Pulsed electric field induced stress in plant systems

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Abstract

Plants produce a wide range of complex phytochemicals in response to different abiotic stressors. Polyphenolic compounds are one of the largest groups of secondary metabolites responsible for the plant resistance. They play a major role in plant adaptation to strained environmental conditions and contributing to color, flavor and taste of the foods. Due to their high antioxidative capacity and positive influence on human health, an increased interest in the consumption of food rich in natural antioxidants has been observed. To increase concentration of polyphenolic compounds through stress induction, pulsed electric field (PEF) treatment has been applied on fruit tissue during postharvest storage. Changes in total polyphenol (TP) concentration, activity and concentration of selected enzymes and impact on the cell membrane permeabilization have been investigated during the stress reaction period after imposed stress.

The results showed that application of low treatment intensities induced increased accumulation of polyphenolic compounds in different fruits. 65 % larger TP concentration was observed in apples after 300 V/cm electric field treatment during stress reaction period of 48 h. *De novo* synthesis of phenolic compounds were suggested due to no detrimental impact on the cell membrane and due to changes in TP accumulation during the stress reaction period. After induction of irreversible membrane permeabilization at 1200 and 4000 V/cm, 88 % lower TP concentration was observed after a stress reaction period of 24 h. Loss of phenolic compounds was attributed to large disintegration of cellular material and leakage through damaged cell membrane. Oxidative processes were suggested based on the strong deterioration and excessive browning of apple tissue. Similar observations were made after PEF treatment of different fruits, indicating *de novo* synthesis of protective substances at optimal stress intensity. An impact of the initial physiological fruit fitness and the attained PEF intensity on the induced stress response has been observed. Furthermore, a distinguishable response in terms of polyphenol accumulation in the multicellular fruit tissue was observed, supporting cell size dependant impact of PEF treatment. Due to complexity of metabolic responses in multicellular fruit tissue and diversity of environmental factors that might mask impact of PEF stress, cell culture suspensions were used as model systems of corresponding higher plants. The analysis of physiological fitness and cell viability has been determined during a cultivation period in a controlled environment after imposed stress to observe the occurrence of sublethal injury. To investigate the effect of the stressor on the cells through

changes in secondary metabolism, polyphenolic concentration was analyzed simultaneously with enzymes involved in polyphenol synthesis and oxidation. Increased phenylalanine ammonia-lyase (PAL) activity immediately after PEF treatment preceded increase in TP concentration observed 9 h after imposed stress, indicating *de novo* synthesis of polyphenolic substances. Even though a direct correlation of TP accumulation and polyphenol-oxidase (PPO) activity was not found, exposure to PEF treatment revealed that a phenolic oxidation system might be involved in the plant resistance together with phenylpropanoid biosynthesis. The obtained results represent evidence of the marked effect of low intensity PEF treatments on changed metabolic processes, followed by secondary metabolite synthesis.

Zusammenfassung

Pflanzen produzieren eine Reihe von komplexen Phytochemikalien als Reaktion auf verschiedene abiotische Stressoren. Polyphenolische Verbindungen sind dabei die größte Gruppe von Sekundärmetaboliten mit einer Bedeutung für die Stressreaktion von Planzen. Sie spielen eine wesentliche Rolle in der Anpassung der Planze an Stressbedingungen und tragen zu Farbe, Aroma und Geschmack von Lebensmitteln bei. Aufgrund ihrer hohen antioxidativen Kapazität und ihres positiven Einflusses auf die Gesundheit wächst das Interesse am Verzehr von Lebenmitteln mit einem hohen Gehalt an natürlichen Antioxidantien. Zur Erhöhung der Konzentration an Polyphenolen durch Stressinduktion wurde die Anwendung gepulster elektrischer Felder (engl. Pulsed electric fields PEF) in der Nach-Ernte Lagerung untersucht. Veränderungen im Gesamtpolyphenolgehalt, in der Aktivität und Konzentration ausgewählter Enzyme sowie der Einfluss auf die Permeabilisierung von Zellmembranen wurden in der Reaktionszeit nach Stressauslösung untersucht.

Die Ergebnisse zeigen, dass die Anwendung niedriger PEF-Behandlungsintensitäten eine Anreicherung von polyphenolischen Verbindungen in verschiedenen Früchten induziert. Ein um 65 % höherer Polyphenolgehalt wurde in Äpfeln nach einer Behandlung mit 300 V/cm innerhalb einer Stressreaktionszeit von 48 Stunden festgestellt. De novo Synthese phenolischer Verbindungen wird dabei als Ursache angesehen, da keine Zerstörung der Zellmembran aber eine Veränderung der Gesamtpolyphenol-Akkumulation während der Stressreaktionszeit beobachtet wurde. Nach der Induktion einer irreversiblen Membranpermeabilisierung bei 1200 und 4000 V/cm kam es zu einer Verringerung der Gesamtpolyphenolkonzentration um 88 % während der Reaktionszeit von 24 Stunden. Die Abnahme phenolischer Verbindungen wird dabei auf die umfangreiche Zerstörung des Zellmaterials und die Auslaugung durch Zellmembranschädigung zurückgeführt. Oxidative Prozesse werden als Ursache angesehen, da ein starker Abbau und eine entsprechend starke Bräunung des Apfelgewebes auftraten. Ähnliche Beobachtungen einer de novo Synthese von Schutzsubstanzen bei optimaler PEF-Intensität konnten nach der PEF-Behandlung von verschiedenen Früchten gemacht werden. Ein Einfluss vom ursprünglichen physiologischen Zustand und der angewendeten PEF-Intensität auf die induzierte Stressantwort konnte beobachtet werden. Weiterhin wurde eine differenzierte Stressantwort bzgl. der Polyphenolakkumulation im vielzelligen Fruchtgewebe festgestellt

und damit die zellgrößenabhängige Wirkung von PEF untermauert. Aufgrund der Komplexität der metabolischen Reaktion in vielzelligem Fruchtgewebe und der Vielschichtigkeit der Umweltbedingungen, die einen möglichen PEF-Effekt maskieren, wurden Zellsuspensionskulturen als Modellsysteme entsprechender höherer Pflanzen genutzt. Die Analyse der physiologischen Fitness und Zellvitalität wurde während der Kultivierung unter kontrollierten Umweltbedingungen nach Stressinduktion durchgeführt, um das Auftreten sublethaler Schäden zu detektieren. Polyphenolkonzentration und für die Polyphenolsynthese und –oxidation relevante Enzyme wurden untersucht um den Effekt des Stressfaktors auf die Zellen und den Einfluss auf Veränderungen des Sekundärmetabolismus zu ermitteln. Eine erhöhte PAL-Aktivität unmittelbar nach PEF-Behandlung gefolgt von einer Erhöhung des Gesamtpolyphenolgehaltes 9 h nach der Stressauslösung konnte als Indiz für die de novo Synthese polyphenolischer Substanzen festgestellt werden. Obwohl eine direkte Korrelation zwischen Gesamtpolyphenolakkumulation und PPO-Aktivität nicht festgestellt werden konnte, zeigte die Behandlung mit PEF, dass das phenolische Oxidationssystem zusammen mit der Phenylpropanoid-Biosynthese in die Pflanzenstressreaktion involviert ist. Die Ergebnisse geben einen Hinweis auf einen deutlichen Effekt einer PEF-Behandlung mit niedriger Intensität auf den Metabolismus und die nachfolgende Synthese von Sekundärmetaboliten.

