Permeabilization of Plant Tissues by Monopolar Pulsed Electric Fields: Effect of Frequency

Suvaluk Asavasanti, William Ristenpart, Pieter Stroeve, and Diane M. Barrett

Abstract Pulsed electric fields (PEF) nonthermally induce cell membrane permeabilization and thereby improve dehydration and extraction efficiencies in food plant materials. Effects of electrical field strength and number of pulses on plant tissue integrity have been studied extensively. Two previous studies on the effect of pulse frequency, however, did not provide a clear view: one study suggested no effect of frequency, while the other found a greater impact on tissue integrity at lower frequency. This study establishes the effect of pulse frequency on integrity of onion tissues. Changes in electrical characteristics, ion leakage, texture parameters, and percent weight loss were quantified for a wide range of pulse frequencies under conditions of fixed field strength and pulse number. Optical microscopy and viable-cell staining provided direct visualization of effects on individual cells. The key finding is that lower frequencies ($f < 1$ Hz) cause more damage to tissue integrity than higher frequencies ($f = 1$ to 5000 Hz). Intriguingly, the optical microscopy observations demonstrate that the speed of intracellular convective motion (that is, cytoplasmic streaming) following PEF application is strongly correlated with PEF frequency. We provide the first *in situ* visualization of the intracellular consequence of PEF at different frequencies in a plant tissue. We hypothesize that cytoplasmic streaming plays a significant role in moving conductive ionic species from permeabilized cells to the intercellular space between plant cells, making subsequent pulses more efficacious at sufficiently low frequencies. The results suggest that decreasing the pulse frequency in PEF may minimize the number of pulses needed to achieve a desired amount of permeabilization, thus lowering the total energy consumption.

Keywords: cytoplasmic streaming, electrical properties, pulse frequency, tissue integrity, viability staining

Practical Application: PEF cause pores to be formed in plant cell membranes, thereby improve moisture removal and potential extraction of desirable components. This study used *in situ* microscopic evaluation of onion cells, as they were pulsed with electric fields at different frequencies, to determine whether frequency was an important parameter. We illustrate that membranes were more effectively broken at lower frequencies as compared to higher frequencies. Application of this information will allow for improved design of PEF systems for more energy efficient dehydration or extraction of plant tissues.

Introduction

The application of pulsed electric fields (PEF) for short durations of a few to several hundred microseconds is a nonthermal technology that is capable of inducing cell membrane permeabilization through a phenomenon called "electroporation." Many food-processing applications have been developed based on the loss of cell membrane integrity following PEF treatment. Initially, most of these efforts were focused on microbial inactivation in liquid foods and beverages (Qin and others 1998; Picart and Cheftel 2003; Aronsson and others 2005). Recently, more interest has been paid to PEF pretreatment of biological materials, such as potato, carrot, radish, apple, sugar beet, and so on, to expedite the rate of dehydration and increase the extraction yield (Angersbach and Knorr 1997; Rastogi and others 1999; Angersbach and others 2000; Bajgai and Hashinaga 2001; Bazhal and others 2001; Taiwo and others 2002; Kulshrestha and Sastry, 2003; Lebovka and others 2003a, 2003b, 2004). The advantage of PEF over traditional thermal processing is that it may conserve nutritional values of fruits and vegetables, while retaining the flavor, color, and taste of fresh food materials. However, industrial use of PEF for nonthermal processing of heterogeneous food materials is limited due to a lack of clear understanding about the mechanism of electrical breakdown processes in whole plant tissues and optimal PEF parameters.

The basic mechanism of electroporation has been studied primarily at the single-cell level in animal, plant, and microbial cells (Pavlin and others 2005). PEF effects are more complex in multicellular systems. The presence of different cells with varying electrical and topological properties within multicellular tissues has a pronounced influence on the effectiveness of electroporation (Lebovka and others 2001). Despite the complexity, at least 6 studies suggest that PEF results in an increase in the electrical

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conductivity and permeability of the whole plant tissue sample (Knorr and Angersbach 1998; Lebovka and others 2001, 2002; Fincan and Dejmek 2002, 2003; Asavasanti and others 2010). Further studies indicated that PEF causes a nonthermal rupture of the cellular membranes, thereby removing the cellular turgor component of texture and affecting the viscoelastic properties of the tissue (Fincan and Dejmek 2003; Lebovka and others 2003a, 2003b; Lebovka and others 2005). Because integrity of the cell membrane is important to the overall quality of fruits and vegetables, changes in electrical and physical properties, such as electrical conductivity, ion leakage, texture, weight loss, have been widely used to evaluate plant tissue integrity (Knorr and Angersbach 1998; De Andrade and others 1999; Arevalo and others 2003; Lebovka and others 2004). Using these measures, it has been shown that membrane permeabilization is dependent on pulse amplitude (electric field strength E), pulse shape, pulse duration (t_i), and pulse number (n) (Zimmermann and others 1974; Kanduser and Miklavcic 2008; Vorobiev and Lebovka 2008). Increasing the electrical field strength, pulse duration, or number of pulses can enhance both the degree of membrane rupture and increase the density (that is, number and size) of pores in the membrane and cell wall (Zimmermann 1986; Weaver and Chizmadzhev 1996; Knorr and Angersbach 1998; Fincan and Dejmek 2002; Arevalo and others 2003; Bazhal and other 2003b; De Vito and others 2008; Asavasanti and others 2010).

Little is known, however, about the effect of pulse frequency (f) on plant cell membrane electroporation (Vorobiev and Lebovka 2008). Earlier studies have been carried out primarily with artificial animal membranes, lipid vesicles, microbial, and animal cell suspension cultures or with plant protoplasts. These studies suggest that lower pulse frequencies may cause more damage to the cell because there is more time for charging the cell membranes between pulses, thereby facilitating pore formation (Bruhn and others 1997; Vernhes and others 1999, 2002; Bilska and others 2000; Evrendilek and Zhang 2005; Loghavi and others 2008). Vernhes and others (1999) observed biphasic dependence of cell viability on pulse frequency in animal cell culture tissues. Cell viability increased when frequency increased from 0.5 to 10 Hz, then decreased at frequencies higher than 10 Hz. These authors also reported that increasing pulse frequency (>10 Hz) increased PEF-induced permeabilization. In contrast, Evrendilek and Zhang (2005) reported that the efficiency of the PEF-induced pasteurization of *Escherichia coli* O157:H7 in both apple juice and skim milk decreased as the pulse frequency increased from approximately 40 to approximately 167 kHz. Despite the relative simplicity of cell suspensions in comparison to animal or plant tissues, the effect of pulse frequency on cell integrity and viability is still unclear. Moreover, it is not clear that the effect of pulse frequency on single animal or microbial cell suspensions is necessarily the same as for plant tissues. Intact tissues are necessarily more complex, since they are composed of heterogeneous distributions of cells with adjacent cell walls attached to each other, and with plasmodesmata providing cytoplasmic connections between cells. Both of these characteristics, as well as the close proximity of one cell to another within the tissue matrix, may affect electroporation. As discussed in recent reviews by Kanduser and Miklavcic (2008) and Vorobiev and Lebovka (2008), the effect of pulse frequency on plant tissues is not clear.

Indeed, only 2 studies have directly examined the effect of pulse frequency on plant tissue permeabilization (Lebovka and others 2000, 2001; see also Table 1). One study reported that changing the pulse frequency between 10 and 1000 Hz had no effect on electroporation efficacy in apple tissue, while the other study showed that a frequency of 0.2 Hz caused significantly more tissue damage than 100 Hz. This latter result is intriguing, because it suggests that very low frequencies might be optimal for inflicting tissue damage. Since only 2 frequencies were examined, however, an optimal frequency was not established. Knowledge of the influence of pulse frequency on plant tissue integrity and optimal frequency level will make it possible for food processors to improve the efficiency and reduce the energy consumption of PEF processes applied to plant tissue dehydration or extraction.

Lebovka and others (2001) also proposed a model for the effect of PEF on plant, in terms of what they called a "correlated percolation phenomenon," where membrane resealing and "moisture transport" after PEF are taken into consideration. These authors suggested that, for high pulse frequencies, the pulse repetition time may not be long enough for pores to expand; therefore, relatively less tissue is damaged. Apple slabs treated at 0.02 Hz yielded more brown spots, which were interpreted as regions of permeabilized and more conductive channels in the tissue after PEF. In contrast, the sample treated at 100 Hz had less brown spots. Because only 2 frequency levels (that is, 0.02 and 100 Hz) were examined, however, the effect of pulse frequency is unclear. More fundamentally, the details of the "moisture transport" phenomenon are unclear.

An important transport process in plant cells, heretofore unconsidered in the context of PEF, involves a convective phenomenon known as "cytoplasmic streaming." In living cells, molecular motor proteins (including, for example, kinesin and myosin) pull organelles, plastids, and vesicles along bundled actin microtubules, thereby inducing fluid motion throughout the cell (also known in older literature as "cyclosis"). This convective fluid motion plays a significant role in metabolism and distribution of molecules and proteins across organelle membranes (Pickard 2003; Taiz and Zeiger 2006; Goldstein and others 2008; Verchot-Lubicz and Goldstein 2010). Movement of these subcellular organelles inside the plant cell cytoplasm induces fluid flow both inside the cytoplasm and inside the vacuolar, which may comprise a large

Table 1–Summary of previous studies on effect of pulse frequency on integrity of PEF-treated plant tissues.

