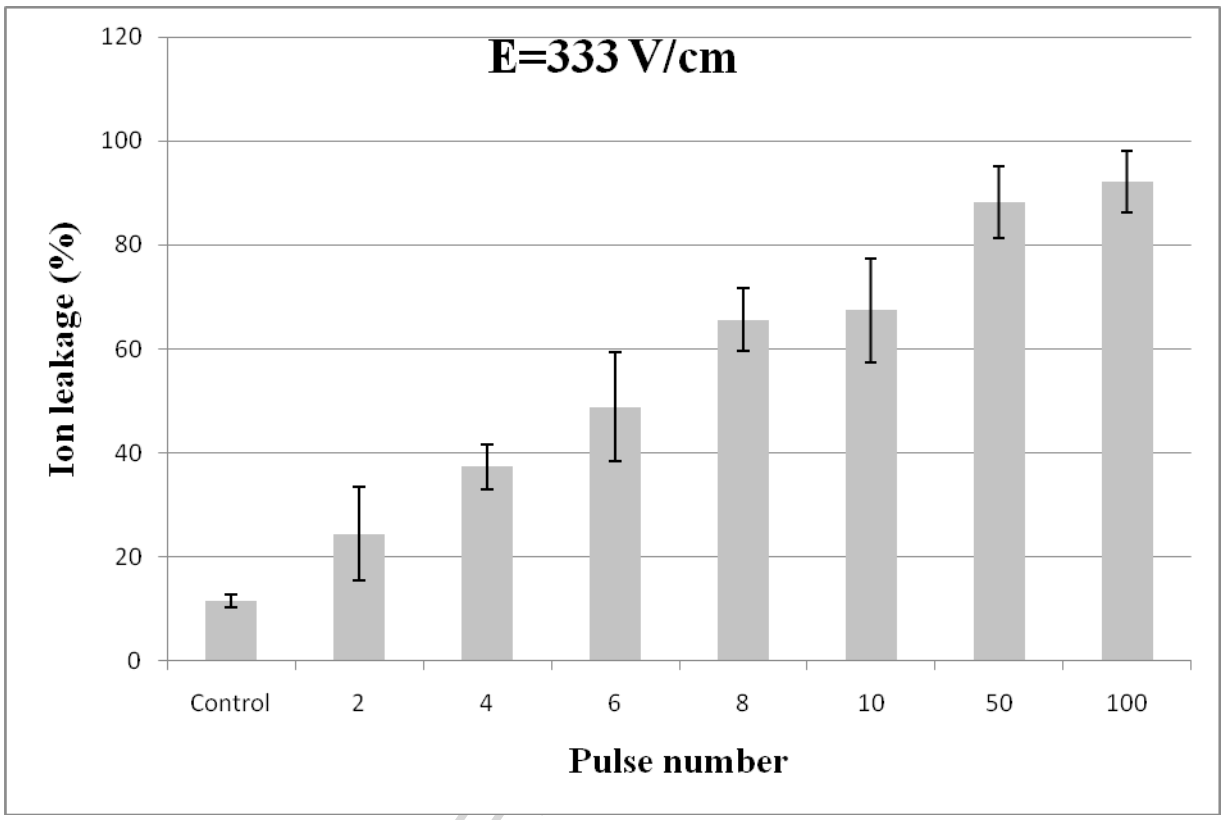


Figure 4.