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List of abbreviations

A	extinction
c	molar concentration (mol/L)
C	capacitor/capacity (F)
CDI	cell disintegration index
d	interelectrode distance (m)
E	electric field strength (kV/m)
E_a	enzyme activity
E_{crit}	critical electric field strength (kV/m)
EDTA	ethylene di-amino tetra acetic acid
f	frequency (Hz)
f_{nec}	necessary frequency (Hz)
GAE	gallic acid equivalents
I	current (A)
K_h	electrical conductivity of untreated material in a high frequency field (3 to 50 MHz)
K'_h	electrical conductivity of PEF treated material in a high frequency field (3 to 50 MHz)
K_l	electrical conductivity of untreated material in a low frequency field (1 to 5 kHz)
K'_l	electrical conductivity of PEF treated material in a low frequency field (1 to 5 kHz)
m	sample mass (kg)
$\dot{m}$	mass flow rate (kg/sec)
n	pulse number
nd	not detected
PAL	phenylalanine ammonia-lyase
PEF	pulsed electric field
PMSF	phenylmethylsulfonyl fluoride
POD	peroxidase
PPO	polyphenol-oxidase
PVPP	polyvinylpolypyrrolydon
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	room temperature (°C)
T	temperature (°C)
τ	pulse duration (µs)
t_{PEF}	total treatment time (s)
TP	total polyphenol concentration (mg GAE/g)
TP_{PEF}	total polyphenol concentration of PEF treated samples
$TP_{untreated}$	total polyphenol concentration of untreated samples
TTC	2,3,5-triphenyltetrazolinum chloride
U_o	initial electric potential from generator (kV)
$\dot{V}$	volume flow rate (m^3/sec)
W_{spec}	specific energy input (kJ/kg)
W_{pulse}	energy input per pulse (J)
Z	impedance (Ω)

1. Introduction and Objective of the Work

The Pulsed Electric Fields (PEF) treatment is a non-thermal food processing method that has attracted significant interest as a novel way of food preservation and cell disintegration of plant and animal raw materials. However, due to its specific impact on biological cell, PEF technology offers alternative concepts based on reversible membrane permeabilization. Permeabilization of the cell membrane takes place when biological cell is exposed to the external electric field since the conductivity of extracellular and intracellular media is several orders of magnitude larger than conductivity of the cell membrane (Kanduser & Miklavcic, 2008). For the given value of PEF treatment intensity, cell viability will be preserved and reversible breakdown takes place (Zimmermann, Pilwat & Riemann, 1974; Angersbach, Heinz & Knorr, 2000). However, if the treatment intensity exceeds a threshold, irreversible permeabilization occurs resulting in cell membrane disintegration and irreversible loss of its function. Even though exact mechanism of pore formation is till date not elucidated, a key concept of permeabilization is facilitation of molecular transport through a dynamic pore, which may cause a significant chemical or osmotic stress of a cell (Weaver & Powell, 1989). Such stress leads to secondary processes that affect the cell and its normal metabolic activity. Alterations of plants metabolic activity have been reported after exposure to different abiotic stressors (Basra & Basra, 1997). The trigger signals of abiotic stressors often promote the formation of free radicals such as reactive oxygen species, which have been suggested to induce polyphenol synthesis (Beck, Fettig, Knake, Hartig & Bhattarai, 2007). Furthermore, post stress destabilizations of biomembranes through changes in ion concentration, transmembrane potential and concentration gradient have been suggested to alter metabolic activity. Physiological events of defense response after plant exposure to abiotic stress are manifested through *de novo* synthesis of phenolic compounds, plant secondary metabolites, recognized as effective antioxidant and anti-inflammatory substances (Dixon & Paiva, 1995; Cisneros-Zevallos, 2003; Larcher, 2003; Pociecha, Plazek, Janowaik & Zwierzykowski, 2009a). An increased interest in the consumption of food rich in natural antioxidants has been observed in the last decade, due to their positive influence on human health (Reyes & Cisneros-Zevallos, 2003).

Due to the specific effect on biological cell membrane, induction of stress by PEF treatment in plant tissue was assumed to induce a similar defence response. The main focus of this study was to investigate possible polyphenol accumulation in higher plants

and in plant cell cultures and to separate impact of PEF on polyphenol accumulation through *de novo* synthesis after reversible membrane permeabilization from easier release of polyphenolics trapped in the cell interior after irreversible membrane permeabilization. Production of secondary metabolites may enhance the nutritional value or alter the phenolic profile of food. Therefore, an increase in TP concentration in stressed products will be considered within this thesis as a positive effect which offers a potential to increase health benefit properties.

Fruits during postharvest storage retain metabolic activity of harvested organs and react to external abiotic stressors, which results in compositional change (Rizzini, Bonghi & Tonutti, 2009). Different postharvest strategies have been developed in order to retard deterioration processes and to prolong the preservation times by finding new fresh-keeping mechanisms. This study proposes a concept based on application of postharvest abiotic stress by PEF treatment in order to enhance the nutraceutical content of fresh fruits. Additionally to possible accumulation of polyphenolic compounds, parameters concerning PEF impact on plant tissue need to be examined. Determination of the cell membrane permeabilization of biological tissue material will be determined by impedance measurements in order to gain insight in disintegration degree post PEF treatment. Polyphenolic compounds can be converted by polyphenol oxidase (PPO) into more reactive species and PPO has been linked to defense responses caused by diverse external stressors (Rivero, Ruiz, Garcia, Lopez-Lefebre, Sanchez & Romero, 2001; Thipyapong, Stout & Attajarusit, 2007). Therefore, PPO activity was assigned as additional stress indicator. To determine possible stress related changes, PEF treated and corresponding untreated tissues need to be kept for chosen stress reaction period after imposed stress, to allow time for response on strained conditions. Furthermore, temperature is a very important factor affecting postharvest life since it has profound effect on biological reactions and will be considered during this study.

In order to investigate stress response activity and possible mechanisms involved, plant cell culture suspensions of corresponding higher plants will be used as model systems to eliminate uncontrollable environmental interactions. Due to similarity of metabolic processes comparable to parent plant and controlled growth conditions, cell cultures are suitable for investigation of PEF impact on polyphenolic accumulation. The first aim was to maintain a stable cell line with representative growth characteristics and to choose a suitable growth phase to induce PEF stress. To detect reactions on PEF as external stressor impact on the cell membrane will be studied, together with different

growth parameters and cell viability. Phenylalanine ammonia-lyase (PAL) is the key enzyme of phenylpropanoid metabolism, through which all phenylpropanoids are formed (Dixon & Paiva, 1995) and therefore chosen marker for polyphenol *de novo* synthesis. Systematic considerations of chosen parameters will be shown after PEF treatment of low intensities. It will be shown that PEF may affect cell metabolic activity of plant tissue without inducing permanent damage.