	PEF condition							
Sample	Frequency (Hz)	Field strength (V/cm)	Pulse shape	Pulse duration (μs)	Nr of pulses	Treatment time*	Report on effect of pulse frequency	Source
Apple tissue	10 to 1000	200 to 2200	Monopolar nearly rectangular	100	1 to 100000	0.1 to 1000 ms	Pulse frequency did not influence electroporation effect.	Lebovka and others (2000)
Apple tissue	0.02 and 100	500	Monopolar nearly rectangular	1000	10	10 ms	Low pulse frequency resulted in more tissue damage.	Lebovka and others (2001)

* Total time that the electric field is applied.

percentage of the cell (Verchot-Lubicz and Goldstein 2010). In plant cells, observed flow patterns include unidirectional streaming, fountain streaming, and spiral rotational streaming. Early studies often associated the existence of cytoplasmic streaming with cell viability; loss of membrane permeability results in cessation of cytoplasmic streaming (Angnes von and others 1995; Tirlapur and König 2002).

The objective of this study is to determine the effect of frequency on PEF efficacy in permeabilization of onion tissues. Electrical and physical properties of onion tissues were evaluated after PEF treatment to determine changes in tissue integrity. Neutral red (NR) dye was used as an indicator in the determination of cell viability of onion tissues after PEF treatment. Finally, we perform the first *in situ* investigation of the effect of pulse frequency on cytoplasmic streaming and mass transport inside PEF-treated onion epidermal cells.

Materials and Methods

Sample preparation

Spanish yellow onions (*Allium cepa*) were obtained from Gills Onions (Oxnard, Calif., U.S.A.) and kept in cold storage at 4 °C up to 3 mo. The size of the onion bulbs was selected between 7.5 and 9 cm dia in order to standardize maturity. Prior to sampling, onion bulbs were equilibrated to room temperature (approximately 25 °C) before removing the papery outer scales and the 1st fleshy scale. The 3rd fleshy scale was cut into 2 cm dia disks along its mid section with a core borer. The inner epidermal cell layer was removed and the onion disks were rinsed with deionized water for 60 s. Excess water was removed by gentle blotting with a tissue (Kimwipes, Kimberly-Clark Global Sales, Inc., Dallas, Tex., U.S.A.). To ensure good contact between electrodes and onion tissues, samples with thickness of approximately 3 mm were carefully selected to match the depth of the well in the sample holder. Average sample disk thickness was obtained by measuring the sample thickness at 5 different locations around the disk periphery with digital calipers (Model CD-6B, Mitutoyo Corp., Tokyo, Japan).

Pulsed electric fields

A Plexiglas sample holder assembled with 2 2 cm dia flat stainless steel electrodes was used as shown in Figure 1A. Prior to placing an onion disk into the sample holder, 1 mL of isotonic solution was added to the well to ensure good electrical contact between the sample and the electrodes. Note that the isotonic concentration was determined by weight gain or loss experiments, as described in Saltveit (2002) and Gonzalez and others (2010b). The isotonic concentration of mannitol for each lot of onions varied slightly, ranging from 0.2 to 0.3 M. The isotonic concentration was determined for each lot of samples in 3 replicates.

An onion sample of the same thickness as the well depth was placed between the 2 electrodes of the sample chambers with the convex plane facing down. Isotonic solution was added to fill the overhead space of the well before the top part of the sample holder was assembled. A small hole in the bottom part of the sample holder provided an overflow exit for excess air and solution. To ensure an airtight condition at atmospheric pressure, a constant sealing force was applied to the o-ring gaskets between the top and bottom electrodes using an Arbor press with a fixed deadweight. Because of the small hole, the sample itself was at atmospheric pressure.

PEFs were applied using the system described by Asavasanti and others (2010). The PEF system consisted of a high-voltage power supply (PowerPAC HV, Bio-Rad, Hercules, Calif., U.S.A.), a function generator (model 33220A, Agilent, Santa Clara, Calif., U.S.A.), a PEF generator, a sample holder, and an oscilloscope (model TDS1012B, Tektronix, Beaverton, Oreg., U.S.A.) for signal monitoring. The PEF generator provided monopolar positive pulses of rectangular shape with a pulse width $t_i = 100\ \mu s$. The main range of frequencies investigated in this study was from 0.1 to 1000 Hz. Frequencies lower and higher than this range were occasionally evaluated. To determine the effect of pulse frequency on onion tissue integrity, 8 levels of pulse frequency f from 0.01 to 5000 Hz were chosen: 0.01, 0.1, 1, 10, 100, 1000, 2000, and 5000 Hz. A 9th set of experiments, with no applied field, served as a control. Experiments were carried out using a constant electric field strength of 333 V/cm (that is, an applied potential of 100 V over a distance of 0.3 cm between 2 electrodes) with a fixed number of pulses, $n = 10$ pulses. These parameters were selected based on the results of our previous study (Asavasanti and others 2010), which showed that these PEF conditions maximized the tissue damage. The total PEF time t_{PEF} was 1000 μs for all conditions, while the total processing time, that is, $t_{totel} = (n - 1)/f + t_i$ approximately $(n - 1)/f$ when $1/f \ll t_i$, varied from approximately 2 ms to 15 min. For the control samples, an onion disk was placed into the sample holder for 2 min without applying PEF and then analyzed identically to the PEF-treated samples.

Finally, to demonstrate the possible energy minimization by reducing the number of pulses at lower frequencies, different combinations of pulse frequency and number of pulses were explored. Onion disks were treated at different low pulse frequencies (that is, 0.01, 0.1, and 0.5 Hz) and the number of pulses was varied from 0 (control) to 10 pulses.

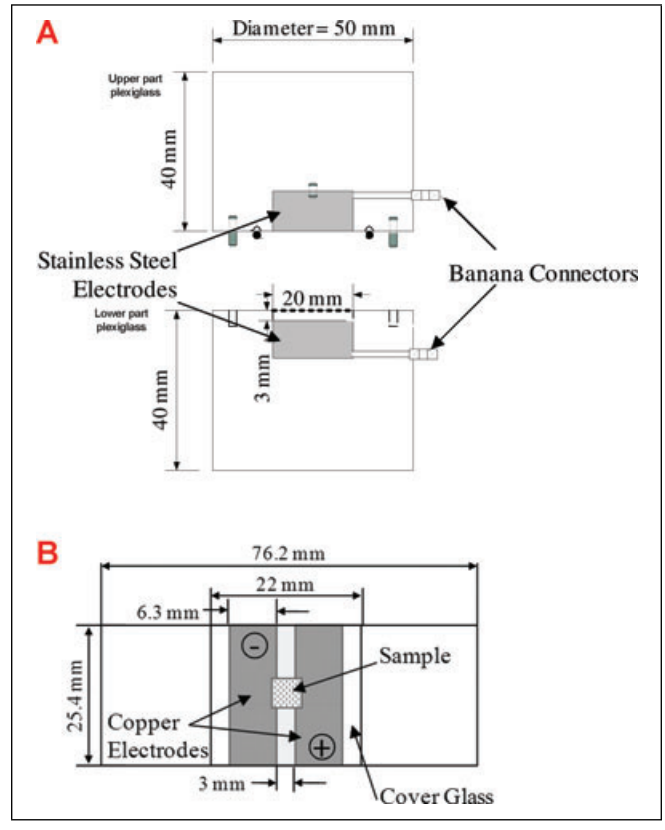

Figure 1–Schematic diagram of PEF treatment chamber for: (A) onion bulb scale internal tissue (parenchyma), and (B) onion epidermis. Each treatment chamber is connected to the PEF generator unit during the treatment and to the LCR meter during electrical characteristic determination.

Electrical characteristics and conductivity disintegration index determination

The electrical characteristics of onion tissue before and after PEF were measured with an Inductance (L), Capacitance (C), and Resistance (R) analyzer or a LCR meter (model 4284A, Hewlett-Packard/Agilent Technologies, Inc., Santa Clara, Calif., U.S.A.) at a frequency of 20 Hz. Kulshrestha and Sastry (2003) suggested low-frequency measurements for examining the effects of PEF on cell membranes, because living tissues, such as plant cells, have dielectric dispersions in the low-frequency region due to the effects of cell membranes from ionic conduction and membrane charging relaxation mechanisms that do not occur at high frequencies. The applied potential across the sample was set at 1 V. The measurement parameters included resistance R (Ω), impedance Z (Ω), and conductance G (S). At least 15 samples were used to determine the electrical characteristics of onion disk treated at $f = 0.01$ to 5000 Hz and controls ($f = 0$ Hz).

The degree of tissue damage was obtained from the electrical conductivity disintegration index Z^* (Lebovka and others 2002)

$$Z^* = \frac{\sigma - \sigma_i}{\sigma_d - \sigma_i} = \frac{(G - G_i) \times L/A}{(G_d - G_i) \times L/A} = \frac{G - G_i}{G_d - G_i} \quad (1)$$

where σ is the measured electrical conductivity (S/cm), L is the sample thickness (m), A is cross-sectional area of the sample (m^2), L/A is the cell constant (cm^{-1}), and the subscripts "i" and "d" refer to the conductivities of intact and completely ruptured tissue, respectively. Complete rupture of the onion tissue was accomplished by 2 cycles of freezing (−18 °C) and thawing at ambient temperature (Palta and others 1977a, 1977b; Gonzalez and others 2010b). Our preliminary study on onions indicated that the average conductivity of intact (G_i) and ruptured (G_d) onion tissues are 227 ± 85 μS and 1620 ± 420 μS, respectively. From Eq. (1), $Z^* = 0$ for intact tissue (control, no PEF) and $Z^* = 1$ for completely ruptured tissue (after 2 successive freeze/thaw cycles).