2. Literature Review

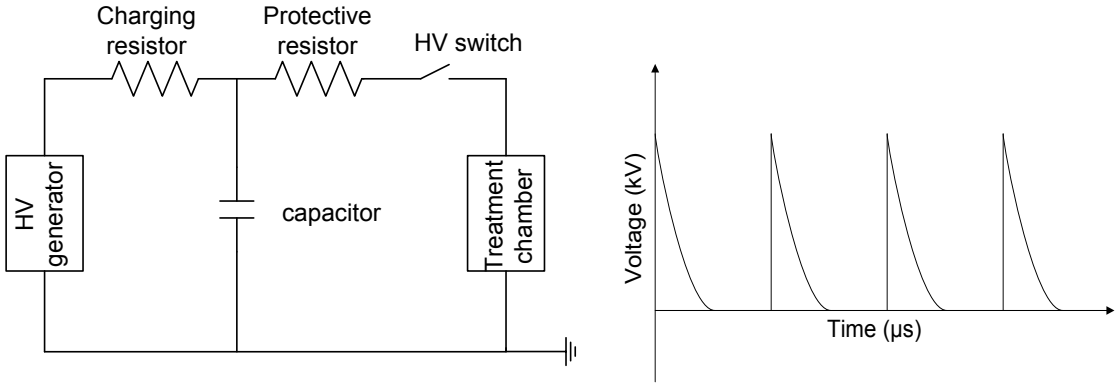
Pulsed Electric Field (PEF) processing has been investigated in recent decades for different applications in the field of medicine, food- and biotechnology. Inducing specific impact on the biological cell membrane, this emerging non-thermal technology attracted significant interest as a novel way of food processing and preservation. PEF also offers a potential to induce stress reactions in plant systems by the application of low-intensity treatments. Practical application of an external electric field requires sufficient strength in order to induce the desirable effect. PEF operating characteristics are strongly dependant of equipment design, of each other and of product characteristics. Therefore, a single specific factor can be hardly adjusted without modifying other related factors. In order to design an effective PEF unit with desirable technical factors, equipment design should be carefully considered together with operating characteristics.

2.1. Physical aspects of a Pulsed Electric Field (PEF) treatment

2.1.1. Treatment unit

The generation of high intensity electric field pulses requires PEF generating system which consists of a high voltage power supply, capacitor or a network of capacitors and a high voltage switch. A key element of the PEF system is the treatment chamber, where generated high voltage pulses are applied to product placed between electrodes (Barsotti, Merle & Cheftel, 1999a). A high voltage DC generator transforms low voltage electric power (usually 220 or 440 V alternating current) to a high voltage direct current power. Once the high voltage has been generated, an energy storage device (capacitor or network of capacitors) is charged across a charging resistor to the required level. The power is released through a pulse forming network, resulting in a defined pulse shape, which is delivered to the electrodes in the treatment chamber. The generation of high intensity electric field pulses is realized by repetitive charging and discharging of capacitor(s) which accumulate electrical energy (Toepfl, Heinz & Knorr, 2005). Slow charging and very fast discharging of the capacitor is required since the pulse width is short in comparison to the time between pulses and can be achieved through a large number of high-power switching devices (Toepfl, 2006). Maximal repetition rate and maximal current and voltage rate are determined by the type of the switching device. In order to prevent a breakdown of the switching system, a protective resistor is necessary to be included in the electrical circuit. A basic electrical circuit diagram for generation of

exponential decay pulses is shown in Figure 2-1. To measure delivered voltage and observe pulse shape and width, which are giving information about the treatment intensity, an oscilloscope can be included in the PEF treatment system together with a low value resistance placed in series with the treatment chamber.



(A) (B)

Figure 2-1: Simplified electrical circuit diagram of the pulsed electric field system (A) for the generation of exponential decay monopolar pulses (B).

Various types of batch or continuous treatment chambers have been developed up to date for different applications (Barbosa-Cánovas, Góngora-Nieto, Pothakamury & Swanson, 1999; Lelieveld, Notermans & Haan, 2007; Huang & Wang, 2009; Jaeger, Meneses & Knorr, 2009a). All of them consist of at least two electrodes (one of them connected to the pulse generator over the high voltage switch and the other one to the ground), which are separated by electric insulating materials to ensure the electric potential between them. The distribution of applied voltage depends highly on the electrode and insulator geometry and on the alignment of those components, influencing some of the treatment characteristics (Toepfl, 2006; Jaeger et al., 2009a; Meneses, Jaeger, Motritz & Knorr, 2011). The simplest alignment of electrodes to achieve most uniform electric field distribution and avoid electrical and thermal hot spots is a parallel plate electrode configuration (Jeyamkondan, Jayas & Holley, 1999). It can be employed in batch and continuous systems. Other most common types of electrode alignments used in continuous flow operations are coaxial cylindrical and collinear electrode configuration (Toepfl, 2006). Due to different electrode arrangement inside coaxial and collinear treatment chambers, the distribution of electric field is not homogenous and should be considered when designing a treatment chamber for a specific product (Jaeger, 2011).

Batch systems are commonly used on laboratory scale, due to the need to dismantle the treatment chamber every time a new batch of product is processed. For

efficient treatment on an industrial scale continuous systems are proposed, with high flow rate capacity and easy integration in already existing processing lines. In the last couple of years evaluation of the treatment and chamber design for process optimization on industrial scale has been intensively studied (Barbosa-Cánovas & Sepulveda, 2005; Toepfl, 2006; Lelieveld et al., 2007; Jaeger, Balasa & Knorr, 2008; Morales-de la Pena, Elez-Martínez & Martin-Belloso, 2011; Toepfl, 2011; Jaeger, Schulz, Lu & Knorr, 2012). To allow successful industrial application the systematic process of scale up is necessary. This process next to the treatment chamber design and evaluation of PEF treatment parameters includes validation of the food safety and quality, operation and overall control of the PEF system, equipment cleanability, investment and production costs. Due to the latest development of scalability techniques, PEF systems for an industrial application of microbial decontamination (equipment capacities of up to 10000 L/h) and cell disintegration (up to 25 t/h) are available today (Bluhm & Sack, 2009; Huang & Wang, 2009; DIL, 2011; Toepfl, 2011).