Electrolyte leakage determination

Control and PEF-treated onion disks were covered with damp tissue paper to avoid moisture loss and chilled overnight in the refrigerator (Saltveit 2002; Gonzalez and others 2010b). To determine an electrolyte leakage profile, chilled samples were equilibrated at room temperature for 1 h prior to the measurement. Two onion disks were placed into a 50-mL plastic tube containing 20 mL of an isotonic solution (0.2 M mannitol) preequilibrated at 25 °C. The electrical conductivity (σ) of each sample, maintained at 25 °C in a shaking circulating water bath (Lindberg/Blue Model SWB1122A-1, Thermo Fisher Scientific, Waltham, Mass., U.S.A.), was measured in μS/cm by a conductivity meter (Accumet portable AP65, Fisher Scientific Pte Ltd., Pandan Crescent, Singapore), periodically for up to 300 min. The timing started when the 2 disks for each sample were placed into the tube. The interval between measurements was 5 to 15 min at the beginning of the run, and longer (60 min) toward the end. After the final measurement, the centrifuge tubes were capped and placed in a freezer overnight at −18 °C. The next day, the frozen samples were removed from the freezer and allowed to warm to room temperature, 25 °C. After a 2nd overnight freeze/thaw cycle, a conductivity measurement was taken and used as the "*Total Conductivity.*" Electrolyte leakage at any time t was calculated as "*% Ion Leakage*" as shown in Eq. (2).

$$\%Ion\ Leakage(t) = \frac{Conductivity(t)}{Total\ Conductivity} \times 100 \quad (2)$$

A preliminary study showed that the relationship between electrical conductivity and time follows an asymptotic curve as suggested by Saltveit (2002). Initially, the rate of electrolyte leakage increased rapidly and then gradually leveled off after 240 min (data not shown). For this reason, 300 min was used as the standard time to evaluate the percent ion leakage of all samples. Average percent ion leakage was obtained from at least 5 samples.

Texture measurement

Puncture tests were performed to 90% deformation of the original onion scale thickness using a 2 mm dia flat-tipped cylindrical probe with a Texture Analyzer (model TA.XT2i plus, Texture Technologies Crop., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, U.K.) and a 5 kg load cell. The test speed was set at 1.0 mm/s. Texture parameters measured were maximum force (N), gradient (from initial point to 20% maximum force) (N/mm), and number of peaks after the maximum force (threshold force = 0.1 N). These parameters were selected based on previous work by Gonzalez and others 2010a, 2010b in our research group, suggesting that maximum force, gradient, and number of peaks could be used as indicators of the hardness of the cells and intactness of the membranes. Fifteen disks of onion were used for each data point.

Percent weight loss determination

Weights of onion disks were measured with an analytical balance (model Adventurer Pro AV812, Ohaus Corp., Pine Brook, N.J., U.S.A.) before and after PEF. After treatment, disks were removed from the sample holder and gently blotted dry with a tissue (Kimwipes). Weight loss was calculated and expressed as percent change in weight after PEF (W) over an initial sample weight (W_i) as shown in Eq. (3).

$$\%\text{Weight loss} = (W_i - W)/W_i \times 100 \quad (3)$$

Average percent weight loss was calculated from at least 4 replicates.

Viability staining and light microscopy

Control and PEF-treated onion disks (approximately 2 cm in diameter) were trimmed into square pieces (approximately 5 mm long × 5 mm wide) using a razor blade. Then, the samples were mounted on a specimen holder with instant glue and allowed to harden for 5 min. To prevent the sample from drying, a piece of Parafilm M® (American National Can Com., Norwalk, Conn., U.S.A.) was placed on the upper surface of the onion piece. Sections were obtained using a Vibratome 1000 Plus (The Vibratome Co., St. Louis, Mo., U.S.A.). The moving speed of razor blade and amplitude of vibration were adjusted to level 6 to 7 (Gonzalez and others 2010a). From each piece, 2 sections of 400-μm thickness were obtained by sectioning the specimen perpendicular to both epidermises. These sections were then used in staining and microscopic analysis.

NR dye (Standard Fluka, Sigma-Aldrich, St. Louis, Mo., U.S.A.) solution was freshly prepared each time before staining the samples. An NR stock solution was prepared by diluting 0.5% NR in acetone for 15 min, then filtered twice using Whatman filter paper nr 1 (Whatman Intl. Ltd., Springfield Mill, Maidstone, Kent, U.K.). The filtered stock solution was diluted to 0.04% in 0.3 M mannitol-0.01 M HEPES (N-[2-hydroxyethyl] piperazine-N′-[2-ethane-sulfonic acid]) (Sigma-Aldrich) buffer, pH 7.8, and used as the dyeing solution.

Prior to staining, samples were rinsed in deionized water to remove cellular debris and cell contents released by the mechanical damage of sectioning. Onion specimens were transferred to porcelain wells containing the dye solution and incubated at room temperature for 2 h. The specimens were rinsed twice for 5 min each time in 0.3 M manitol-0.01 M HEPES buffer solution. To obtain images of stained cells, 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 (Model BHS, Olympus System Microscope, Tokyo, Japan) at 4.0× objective magnification. A color digital camera (Olympus MicroFire, Olympus America, Melville, N.Y., U.S.A.) attached to the microscope was used to capture images via an interface of the Olympus MicroFire software, V.2.3 (Optronics, Goleta, Calif., U.S.A.). Color photomicrographs (1600 × 1200 pixel resolution, white balance corrected) were captured from 2 selected areas, which were positioned between and equidistant from 2 vascular bundles and 4 to 5 cell rows away from the outer epidermis, within the parenchyma tissue of each specimen (Gonzalez and others 2010a).

The entire range of selected frequencies (that is, control, 0.1 Hz, 1 Hz, 1 kHz, and 5 kHz) was replicated on 2 separate days to be able to make a qualitative comparison among micrographs obtained from the different frequency experiments. Fresh samples were stained immediately after standard sample preparation steps, while control samples were placed in the sample holder without applying PEF for 2 min prior to staining. Four replicate disks were used for each treatment, and from each disk, 2 micrographs were obtained. One of 8 images was selected as a representative of the group for inclusion in the manuscript. The power level of the lighting source was fixed at the recommended photo setting (8 to 9) of the microscope.

Cytoplasmic streaming investigation

The abaxial epidermal peel was manually stripped from the concave surface of the 2nd outermost scale of the onion bulb and cut into 5 × 5 mm sections. The sections were rinsed with deionized water for 1 min to remove onion flesh debris prior to mounting to a special PEF treatment chamber (Figure 1B). The chamber consisted of 2 parallel copper electrodes attached to a glass slide; the distance between the 2 electrodes was 3 mm. The section was oriented such that the long axes of the cells were parallel to the electrodes. Isotonic solution was used as a mounting media and a cover glass was carefully placed on top of the chamber to avoid entrapment of air bubbles.

The sample was observed in a phase contrast, transmitted light microscope at an objective magnification of 20× (Leica DMI3000B, Meyer Instruments Inc., Houston, Tex., U.S.A.). The video was captured from the mid section of the sample, that is, about 8 cells down and 8 cells away from the electrode, for at least 130 s with a CMOS color USB camera (EO-3112C, Edmund Optics Inc., Barrington, N.J., U.S.A.) at a frame rate of 12 frames per second and a pixel resolution of 1280 × 1024. Five frequency levels were examined, that is, 0 Hz (control), 0.1 Hz, 1 Hz, 100 Hz, and 1 kHz. PEF was applied 30 s after the video was initiated. The initial 30 s of each video serves as the "control" for each particular replicate. At least 3 replicates were performed for each pulse frequency. Quantitative information about the cytoplasmic speed profiles of epidermis subjected to different pulse frequencies was determined using custom image analysis routines in MATLAB version 7.2.0.232 (Mathworks Inc., El Segundo, Calif., U.S.A.). For each treatment, an average speed was estimated from 2 set of videos, in which a total of 15 selected areas of about 60 × 60 pixels^2 were analyzed. Three areas were selected to represent the cytoplasmic streaming speed profile within each cell and a total of 5 cells were analyzed. At least 10 videos were analyzed for the cytoplasmic streaming speed profile, but qualitative information about the effect of pulse frequency on cytoplasmic streaming was obtained from at least 4 videos per treatment. To determine the effect of pulse frequency on cytoplasmic streaming, a normalized streaming speed $U(t)$ was calculated as:

$$U(t) = u(t)/u_0 \tag{4}$$

where $u(t)$ is the cytoplasmic streaming speed at time t and u_0 is the time-averaged cytoplasmic streaming speed prior to application of PEF for each particular sample, that is, the first 30 s of each video. In this fashion, natural variability in the cytoplasmic streaming speed different cells and different tissues is accounted for. To compare the effects of different frequencies on cytoplasmic streaming, the "half-life" (t_{50}), or the time required for the streaming speed to decay to 50% of its initial value, was determined. Because the cytoplasmic streaming speed may be affected by light (Shimmen 2007), the control samples (with no PEF applied) were exposed to the same lighting conditions.