2.1.2. Processing characteristics

Pulse shape and width depends on the configuration of the discharge circuit, on the type of the switch and on the electrical properties of the media (product) placed between the electrodes. Most commonly used pulse shapes are exponential decay and rectangular (square) pulses. The electric field pulses may be applied as monopolar (only positive pulses) or bipolar, which produce alternating changes in the movement of charged molecules (positive and negative pulses). During an exponential decay pulse the voltage rises very quickly, exceeds beyond the effective voltage and slowly decays, whereas during rectangular pulse the voltage remains relative constant after reaching the peak value for a defined period of time before descends to zero (Toepfl et al., 2005). Efficiency of PEF inactivation using different pulses wave forms have been demonstrated, showing sufficient microbial inactivation (Qin, Zhang, Barbosa-Cánovas, Swanson & Pedrow, 1994; De Haan & Willcock, 2002; Kotnik, Pucihar, Rebersek, Miklavcic & Mir, 2003). PEF disintegration of plant tissue was shown to be effected by pulse protocol. Larger disintegration of onion tissue was observed when larger pulse width was applied (Ersus, Oztop, McCarthy & Barrett, 2010). Some authors reported that larger time between pulses increased the degree of tissue disintegration, which was explained through the resealing processes that might have masked permeabilization at long time intervals between pulses (Vorobiev & Lebovka, 2008). Therefore, to define the characteristics of a high voltage

pulse and the efficiency of PEF treatment, beside pulse shape and pulse intensity, pulse protocol (which includes pulse duration and time between pulses) should be considered.

The electric potential across the treatment chamber is defined by the electric field strength (E). If the media (and/or product placed in the treatment chamber and exposed to the electric field pulses) is homogenous, the electric field strength is in the case of parallel plate electrode configuration given by following equation:

$$E = \frac{U}{d} \qquad [\text{kV/m}]$$

Equation 2-1

where U represents delivered voltage to the treatment chamber and d is the interelectrode distance.

The duration of PEF treatment is defined by the exposure time to the electric field and is characterized through total treatment time (t_{PEF}), shown in Equation 2-2. Time in which the electric field remains at the certain level (τ) represents pulse width of each single pulse, multiplied with the number of pulses (n).

$$t_{PEF} = n * \tau \qquad [\text{sec}]$$

Equation 2-2

Thereafter, the treatment time increases either with the number of pulses or with the pulse duration. The time at which the electric field remains constant (τ) during exponential decay pulse corresponds to the time required for a given pulse to decay to 37 % of its peak voltage (Barsotti et al., 1999a; Ho & Mittal, 2000), while during rectangular pulses voltage remains constant for the whole duration of a pulse (Toepfl, 2006). Since the pulse protocol was shown to exhibit impact on PEF disintegration (Asavasanti, Stroeve, Barrett, Jernstedt & Ristenpart, 2012), it should be together with total treatment time considered for identification of a PEF treatment. The number of pulses per second corresponds to a repeat frequency f, measured in hertz [Hz].

In order to compare PEF to traditional processing treatments, energy consumption is shown to be a useful instrument for treatment intensity evaluation and can be expressed as specific energy input (W_{spec}). It is characterized as pulse delivered energy from the capacitors which dissipates in the treated product, and can be delivered by different combinations of current, voltage and pulse width (Toepfl, 2006). The specific energy input in batch PEF treatment systems is determined by the following equation:

$$W_{spec} = \frac{W_{pulse} * n}{m} \qquad [kJ/kg]$$

Equation 2-3

where W_{pulse} represents the energy per pulse, n the number of pulses and m the mass of the treated sample. In PEF generating systems, releasing of the energy stored in capacitors dissipates in a protective resistor and in the treatment chamber. The energy per pulse leaving the capacitors can be estimated through the following equation:

$$W_{pulse} = \frac{1}{2} * C * U_o^{\,2} \qquad [kJ/kg]$$

Equation 2-4

where C denotes the capacity of the set of capacitors and U_o the initial charge voltage.

In continuous PEF treatment systems the specific energy input can be calculated based on the energy delivered per pulse (W_{pulse}), the pulse frequency (f) and the mass flow rate ($\dot{m}$):

$$W_{spec} = \frac{W_{pulse} * f}{\dot{m}} \qquad [kJ/kg]$$

Equation 2-5

The frequency necessary to affect each passing volume (f_{nec}) with a certain number of pulses (n), where the volume flow rate ($\dot{V}$) and the volume of sufficient treatment is known (V_{treat}), assuming plug flow, can be calculated from the following equation:

$$f_{nec} = \frac{n * \dot{V}}{V_{treat}} \qquad [Hz]$$

Equation 2-6

Since specific energy input is in direct dependence with the treatment chamber design, the electric field strength, the treatment time and the product conductivity, it can not be used per se to evaluate effectiveness of the PEF treatment without considering aforementioned factors.

The energy consumption represents an important parameter that should be addressed to maintain the non thermal characteristics of PEF treatment. When applying electrical energy to the product, ohmic heating will take place, which may cause temperature increase in treated product. To avoid thermal effects when using very high energy inputs during PEF processing, the treatment chambers can be equipped with a cooling system. Furthermore, synergetic effect of treatment temperature on PEF treatment

efficiency has been reported (Pagan, Condon & Raso, 2005; Toepfl et al., 2005) and therefore aforementioned difficulty can be utilized as an advantage during processing while maintaining thermal border conditions.

2.1.3. Product characteristics

Complex chemical and physical characteristics of products subjected to PEF treatment are important factors of individual nature that can strongly influence the efficiency of PEF processing. One of the factors of the greatest relevance is ionic strength, which is responsible for the electrical conductivity of the treated product. Conductivity strongly depends on the kinetic state of an ionic solution and is linearly proportional to the temperature. As mentioned earlier, an increase in temperature of treated product/media during PEF processing might occur due to ohmic heating. Thereupon, an increase in the electrical conductivity will follow temperature increase, which will result in decrease of electric field intensity delivered to the product. Specific range of product conductivity is required to achieve effective PEF processing. For permeabilization of biological cells a minimal conductivity is necessary, since certain amount of ions are needed in treated media to establish transmembrane potential. However, when the conductivity is to high, it may limit the possibility of non thermal processing and it may present a difficulty when trying to achieve the supercritical field strength, due to a lower generated peak field strength across the treatment chamber (Toepfl et al., 2005). Since conductivity of most "food stuff" can not be modified without changing other important product properties, from an engineering point of view, treatment chamber design and electrode configuration with high load resistivity can diminish this effect and avoid thermal and electrical hot spots (Gerlach, Moritz, Alleborn, Baars & Delgado, 2008).

The dielectric strength represents another important parameter related to product property, which defines maximum electric field strength that the product can withstand intrinsically without experiencing dielectric breakdown (Barbosa-Cánovas & Sepulveda, 2005). Air bubbles or suspended particles present in treated media or entrapped within the product limit the treatment intensity and may cause dielectric breakthrough, as a high current flows through the bubble due to different dielectric properties. When processing nonhomogenous products (at a macroscopic level) that comprise parts of different dielectric strengths, maximal applicable treatment intensity is determined by the part with the lowest dielectric strength. Furthermore, the composition of treated product/media (the content of proteins, polysaccharides or macromolecules) was reported to have protective effect on microorganism inactivation (Zhang, Chang & Barbosa-Cánovas, 1994; Grahl &

Märkl, 1996; Martín-Belloso, Vega-Mercado, Qin, Chang, Barbosa-Cánovas & Swanson, 1997; Jaeger, Schulz, Karapetkov & Knorr, 2009b) and should be considered during the evaluation of microbial safety after PEF treatment.