Statistical analysis

Data were analyzed using SAS 9.1 software (SAS Institute Inc., Cary, N.C., U.S.A.). The effect of pulse frequency on onion tissue integrity was determined for a fixed electric field strength and number of pulses. One-way analysis of variance and Duncan's multiple range tests were used to determine statistically significant differences among treatments ($P < 0.05$).

Results and Discussion

Effect of pulse frequency on electrical characteristics and conductivity disintegration index

The effects of pulse frequency on the resistance R, impedance Z, conductance G, and electrical conductivity disintegration index Z^* of onion tissues treated with PEF at $E = 333$ V/cm, $n = 10$ pulses, and $t_i = 100$ μs are shown in Figure 2 and 3, respectively. For all frequency levels ($f = 0.01$ to 5000 Hz), R and Z values of the PEF-treated onion tissues are approximately 30% to 80% lower than the controls, whereas the G value of the PEF-treated onion tissues is approximately 25% to 350% higher than the controls. In biological tissues, previous investigators have found that pores, formed in the cell membranes upon electric field exposure, result in a drop in electrical resistance as ions may more easily pass through the electroporated membrane (Coster 1965). The resistance of PEF-treated onion tissues decreases from approximately 5 kΩ (control) to about 1 kΩ for $f = 0.01$ and 0.1 Hz. At higher frequencies ($f = 1$ to 5000 Hz), the resistance of the onion tissues drops by approximately 2 to 3 kΩ (Figure 2). The results indicate that lower frequencies result in less resistance, and therefore greater tissue damage than higher frequency applications. Figure 3 shows that low pulse frequencies ($f < 1$ Hz) yielded Z^* approximately 0.45, which is significantly higher ($P < 0.05$) than controls ($Z^* = 0$) and samples treated at higher pulse frequencies of between 1 and 5000 Hz (Z^* approximately 0.1 to 0.2). Above 10 Hz, increased pulse frequency has no significant effect on onion tissue integrity; Z^* remained approximately constant ($P < 0.05$). An increase in Z^* indicates increased permeability of the cell membrane and loss of plant tissue integrity, which results from PEF-induced cell membrane permeabilization or breakdown

(Zimmermann 1986; Lebovka and others 2000; Kulshrestha and others 2008).

The results show that the electrical characteristics of onion bulb scale ground tissue are highly affected by the pulse frequency; as the pulse frequency increases, the R and Z values increase, while Z^* values decrease (Figure 2 and 3). Our findings are qualitatively in agreement with the previous studies on effect of pulse frequency on plant tissue, listed in Table 1. Lebovka and others (2000) also reported that pulse repetition times within the interval of 1 to 100 ms (or frequency of 10 to 1000 Hz) had the same influence on thin apple slices treated with different electric field strengths ranging from 0.2 to 2.0 kV/cm, $t_i = 100\ \mu s$ at 25 °C. Lebovka and others (2001) found that PEF at 0.02 Hz resulted in more damage, that is, higher relative conductivity, in apple tissue than at 100 Hz. PEF-induced permeabilization of biological tissues has been described as a correlated percolation phenomenon controlled by different key processes, for example, electroporation of cellular membranes, time-dependent expansion of the pore size (in range of hundreds of μs to ms), resealing of membranes (in range of several seconds to several minutes), and moisture transfer inside the tissue structure (Zimmermann 1986; Tsong 1992; Bazhal and others 2003a; Kanduser and Miklavcic, 2008).

Lebovka and others (2001) proposed a hypothesis that in case of low pulse frequencies when the duration between 2 consecutive pulses (Δt) exceeded the time required for resealing and moisture transport, the damage process had a correlated percolation pattern. On the other hand, in the case of high pulse frequencies, there was not enough time for pores to expand or moisture transfer process to complete; thus, the damage process had a random character. Although simulated percolation patterns showed similar effect of pulse frequency to the photographs of PEF-induced damage in apple slabs, details concerning the structure of cellular material, and the heterogeneous electrophysical properties within the sample were not taken into account. Although the mechanism of PEF-induced damage in plant tissues is not yet completely understood, our study at 8 difference pulse frequencies provides additional evidences to support the conclusion that the lower pulse frequencies ($f < 1$ Hz) cause more damage to plant tissue integrity than the higher frequencies.

Effect of pulse frequency on electrolyte leakage profile and percent ion leakage

Figure 4 illustrates that control samples have an ion leakage value of approximately 10% at 300 min, which is most likely due

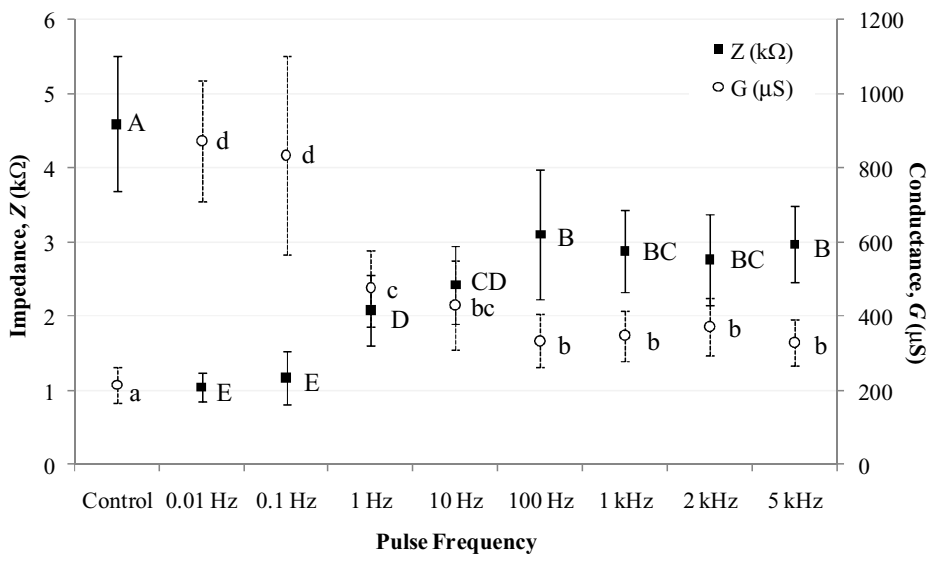

Figure 2–Effect of pulse frequency on impedance Z and conductance G of onion tissue ($E = 333$ V/cm, $n = 10$, $t_i = 100\ \mu$s). Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$). Statistical comparisons are done separately for each electrical characteristic as shown in different cases.

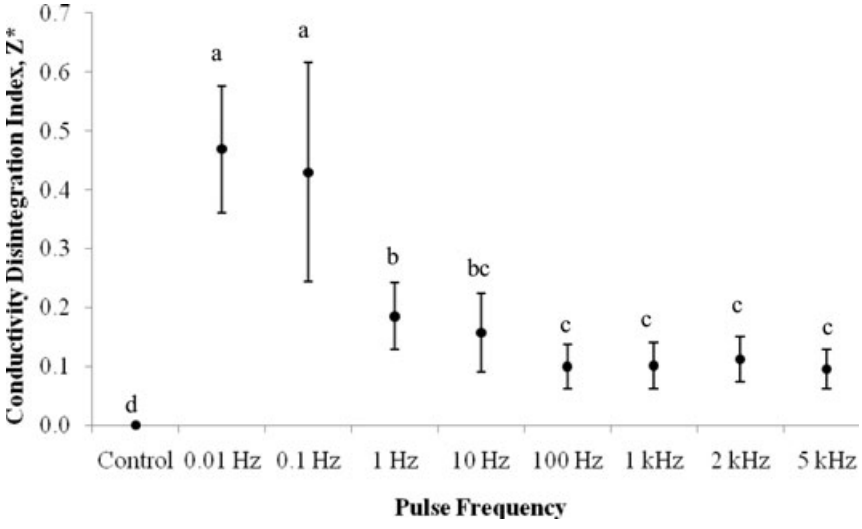

Figure 3–Effect of pulse frequency on conductivity disintegration index Z^* of onion tissue ($E = 333$ V/cm, $n = 10$, $t_i = 100\ \mu$s). Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$).

to leakage of electrolytes from cells cut during sample preparation and/or from the apoplasmic region of the tissue. At all frequency levels of interest, percent ion leakage of the onion tissues treated by PEF is significantly higher than that of the controls ($P < 0.05$). The highest percent ion leakage in onion tissue (approximately 80%) is observed at relatively low pulse frequencies of 0.01 and 0.1 Hz. At increased pulse frequencies, a decreased percent of ion leakage is observed. Increasing pulse frequency from 0.1 to 1 Hz or 10 Hz results in a significant decrease in percent ion leakage from approximately 80% to approximately 50% or approximately 40%, respectively ($P < 0.05$). However, above 10 Hz, increasing the pulse frequency has no significant effect on percent ion leakage ($P < 0.05$), and the average percent ion leakage at higher frequencies ranges between 30% and 45% (Figure 4). These results are consistent with the electrical characteristics determination, that is, Z^* reduces as frequency increases (Figure 3). At low frequencies, onion tissue becomes more ruptured and the absence of intact membranes results in more electrolyte leakage from the cells.

Measurement of electrolyte leakage from plant tissues into a surrounding solution has long been used to evaluate the intactness and permeability of cell membranes (Murray and others 1989; Vasquez-Tello and others 1990; Saltveit 2002). Numerous applications of this method have been made, including quantifying chilling injury in cucumbers (Kuo and Parkin 1989) and tomatoes (Saltveit 2002), mechanical damage to popcorn kernels (Goneli and others 2007) and tomatoes (Milczarek and others 2009), and high pressure and thermally treated onions (Gonzalez and others 2010b). In general, electrolytes will follow concentration gradients from a high concentration inside the cell to a lower concentration outside in the mannitol solution. Distilled water has conductivity in the range of 0.1 to 1 μS/cm, while the conductivities of solutions in equilibrium with plant tissues are about 1 to 2 order of magnitude larger (Milczarek and others 2009). The efflux of electrolytes is driven by passive diffusion and convection (that is, liquid flow due to movement of plastids and organelles in the cytoplasm, aka. "cytoplasmic streaming"), while electrolyte influx is due to active transport. Loss of tissue integrity, as indicated by electrolyte leakage, may result from either an increased efflux due to damage to the plasma membrane, or a decreased influx due to damage to the active transport system (Palta and others 1977a). In this study, we interpret an increase in percent ion leakage as a PEF induced increases in permeability of the plasma membrane and thus ion leakage.

Effect of pulse frequency on texture properties

Previous studies (Lebovka and others 2004; De Vito and others 2008; Asavasanti and others 2010; Gonzalez and others 2010a, 2010b) showed that changes in plant tissue integrity after thermal, high-pressure, and PEF processing can be quantified by measurement of texture properties. Figure 5 illustrates the effects of pulse frequency on maximum force, initial gradient (up to 20% of maximum force), and number of peaks following the maximum force in onion tissues subjected to PEF. There was no significant change in maximum force observed ($P < 0.05$) at any of the frequency levels applied. Average maximum force was approximately 6 N. From a sensory standpoint, maximum force represents the hardness of the food sample (Bourne 2002). Thus, there is no difference in the hardness of onion tissue treated with PEF at different frequencies.

It may be that at the selected level of field strength and number of pulses, PEF has little to no effect on the cell wall structure (Azencott and others 2007). Although PEF causes loss of cell membrane integrity (and subsequent loss in turgor pressure), tissue response to deformation applied during the texture test does not depend solely on the turgor pressure. The texture profile obtained is a combination of the cell wall properties and the turgor component. However, the contribution of these 2 components is not well understood (De Vito and others 2008). This finding is similar to that published in our previous study (Asavasanti and others 2010), suggesting that there is no significant change in maximum force in onion tissues treated by PEF at different electric field strengths and pulse numbers, but it contradicts the results of Bazhal and others (2003c). Based on the porosity measurements of PEF-treated apple samples with $Z^* = 0.9$ to 1 ($E = 1000$ V/cm; $t_i = 300$ ms; $n = 60$ pulses; $f = 1$ Hz), these authors suggested that PEF affects not only the cell membrane, but also cell wall integrity. They used this as an explanation of the increase in compressibility of apple tissue after PEF, even though there was only a low level of tissue destruction. Previous studies on PEF-induced damage on plant cell walls have reported contradictory results, most likely due to differences in the commodity and tissue structure, intensity of

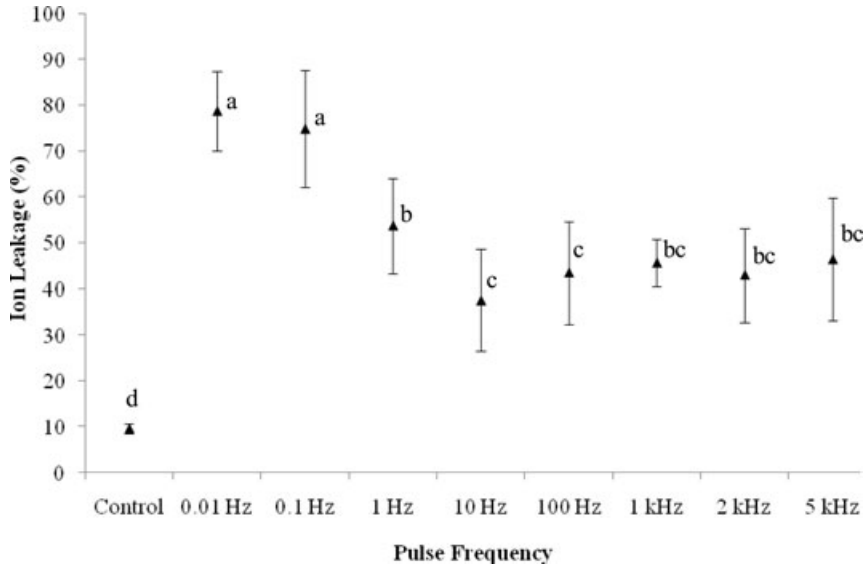

Figure 4–Effect of pulse frequency on percent ion leakage of onion tissue ($E = 333$ V/cm, $n = 10$ pulses, $t_i = 100$ μs). Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$).

the PEF treatment, and the method of texture measurement. The mechanisms of PEF-induced damage on cell walls remains largely unknown.

Other texture properties, however, were affected by the frequency of PEF. Specifically, significant decreases in the number of peaks following maximum force and the slope magnitude ($P < 0.05$) were both observed at lower frequencies. The lowest number of peaks (approximately 2 peaks) is detected when PEF is applied at low frequencies of either $f = 0.01$ or 0.1 Hz. Similarly, the smallest gradient value (approximately 1.6 N/mm) is observed when PEF is applied at either $f = 0.01$ or 0.1 Hz (Figure 5). PEF applications at a frequency of 1 Hz and above yield no significant difference in either number of peaks or gradient value of onion tissues ($P < 0.05$).

The number of peaks present in the texture profile following the maximum force may arise from the puncture probe passing through different layers of intact cells (Gonzalez and others 2010b). Cellular turgor pressure inside intact plasma and tonoplast membranes, together with the structural support of the plant cell wall, impart rigidity, and firmness to plant tissues. The gradient or initial slope of the profile is correlated with stiffness; a larger number of cells with intact membranes result in greater stiffness (Bourne 2002; Gonzalez and others 2010b). The gradient and number of peaks have previously been correlated with membrane integrity and cell viability of high-pressure and thermally processed onions by our group (Gonzalez and others 2010a, 2010b). It has been suggested that fruit firmness, together with the biomechanical properties of most plant tissues, is influenced by cellular turgor pressure (Shackel and others 1991). Products lose their firm or crisp texture on heating because of loss of turgor (Szczesniak 1998). When membrane integrity is lost in onion tissue, the initial gradient and number of peaks following the maximum force derived from the texture profile change significantly. Consequently, texture measurements can be used to evaluate membrane integrity of PEF-treated samples and to distinguish the effect of different pulse frequencies. The significant changes in both number of peaks and gradient determined in this study (Figure 5), particularly at low frequencies, may be interpreted as loss of integrity of the onion cell membranes. Fincan and Dejmek (2002) reported that the cell wall structure seems to be unaffected by PEF, and this may contribute to the maintenance of maximum force values in PEF-treated onion tissues ($P < 0.05$).

Effect of pulse frequency on percent weight loss

The effect of pulse frequency on percent weight loss of PEF-treated onion tissue is illustrated in Figure 6. For onion tissues treated at low frequencies, for example, $f = 0.01$ and 0.1 Hz, weight loss of 6.3 ± 2.9% and 6.4 ± 4.4% are observed, respectively. This amount of weight loss is significantly higher than that measured in the controls and samples treated with PEF at the higher pulse frequencies, that is, approximately 0% weight loss ($P < 0.05$). No significant weight loss is observed in onion tissues treated with PEF from $f = 1$ to 5000 Hz. These results contribute to same conclusion that PEF at low frequencies results in a higher degree of tissue damage and enhanced rate of mass transfer of electrolytes than higher frequencies ($f \geq 1$ Hz).

Many researchers have reported that PEF may enhance the rate of mass transfer and extraction yield in many plant materials (Rastogi and others 1999; Ade-Omowaye and others 2000; Lebovka and others 2007). These authors also determined the Z^* value of the tissues under different conditions and reported that the higher the degree of PEF treated tissue damage, the more rapid the drying process. Most of these studies did not use the most effective pulse frequency, which resulted in high electric field strength and/or more number of pulses required. Using lower pulse frequency may reduce the electric field strength and/or the number of pulses required to obtain the same extraction yield or drying rate.

Unlike other studies, which focused on only the effect of electric field strength and number of pulses while keeping pulse duration and pulse frequency constant, the present study indicates that pulse frequency plays an important role in PEF-induced permeabilization of plant tissues. Lowering pulse frequency to below 1 Hz, while keeping other parameters constant results in a significant increase (approximately 6.5%) in percent weight loss of onion tissue. This finding suggests that extraction yield or dehydration efficiency can be improved simply by PEF application at low pulse frequencies.