2.2. Biological aspects of a PEF treatment

The application of an external electric field has been generally accepted to induce an impact related to local structural changes of the cell membrane in any biological cell: animal, plant or microbial. The permeability of a cell membrane, which separates the interior of the cell from the external environment, can be increased with electropulsation. Many theoretical models have been suggested till date (Zimmermann et al., 1974; Chernomordik, Sukharev, Popov, Pastushenko, Sokirko, Abidor et al., 1987; Schoenbach, Peterkin, Alden & Beebe, 1997; Barsotti, Merle & Cheftel, 1999b; Ho & Mittal, 2000; Teissie, Golzio & Rols, 2005), however, there is still no clear evidence underlying its mechanism of action at the cellular level.

2.2.1. Biological cell material

The cell is a structural and functional unit of every living organism. Its size varies from 0.1 μm to 150 mm (Sinha, 2004) and comes in different shapes depending upon the function. A plant cell consists of a protoplast which includes metabolically inactive substances and protoplasm enveloped by the cell membrane, which is surrounded by the cell wall (Figure 2-2). The cell wall is a rigid structure that provides shape and strength to the cell, metabolically inert and permeable in nature, made of cellulose and pectic substances.

Protoplasm includes cytoplasm and nucleus, representing semi-fluid matter, which is the site of a large number of metabolic processes sensitive to temperature, electric current, shock, light etc. Metabolically active substances (endoplasmatic reticulum (ER), Golgi apparatus, ribosome, mitochondria and plastids) are together with nucleus (the central organ of the cell) and vacuoles (the "store houses" of the cell) enclosed in cytoplasm. The biological protein synthesis takes place at ribosome which together with ER forms rough ER that manufactures membranes and secretory proteins. Smooth ER on the other hand does not hold ribosomes and is responsible for carbohydrate and lipid synthesis (Sinha, 2004). Synthesized lipids are transported around the cell through Golgi apparatus, whose function is to modify proteins delivered from rough ER. For an array of reactions like amino acid and fatty acid synthesis, regulation of calcium concentration in

the cell, mitochondrion plays an important role, including energy production necessary for regulation of cellular metabolism, together with regulation of membrane potential.

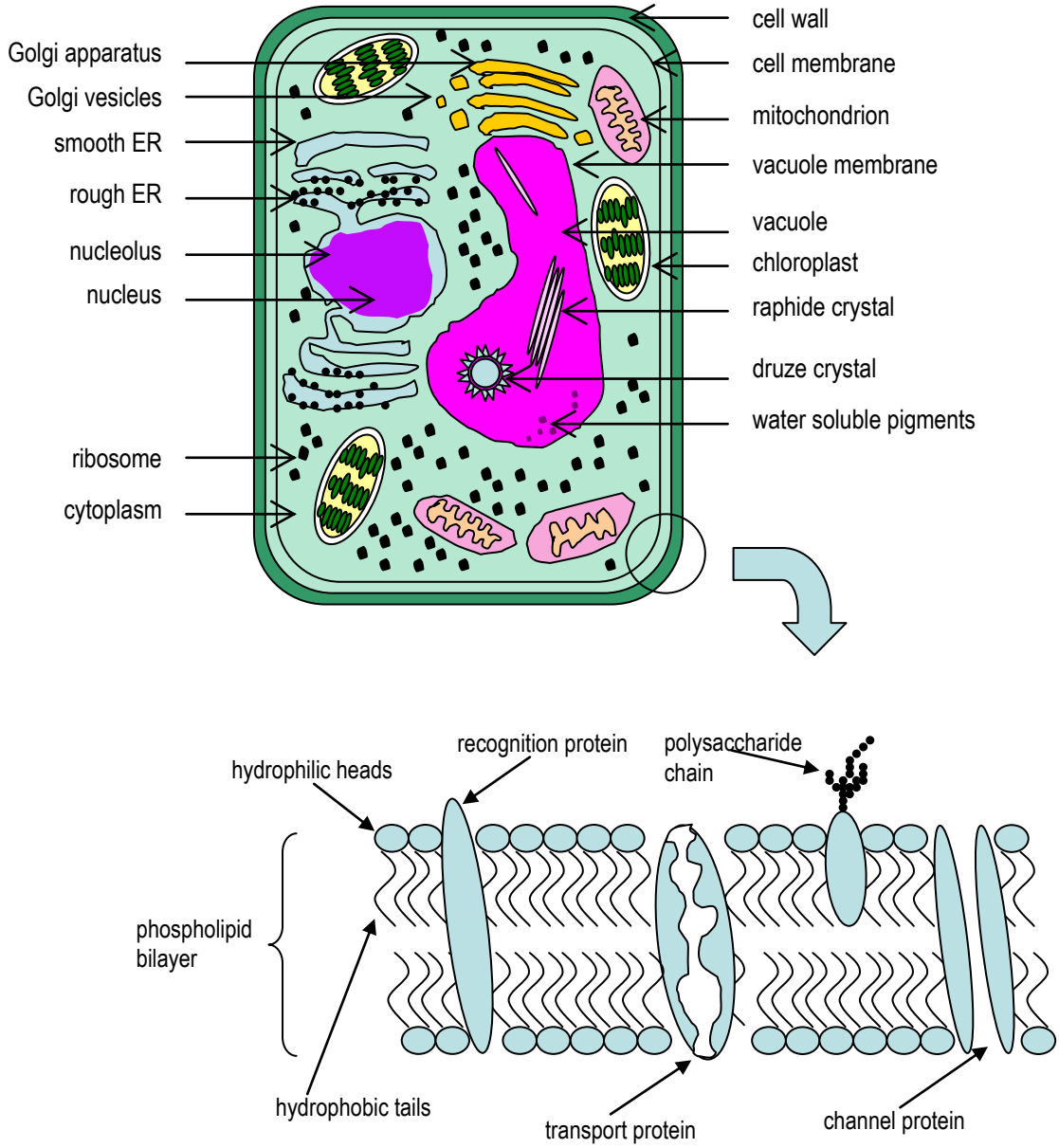


Figure 2-2: The plant cell and membrane structure.

Most of the plant cells usually comprise one large central vacuole surrounded by a differentially permeable cytoplasmic membrane called tonoplast. In young meristemic cells small vacuoles are scattered in the cytoplasm, while in mature cells they fuse together into one large vacuole that can occupy 30 to 80 % of the cell total volume (Alberts, Johnson, Lewis, Raff, Roberts & Walter, 2008). They contain organic and inorganic substances such as: organic acids, soluble carbohydrates, enzymes, alkaloids, anthocyanin pigments, inorganic salts, etc. Aside from the storage of cellular metabolite

byproducts, protective and toxic substances, vacuoles are involved in cellular responses to environmental and biotic factors that provoke stress (Marty, 1999). They are reported to play an important role in cell detoxification and in maintaining turgor pressure against the cell wall. Since tonoplast is involved in regulation of the ion movements, transport of protons from cytosol into the vacuole stabilizes cytoplasmic pH. These aspects show that vacuolar physical and metabolic functions are essential to plant life.

The cell organelle that separates internal from external cell environment is the cell membrane, whose basic functions depend on its molecular structure. It consists of the phospholipid bilayer with nonpolar hydrophobic tails pointing towards the inside of the membrane and the polar hydrophilic heads forming the inner and the outer surface of the membrane (Figure 2-2). Such a molecular orientation is present due to thermodynamic conditions, where lowest free energy state of hydrophilic and hydrophobic interactions should be attained. Through such an arrangement of the lipid bilayer, passive diffusion of hydrophobic molecules is possible; however, it is impermeable to ions and polar molecules. Different types of proteins embedded through the flexible double layer are responsible for transport activities of these substances, which is described by the fluid mosaic model (Singer & Nicolson, 1972). Membrane fluidity depends on the temperature and composition of the bilayer, while permeability depends mostly on the electric charge and polarity of the molecule and to a lesser extent to the molar mass of the molecule.

Membrane proteins do not only support cell normal metabolic activities, but are also responsible for maintaining transmembrane potential (or membrane voltage) which refers to the difference of electric potential (voltage) between the interior and the exterior of a biological cell. Difference of electrical charges on both sides of the membrane is created due to differential mobility of diffusing ions across the membrane.

2.2.2. PEF induced permeabilization

PEF treatment is based on the use of high intensity pulsed electric fields in order to disrupt the cell membrane, which is highly important semipermeable barrier responsible for regulation of many complex metabolic activities including a number of transport mechanisms. The phenomenon of PEF induced permeabilization was studied based on model systems (phospholipid vesicle and planar bilayer) as well as on single cell level (microorganisms), in dense cell suspensions and in complex biological tissues (Sale & Hamilton, 1967; Crowley, 1973; Teissie & Tsong, 1981; Chernomordik et al., 1987; Winterhalter, Klotz & Benz, 1996; Zimmermann, 1996; Kanduser & Miklavcic, 2008). To elucidate the permeability of a cell membrane several hypothesis have been made. All of

them considered the lipid bilayer as the universal basis for cell membrane structure. One of the most accepted theoretical model is the electromechanical model that describes the dielectrical breakdown effect of the cell membrane through mechanical compression (Zimmermann et al., 1974). Intracellular and extracellular environment consists of a conductive media filled with free charges of opposite polarities that are separated by the cell membrane, constituting naturally occurring transmembrane potential of about 10 mV. Exposure to an electric field induces accumulation of cell interior and exterior charges across the membrane and therefore an increase of the initial transmembrane potential. Accumulation of opposite charges on both sides of the membrane raises compression pressure on the membrane and induces membrane thinning that subsequently increases electrostatic attraction (Figure 2-3). When the overall potential exceeds a threshold value of about 200 mV to 1 V, elastic resistance of a membrane laps and locale rupture with pore formation occurs in the sub-microsecond range at a given value of applied field (Crowley, 1973; Tsong, 1991; Zimmermann, 1996; Schoenbach et al., 1997). The permeabilization of a biological cell membrane will occur as long as the induced electric field strength (E) is larger than critical membrane field strength (E_{crit}).

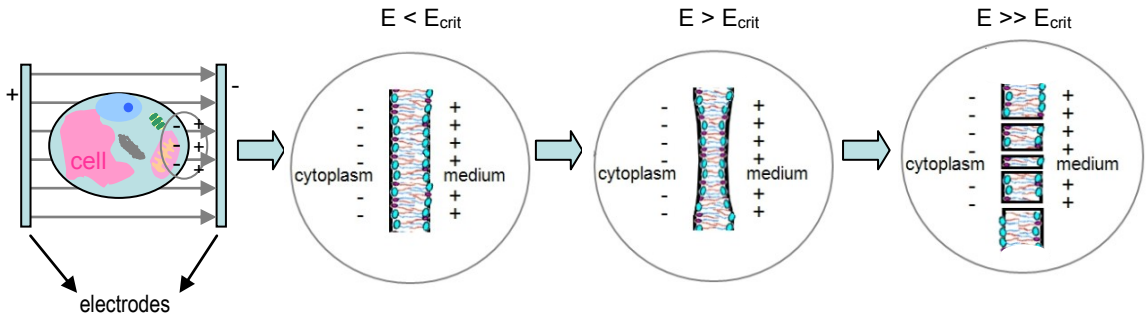


Figure 2-3: Mechanism of PEF induced permeabilization described through electromechanical model.

A biological cell undergoes specific changes when exposed to high voltage electric pulses. Local structural changes and a rapid breakdown of the cell membrane take place, depending mainly on the treatment intensity (electric field strength, pulse duration, number of pulses). This phenomenon, called permeabilization or electroporation, occurs within a very short time range (µs to ms) leading to temporary (reversible permeabilization) or permanent pore formation (irreversible permeabilization), and thus changing membrane properties (Zimmermann et al., 1974).

When low treatment intensity is used, the voltage-induced openings of channels in the cell membrane do not cause irreparable damage (Schoenbach et al., 1997; Teissie et

al., 2005). A flux of ions through the channels alter the ion concentrations close to the cell membrane that results in cell stress, after which cells need some time (from seconds to hours) to recover (Schoenbach et al., 1997). Reversible permeabilization involves creation of a number of metastable pores and actually represents a temporary high conductance state of the cell membrane, while the viability of the cell remains preserved (Dimitrov, 1995; Zimmermann & Niel, 1996; Weaver, 2000; Teissie et al., 2005). It is theoretically explained through rapid creation of small pores where transition from the rupture to the dielectric breakdown can be qualitatively understood in terms of a competition between the kinetics of pore creation and of pore expansion (Weaver, 1995). Increase in PEF treatment intensity will promote formation of large pores or/and increase in number of pores, which are not able to reseal again and permeabilized state remains. Irreversible permeabilization of the cell membrane is associated with mechanical destruction and causes permanent loss of membrane semipermeability and may lead to disintegration of cellular tissue (Crowley, 1973; Zimmermann et al., 1974).

Experimental data indicate that critical electric field strength necessary for cell membrane permeabilization increases as cell radius decreases. Depending on the size and geometry of the cell, critical electric field strength was found in the range of 1 - 2 kV/cm for plant cells (cell size 40 - 200 μm) and 10 - 14 kV/cm for microbial cells (1 - 10 μm) such as *E. coli* (Toepfl et al., 2005). Several additional factors have been reported to affect permeabilization effectiveness: cell orientation and local cell density, membrane fluidity, cytoskeleton and presence of cell wall, external medium composition and osmotic pressure (Teissie et al., 2005; Kanduser & Miklavcic, 2008).