Effect of pulse frequency on cell viability: viability staining

To verify the effect of pulse frequency on degree of PEF-induced damage, as indicated by changes in electrical and physical

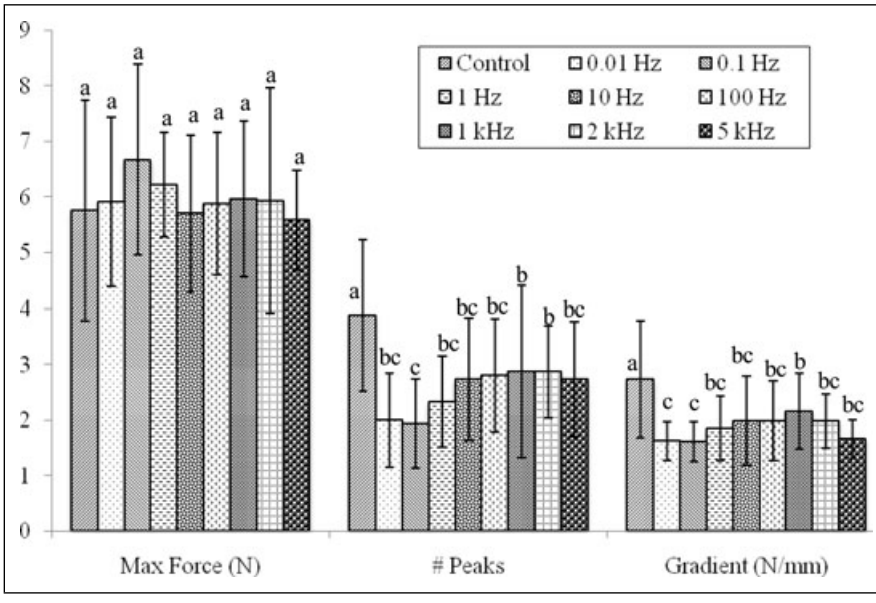

Figure 5–Effect of pulse frequency on texture properties of onion tissue ($E = 333$ V/cm, $n = 10$, $t_i = 100$ μs). Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$). Statistical comparisons are done separately for each textural property.

properties of onion tissue, a viability staining technique was applied to control and PEF samples at $f = 0.1$ Hz, 1 Hz, 1 kHz, and 5 kHz, respectively. These frequency levels were selected because they show significant differences in onion tissue integrity after PEF. Since there is no significant difference in Z^* value, percent ion leakage, texture properties, or percent weight loss in onion tissue treated at either 0.01 or 0.1 Hz, the treatment at $f = 0.01$ Hz was omitted because the time required for this application limits its practical application. Figure 7 shows representative photomicrographs of NR stained onion tissues treated with different pulses frequencies. Control samples contain primarily viable cells, as shown in Figure 7A. Average onion parenchyma cell dimensions are below 400 μm as reported by Gonzalez and others (2010a) and Ersus and Barrett (2010). NR staining distinguishes viable cells from nonviable cells. NR dye can easily diffuse through intact plasmalemma and tonoplast membranes into the vacuole, where it accumulates due to ionization of the dye in the low-pH environment (for example, pH 5 to 5.5) inside the intact vacuole (Horobin and Kiernan 2002; Fincan and Dejmak 2002; Gonzalez and others 2010a). Viable cells are indicated by a concentrated red area, while nonviable cells are interpreted to be those where dye is more diffuse and not concentrated due to ruptured membranes. Damage to cells may result from mechanical damage during Vibratome sectioning of cells that are either greater than 400 μm in diameter, or partially aligned outside of the 400 μm sectioning range. Cells may also become nonviable due to PEF-induced cell membrane permeabilization. The black areas in the micrographs are air bubbles that may be trapped in the extracellular spaces of the cut surfaces during specimen preparation. To mitigate this problem, the specimens were fully submerged in deionized water during sectioning.

PEF at $f = 0.1$ and 1 Hz (Figure 7B and 7C) results in rupture of most of the cells, while greater numbers of cells remain viable in onion tissues treated by PEF at higher frequencies, for example, 1 and 5 kHz (Figure 7D and 7E). A similar method of viability staining was used by Ersus and Barrett (2010). They observed severe tissue damage in parenchyma cells, vascular bundles, and outer epidermal cells treated at $E = 333$ V/cm, $t_i = 100$ μs, $n = 10$ pulses and $f = 1$ Hz, which is similar to the results reported here. The viability staining results further confirm the finding that frequencies below 1 Hz result in more PEF-induced permeabilization of cells than the higher pulse frequencies. PEF at 0.1 Hz results in the highest degree of cell permeabilization, while 5 kHz yields the most intact cells. Our findings are in agreement with the study of macroscopic structural changes of apple slabs after PEF at $E = 500$ V/cm, $t_i = 1$ ms and $n = 10$ at $f = 0.02$ and 100 Hz reported by Lebovka and others (2001). Their photographs show that the lower frequency is associated with more damage to the cellular material than the higher frequency, as indicated by dark brown spots (that is, enzymatic browning) on the sample. The authors hypothesized that brown spots correspond to formation of moisture-saturated and more conductive channels in the cellular

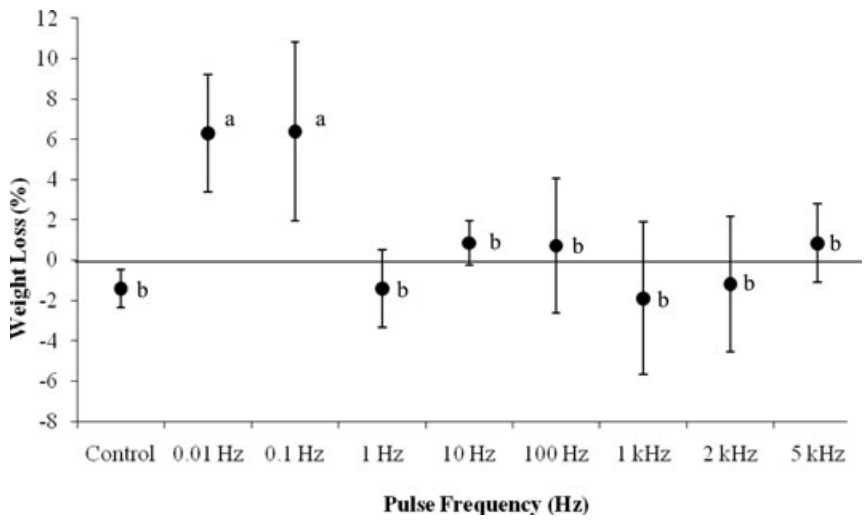

Figure 6–Effect of pulse frequency on percent weight loss of onion tissue treated with PEF ($E = 333$ V/cm, $n = 10$ pulses, $t_i = 100$ μs). Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$).

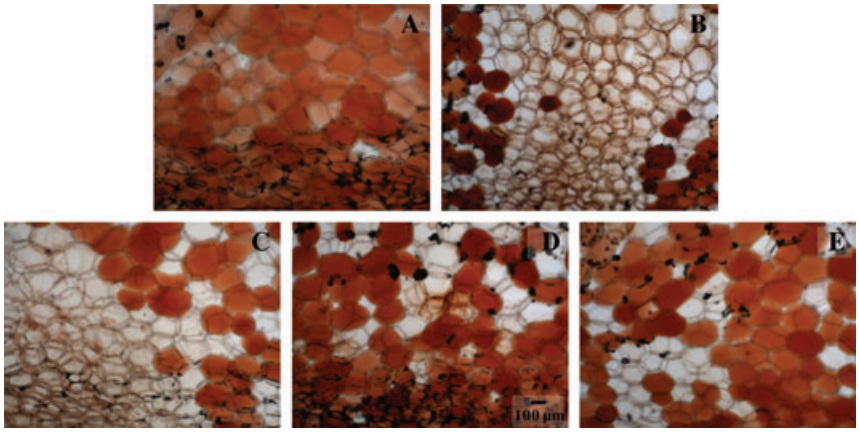

Figure 7–Representative photomicrographs of onion tissue treated with PEF at different pulse frequencies, $E = 333$ V/cm, $n = 10$ pulses: (A) control, (B) 0.1 Hz, (C) 1 Hz, (D) 1 kHz, (E) 5 kHz. Specimen thickness = 400 μm. Scale bar is 100 μm.

structure. Their study yielded qualitative information about PEF-induced cellular damage in apple tissue at only one low- and one high-frequency level. The microscopic analyses presented here of NR stained onion tissue provide the first direct correlation between electrical and physical properties and the cytology of plant tissues subjected to wide range of PEF frequencies (that is, $f = 0.1$ to 5 kHz).

Effect of pulse frequency on cytoplasmic streaming

A representative onion epidermis is shown in Figure 8A. The arrows illustrate the direction of cytoplasmic streaming inside one cell at the particular plane of focus, while the boxes indicate typical locations in which the streaming speed was measured. Because both the magnitude and direction of cytoplasmic streaming varies throughout each cell, multiple locations were used for measuring the speed to obtain spatially averaged quantities. Observed flow directions included unidirectional streaming, fountain streaming (in which the motion near the central axes of the cell is opposite to that near the periphery), and spiral rotational streaming. Here, the streaming speed was calculated as the observed particle displacement per unit time interval for all plastids moving through a given box. The resulting speeds as a function of time were then averaged over 3 boxes each in 5 different cells.