Alternative concepts of pore formation in phospholipid bilayers have been described, from molecular rearrangements during permeabilization to the possibility of pore formation through integral proteins or at the lipid-protein interface (Dimitrov, 1984; Weaver & Powell, 1989; Tsong, 1990; Chernomordik, 1992; Glaser, Leikin, Chernomordik & Pastushenko, 1998). Fluid lipid bilayer membranes are envisioned to undergo specific rearrangements through several different pathways (Figure 2-4). Transport through the membrane associated with diffusion potentials and membrane pumps may be electrically driven due to external electric field and cause greatly enhanced transport of molecules and ionic species through the membrane (Figure 2-4A). Electromechanical model suggest compression and thinning of the membrane (Figure 2-4B), which may lead to transient pore formation (Figure 2-4C and D). Formation of hydrophobic pores (Figure 2-4D) results from the growth of lateral fluctuations of the

positions of phospholipid molecules in the lipid bilayer. They form only in a very small fraction of the total membrane surface. The presence of an induced transmembrane potential provides the free energy necessary for structural rearrangements of membrane phospholipids where reorientation of hydrophilic heads may take place and a hydrophilic pore is formed (Figure 2-4C). Since the formation of hydrophobic pores is energetically more favorable when the pore radius is very small, they are considered as intermediate stages in the formation of hydrophilic pores (Tsong, 1991; Glaser et al., 1998). Furthermore, since functionality of the integrated proteins in lipid bilayer depends on the transmembrane potential, membrane permeabilization might occur through or next to a protein channel (Figure 2-4E). Voltage-sensitive protein channels, which get activated at considerably lower potential (50 mV), in comparison to critical transmembrane potential, might open due to electrical injury. However, protein channel opening does not preclude increase in transmembrane potential to overcritical value for creation of pores on different sites on the membrane.

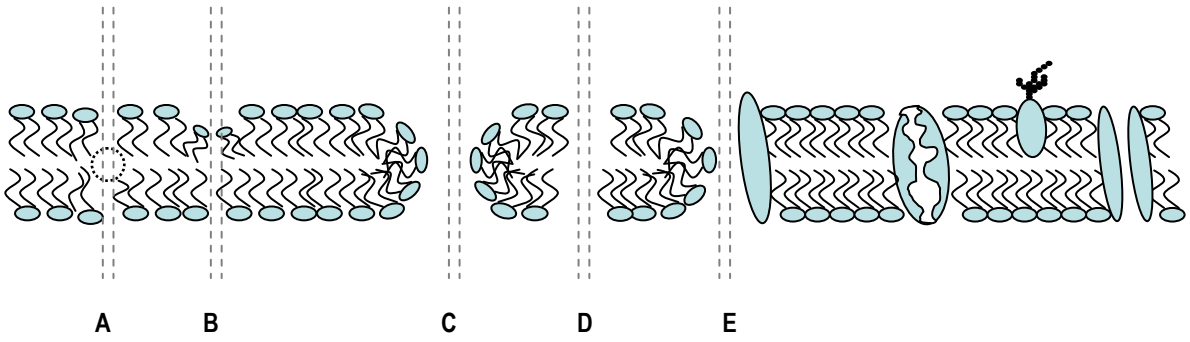


Figure 2-4: Pore formation hypothesis (A) Free volume fluctuation allowing entry of molecules and ionic species; (B) Local membrane compression and thinning; (C) Hydrophilic pore formation ; (D) Hydrophobic pore creation, believed to be a precursor to hydrophilic pore formation; (E) Pore formation next to integral protein.

Teissie *et al*. (2005) suggested a pore development process that occurs during permeabilization through several phases. Charging of the membrane is taking place due to the induced electric field until the critical transmembrane value is reached. Increase of the membrane potential difference occurs after a very short time (microsecond range) and represents the first phase, called *induction step*. At that point a mechanical stress is present, with local defects of the membrane. As long as the electric field remains at an overcritical value, an electromechanical stress remains present with time dependant membrane transition that is explained through the leaky state of the cell membrane in the *expansion step*. A recovery of the membrane organization occurs as soon as the electric field intensity is lower than the threshold value. This process of stabilization represents

following phase indicated as *stabilization step*, where a strong decrease in the flow of polar molecules was observed. However, the cell membrane remains leaky to polar compounds, followed by a process of slow resealing (seconds to minutes range). The *resealing step* represents a recovery of membrane impermeability and was shown to be temperature dependant (Kinosita & Tsong, 1977; Lopez, Rols & Teissie, 1988).

The major gap in the knowledge of the mechanism of electropermeabilization is the lack of information on the transmembrane traffic of polar compounds on molecular level. However, due to recognized specific effect on the cell membrane, PEF treatment represents a versatile method, offering practical application on various biological systems in the field of medicine, food- and biotechnology. Although employment of moderate fields has been lately implemented in food science, most of the studies were done in the range of irreversible membrane permeabilization, with the main objective to induce microbial inactivation or to facilitate extraction of specific constituents and/or to increase drying rate.

2.2.3. Detection of membrane permeabilization

Identification of the permeabilization of cellular tissue after PEF treatment represents a very difficult task, since pore formation is in the submicrosecond range and pore area is in the range of 0.1 % of the total membrane surface (Toepfl et al., 2005). The creation of pores and structural reorganization of the cell membranes have not been directly observed to date. Since PEF treatment induces structural modifications and related functions of the cell membrane, therefore affecting metabolic activities and physiological fitness of the cells, cell membrane permeabilization was investigated through a sequence of events as indirect evidence. Several methods developed for determination of membrane breakdown, for smaller cells with diameter of 1-40 μm (Chernomordik et al., 1987; Weaver & Chizmadzev, 1996; Zimmermann & Niel, 1996; Teissie et al., 2005; Kanduser & Miklavcic, 2008) were demonstrated through:

- measurements of the cell population size distribution in a particle analyzer,
- studies of the uptake or release of different indicator substances (e.g. radioactive isotopes),
- microscopic observation of release of low-molecular weight substances from electroporated cell,
- determination of the spatial changes in the transmembrane voltage and conductance (using voltage sensitive fluorescence dyes), and
- usage of patch clamp techniques.

In the case that cells are large enough for the introduction of microelectrodes, the electroporation can be also determined by measurements of the membrane current-voltage characteristics (Zimmermann & Niel, 1996). Identification of the degree of membrane permeabilization in complex tissue is even more challenging, due to multiphase biological systems, where additional product parameters (as discussed in chapter 2.1.3) interfere with PEF treatment, and consequently influencing observations on cellular level. Several staining methods in combination with microscopic observation were successfully applied on heterogeneous material (Fincan & Dejmek, 2002; Phoon, Galindo, Vicente & Dejmek, 2008; Janositz & Knorr, 2010). A release of plant pigments or ions into the external cell containing media was investigates as indirect indicator of the degree of ruptured cells (Dörnenburg & Knorr, 1993; Saulis, Satkauskas & Praneviciute, 2007). Measurements of the diffusion coefficient in electroporated tissue was suggested as one of the methods to evaluate the damage degree of PEF treatment (Jemai & Vorobiev, 2002).