The normalized cytoplasmic streaming speed profiles for tissues treated with different PEF pulse frequencies are shown in Figure 8B. Here, time equal zero indicates application of the first PEF pulse. Several features of the data are notable. First, the magnitude of the observed streaming velocities prior to PEF, approximately 5 μm/s, is consistent with previous observations where velocities ranged from 1 to 100 μm/s, with 10 μm/s reported as a typical speed (Pickard 2003). This observation suggests that the onion tissues used here are typical with respect to cytoplasmic streaming. Second, the streaming speed in the absence of PEF (that is, the control) was approximately constant in time, throughout the observation period (130 s). This observation indicates that any changes observed in cytoplasmic streaming speed subsequent to PEF are indeed due to PEF rather than some other effect (that is, microscope illumination).

In contrast to the control, samples treated with PEF showed a dramatic decline in the speed of cytoplasmic streaming. We emphasize that no noticeable change in the direction of the cytoplasmic streaming during or after application of PEF was observed;

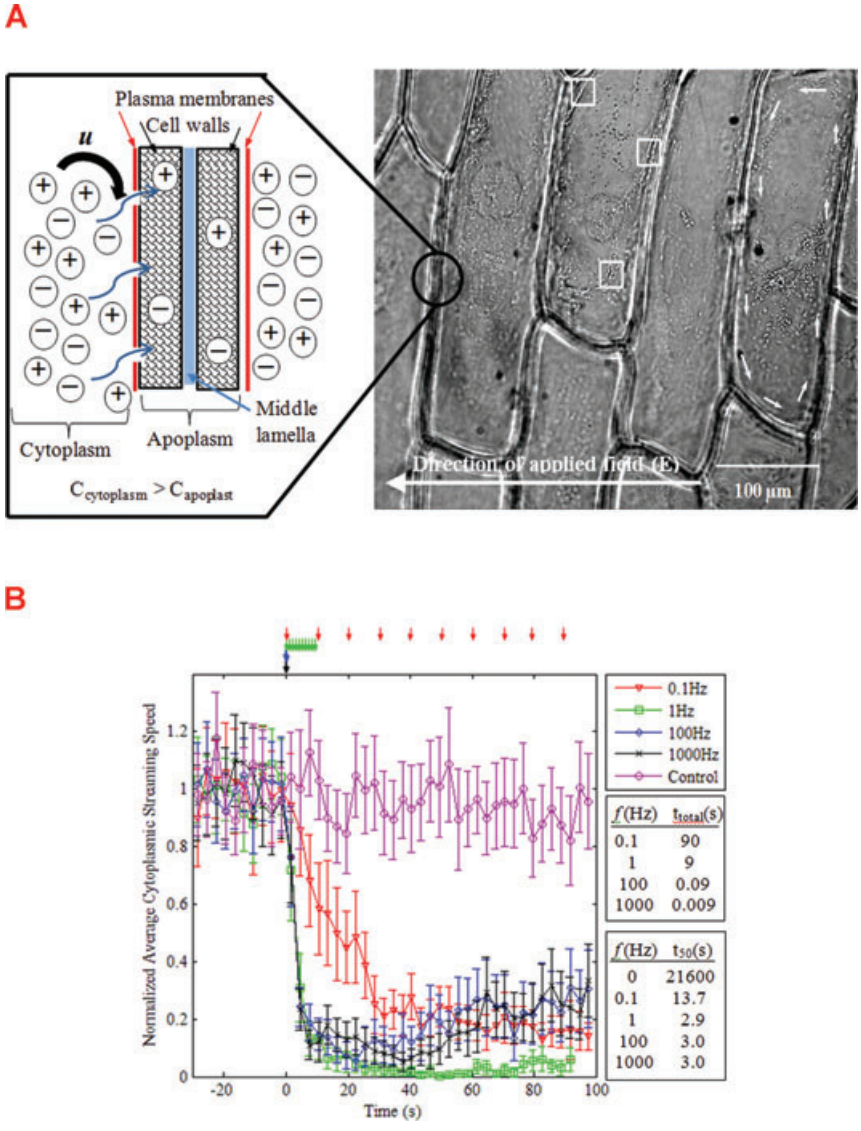

Figure 8–Cytoplasmic streaming of onion epidermis. (A) Representative micrograph of onion epidermis taken with a phase contrast microscope at 20× objective magnification. Scale bar is 100 μm. Square boxes (approximately 60 × 60 pixels^2) represent areas selected for analyzing cytoplasmic streaming speed. Small arrows illustrate direction of cytoplasmic streaming inside the cell at the particular plane of focus. The large arrow indicates direction of the applied field. The enlarged diagram to the left illustrates the interface between 2 adjacent cells, both of which are surrounded by a cell wall and plasma membrane, with the pectin-rich middle lamella between them. The cell on the left has been permeabilized by PEF; the cell on the right has not. Cytoplasmic streaming may enhance the movement of relatively high-conductivity fluid, originating inside the cell in the cytoplasm, through pores in the plasma membrane induced by PEF, and into the lower conductivity extracellular space or apoplasm. (B) Normalized cytoplasmic streaming speed profile of onion epidermis treated with PEF ($E = 333$V/cm, $t_i = 100\mu$s, $n = 10$ pulses) at different frequencies (that is control, 0.1 Hz, 1 Hz, 100 Hz, and 1 kHz). Pulsed electric fields were applied at $t = 0$ s; no PEF applied from $t = -30$ to 0 s. Small arrows on top of the plot indicate time that PEF was applied; these arrows were color coded according to each frequency of treatment. Total treatment time (t_{total}) varies from approximately 0.009 s for 1 kHz treatment to approximately 90 s for 0.1 Hz treatment. "t_{50}" is time that the streaming speed reduces to 50% of its initial value.

the observed speed simply decayed with time following PEF. The rate of decline, however, was sensitive to the PEF frequency. For the low frequency of 0.1 Hz PEF, the average cytoplasmic streaming speed gradually dropped over a period of approximately 30 s to below 80% of its original streaming speed; the measured half-life was $t_{50} = 13.7$ s. However, at higher pulse frequencies, that is, 1 Hz, 100 Hz, and 1 kHz, streaming dropped close to zero almost instantaneously; in these cases t_{50} approximately 3 s. These results were highly reproducible; similar trends with respect to pulse frequency were observed for at least 3 trial replicates (data not shown). Significantly, the transition between rapid speed decay and gradual speed decay occurs at the same frequency range (between 1 and 0.1 Hz, respectively) where the amount of cellular damage increases dramatically (as shown in Figure 2 to 6). When PEF at 0.1 Hz was applied, severe tissue rupture was observed as indicated by higher conductivity disintegration index, percent ion leakage and percent weight loss, significant changes in texture characteristics, and decreases in the number of viable cells (red area in viability staining experiment) in comparison to the onion tissue treated at higher pulse frequencies.

It is widely accepted that the role of cytoplasmic streaming in plant cells is to facilitate mass transport by providing a convective driving force that is faster than molecular diffusion driven by concentration gradients (Pickard 2003, Goldstein and others 2008). Although previous researchers have reported that cytoplasmic streaming is affected by the application of an electrical current through the tissue (Hill 1941; Kishimoto and Akabori 1959; Tazawa and Kishimoto 1968; Simons 1981; Wong and Allen 1985; Angnes von and others 1995; Shimmen 2007), the strong correlation between persistence of cytoplasmic streaming and overall damage to the tissue as a function of frequency has not been previously reported.

The correlation between persistence of cytoplasmic streaming and increased damage at low frequencies suggests that cytoplasmic streaming might play a significant role in increasing the cellular damage at sufficiently low PEF frequencies. Cytoplasmic streaming may enhance the movement of relatively high-conductivity fluid, originating inside the cell in the cytoplasm, through pores in the plasma membrane induced by PEF, and into the lower conductivity extracellular space or apoplasm, as illustrated in Figure 8A. Increasing the conductivity of the extracellular fluid around ruptured cells may create preferable paths for the next pulse to travel and reach more intact cells. This fluid movement may also accelerate the physical damage to the cell membrane initiated by electroporation. PEF at high pulse frequencies results in an abrupt cessation of cytoplasmic streaming, thus there is no longer movement of the fluid within the cytoplasm. Moreover, this abrupt cessation may allow resealing of the cell membrane to begin, which may explain the observation of more intact cells present in onion tissue treated at higher frequency than in the tissue treated at lower frequency.

An approximate scaling analysis based on a cytoplasmic streaming convective time scale (t_d) yields a critical frequency similar to that observed experimentally. Given the average cytoplasmic streaming speed u and the characteristic length of an onion epidermal cell L, the critical pulse frequency may be estimated as,

$$f_{\text{crit},c} = u/L \tag{5}$$

This frequency represents the (inverse) amount of time necessary for the flow to carry material a distance L. Substitution of the appropriate values (u approximately 5 μm/s, L approximately 100 μm) yields a critical frequency of about 0.05 Hz for onion epidermis (or a characteristic convective time of 20 s) that is consistent with our observed critical frequency of approximately 0.1 Hz in the damage characterization experiments.

It is unlikely that the fluid flow penetrates very effectively into the apoplasm, given the high concentration of pectin in the middle lamella and the cell wall itself, both of which impede bulk fluid flow. Thus, the role of cytoplasmic streaming is more likely as a means of enhancing the rate of diffusive flux into the apoplasm following membrane rupture. In other words, the cytoplasmic streaming helps deliver additional ions to the edge of the cell wall near the site of membrane rupture, thus helping maintain a maximal concentration gradient and maximizing the diffusive flux. A diffusive time scale can be estimated as

$$f_{\text{crit},d} = D/l^2 \tag{6}$$

where D is the effective diffusion coefficient of ion in cell wall, which is in an order-of-magnitude of 10^{-8} cm^2/s (Meychik and others 2003), and l is the cell wall thickness, which is approximately 1 to 3 μm for onion epidermis (Lecain and others 1999; Hepworth and Bruce 2004). The quantity $f_{\text{crit},d}$ represents the inverse amount of time necessary for ions to diffuse across the apoplasm. Substitution of characteristic values yields a critical frequency of about 0.1 Hz (or a characteristic diffusion time of 10 s) that is in the same order-of-magnitude as the critical frequency based on convective time scale. Thus, the PEF at a low frequency of 0.1 Hz has the appropriate time period between the pulses for the ions to be redistributed by both convection and diffusion from one side of a permeabilized cell to the other side of the cell and through the apoplasm to adjacent intact cells, as sketched in Figure 8A. When the next pulse is applied the more conductive pathway has been established, thereby promoting damage to adjacent intact cells. The same argument applies for even lower frequencies (0.01 Hz or lower). For frequencies higher than 0.1 Hz, cytoplasmic streaming stops and the time periods are too short for the redistribution of the ions to change the conductive pathway to adjacent intact cells.

Reduction of energy consumption by selecting effective pulse frequency

Selection of an optimal effective pulse frequency in combination with number of pulses applied may allow one to reduce the energy requirements of PEF. Figure 9 shows the effect of pulse number on integrity of onion tissues treated with PEF at different pulse frequencies ($f = 0.01$, 0.1, and 0.5 Hz) at $E = 333$ V/cm and $t_i = 100$ μs. For all frequency levels used in this study, pulse number significantly affects the Z^* value of PEF-treated onion tissues ($P < 0.05$). The lower the number of pulses, the lower the degree of PEF-induced tissue damage. However, at $f = 0.01$ Hz, pulse number could be reduced from 10 to 9 without causing a significant reduction in degree of tissue permeabilization, as indicated by the Z^* values ($P < 0.05$). Using the same electric field strength of 333 V/cm, PEF at lower pulse frequencies, for example, $f = 0.01$ or 0.1 Hz, for only 10 pulses results in approximately the same level (approximately 80%) of ion leakage as that at $f =$ 1 Hz for 100 pulses as reported by Ersus and Barrett (2010). Note that the total processing time is the same, that is, $t = 90$ s, for f $= 0.1$ Hz for 10 pulse and $f = 1$ Hz for 100 pulses, but the total time in which an electric field is applied to the sample is 10 times less in the case of the lower frequency application.

To optimize the energy consumption in PEF, different combinations of pulse frequency and pulse number could be used to obtain

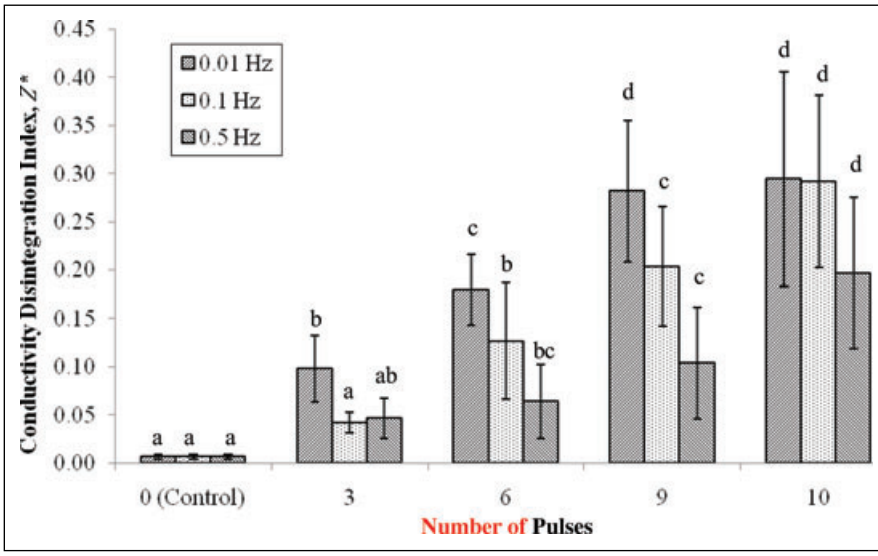

Figure 9–Effect of pulse number on conductivity disintegration index Z^* of onion tissue treated with PEF at $f = 0.01$, 0.1, and 0.5 Hz, respectively ($E = 333$ V/cm, $t_i = 100$ μs). Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$). Statistical comparisons are done separately for each pulse frequency.

similar PEF effects on plant tissue integrity (or pulse equivalent effect). For example, the Z^* value of approximately 0.2 could be obtained by using 3 different combination of PEF parameters: (i) $f = 0.5$ Hz and $n = 10$ pulses; (ii) $f = 0.1$ Hz and $n = 9$ pulses; (iii) $f = 0.01$ Hz and $n = 6$ pulses (Fig. 9). The volume density of the energy input Q during the PEF can be calculated from

$$Q = \int_0^{t_1} \sigma_1 E^2 dt + \int_0^{t_2} \sigma_2 E^2 dt + \cdots \int_0^{t_n} \sigma_n E^2 dt = \sum_{i=1}^{n} \sigma_i E^2 \Delta t \quad (7)$$

where the electrical conductivity of the tissue σ increases with time t owing to PEF-induced tissue damage (Vorobiev and Lebovka 2008). Assuming that all samples have the same initial electrical conductivity and are treated under the same electric field strength E and pulse width Δt, the number of pulses applied n simply indicates the amount of energy consumption.

Approximately 40% reduction in energy consumption can be obtained by reducing pulse frequency from 0.5 to 0.01 Hz; however, the total processing time is significantly increased from 18 to 500 s. In the other case, by changing pulse frequency from 0.5 to 0.1 Hz, approximately 10% reduction in energy consumption can be obtained, while the total processing time is increased slightly, that is, from 18 to 80 s. These results demonstrate the possibility of using lower pulse frequency to minimize the number of pulses required to generate the same degree of tissue damage, thus lowering the energy consumption in PEF. However, using low pulse frequency may increase the total processing time. Different combination of pulse frequency and pulse number should be explored in order to determine the optimal PEF conditions. To further increase the PEF efficiency, the use of higher electric field strength is also recommended (Lebovka and others 2002). For more accurate determination of energy consumption or savings, future work should include a record of the precise conductivity profile of the sample during PEF.

Conclusions

Pulse frequency plays an important role in the PEF-induced damage of onion tissues treated at constant electric field strength, pulse width, and pulse number. The key result of this work is that PEF at frequencies below 1 Hz causes a significantly higher degree of tissue permeabilization relative to higher frequencies, while the amount of damage is independent of frequency at values above 1 Hz. Changes in the electrical characteristics, electrical conductivity disintegration index, percent ion leakage, texture parameters, and percent weight loss indicate that low pulse frequencies ($f <$ 1 Hz) yield a higher degree of tissue permeabilization than higher pulse frequencies ($f = 1$ to 5000 Hz). By means of a viability staining technique using NR dye, we are able to correlate the electrical and physical properties with the viability of permeabilized cells in onion tissue subjected to PEF at different pulse frequencies. The viability cell staining results reinforced our findings that a larger percentage of onion cells are permeabilized at low frequency. Our results show that PEF frequency has a pronounced effect on the persistence of cytoplasmic streaming within onion cells. We are the first to report a strong correlation between persistence of cytoplasmic streaming and overall damage to the tissue as a function of frequency. The critical frequency of 0.1 Hz observed in the damage characterization experiments is qualitatively consistent with both convective and diffusive time scales for fluid transport via cytoplasmic streaming. These observations suggest that cytoplasmic streaming may play a significant role in increasing the plant cellular damage at sufficiently low frequencies but does not solely control the degree of PEF-induced tissue damage. The fluid motion is shown to cease almost instantly at PEF frequencies greater than 1 Hz, in contrast it continues for some time at lower frequencies. We hypothesize that the continuation of cytoplasmic streaming after PEF at low pulse frequencies, such as 0.1 Hz, enhances the transport of high-conductivity fluid inside the permeabilized cells to the extracellular space (cell wall and middle lamella) and creates more conductive pathways for the electrical current. When the next pulse comes, the current travels through these conductive paths and permeabilizes more cells, which results in a higher degree of tissue permeabilization. The liquid movement may also accelerate the physical damage initiated by PEF effect. The critical pulse frequency is below 1 Hz for onion tissue and about 0.1 Hz for onion epidermis that has a characteristic length scale of about 100 μm. However, the critical frequency is dependent on the dimension of the plant cell that varies for different cell types, different tissues, and different plant commodities. More study is needed to determine the effect of cytoplasmic streaming on PEF-induced tissue damage.

Electrical characteristic, ion leakage, texture, weight loss, viability staining, and cytoplasmic streaming results suggest that using

lower pulse frequency can minimize the number of pulses required to achieve a desired amount of permeabilization, thus lowering the total energy consumption. The authors suggest the use of PEF at low frequencies ($f < 1$ Hz) for increased yield and improved efficiency of juice extraction and dehydration processes in food plant materials. Different combinations of pulse frequency and number of pulses should be explored in order to determine the optimal PEF conditions, since using low pulse frequency may increase the total processing time.

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