To identify the quantity of electric field induced membrane rupture, a very useful method which studies the degree of cell permeabilization has been developed (Angersbach, Heinz & Knorr, 1997; 1999). This method is based on the frequency dependant passive electrical properties of biological cell systems (for vegetable and muscle tissue characterized in frequency ranges from 10^3 to 10^7 Hz) and can be quantified as the portion of disintegrated cells. The model describes the conductivity spectra of total intact or total ruptured cells, as well as systems with a combination of ruptured and intact cells (Figure 2-5). When such a complex system (biological tissue sample) is exposed to alternating voltage with increasing frequency, the intensity of a floating current gives information about the impedance (Z) of a measured sample (Equation 2-7), which varies with the frequency. The impedance represents a complex resistance that measures overall opposition of a circuit to current and is composed of two parts: real part defined as ohmic resistance and imaginary part which is influenced by the capacity and the self-induction. Z is given by the equation:

$$Z = \frac{V}{I} \qquad [\Omega]$$

Equation 2-7

where V represents the complex alternating voltage and I the complex alternating current.

The total conductivity of the cell systems to the alternating current frequencies depends on the electrical properties of a biological cell membrane. The intact cell membrane can be regarded as an insulator between intracellular and extracellular conductive media, whereas a single intact cell in surrounding tissue can therefore be

regarded as series of resistors. A ruptured membrane is no longer a dominating insulator (when low frequencies are used) resulting in larger conductivity of measured sample. The typical frequency-conductivity curves of intact and ruptured cells are shown in Figure 2-5.

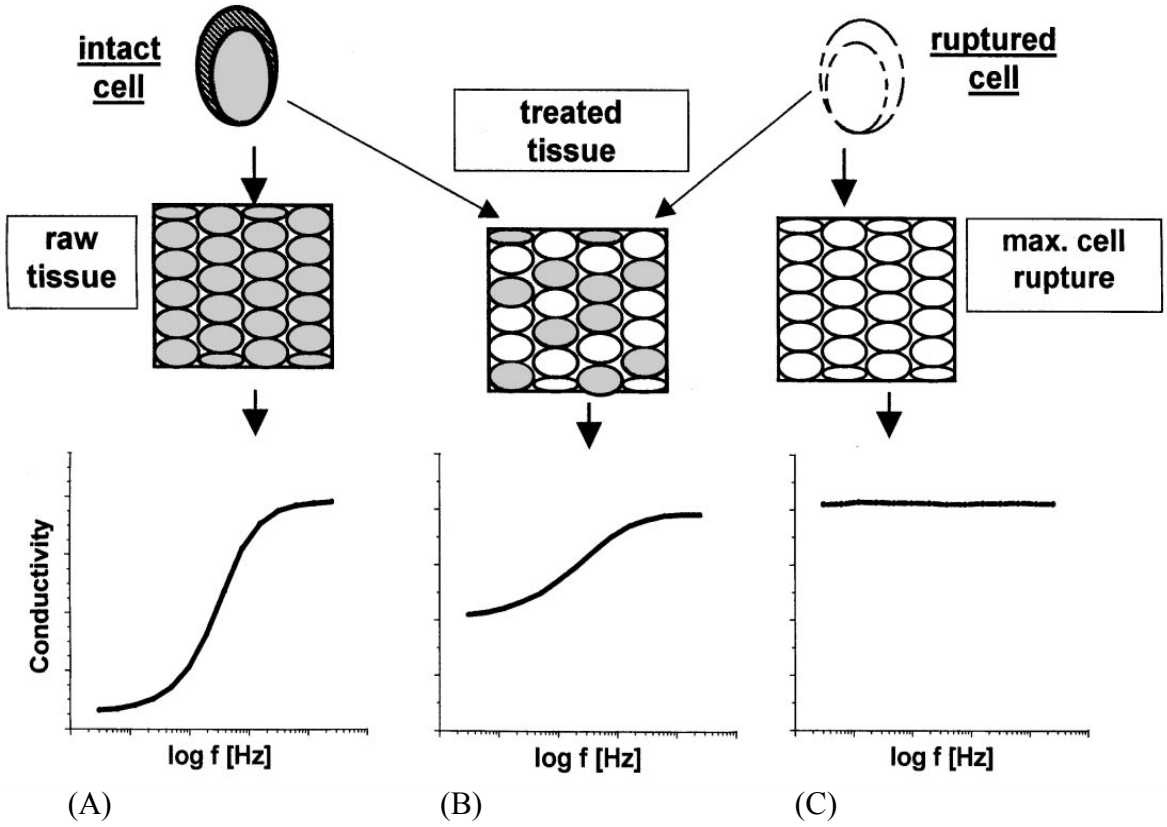


Figure 2-5: Typical frequency dependant electrical conductivity spectra of plant raw tissue within: (A) intact cells, (B) partial ruptured and (C) totally ruptured cells in the frequency range of the measured current of 1 kHz to 50 MHz (Knorr & Angersbach, 1998).

Based on the data of frequency dependant conductivity of intact and ruptured cells, the cell disintegration index (CDI) can be calculated from the following equation:

$$CDI = 1 - \frac{K_h}{K'_h} * \frac{(K'_h - K'_l)}{K_h - K_l}$$

Equation 2-8

where K_l and K'_l are the electrical conductivity of untreated and treated material, respectively, in a low frequency field (1 to 5 kHz), and K_h and K'_h are the electrical conductivity of untreated and treated material, respectively, in a high frequency field (3 to 50 MHz). The cell disintegration index represents the proportion of permeabilized cells and varies from 0 for intact cells to 1 for completely disintegrated cells (Knorr & Angersbach, 1998).

2.3. PEF applications

Due to the permeabilization phenomenon, the pulsed electric field technology has been proposed as a useful tool for many practical applications in several areas. Different cell types exhibit diverse permeabilization effects when exposed to PEF treatment (Weaver, 2000; Vorobiev & Lebovka, 2008). Depending on the intensity of the applied treatment, permeabilization of the cell membrane leads to reversible or irreversible pore formation and disintegration of cellular tissue. Due to its impact on a biological cell, PEF can be utilised in the food industry for various applications: stress induction in plant cells and tissues, alternating nutritional value of food products, changes in texture and moisture of raw materials and inactivation of microorganisms at low temperatures (Table 2-1).

Table 2-1: PEF applications in food technology in dependence of electric field treatment intensity

Processing intensity	*Electric field intensity*	*Electric pulse duration*	*Permeabilization*	*Application*
Low	0.1 – 1.5 kV/cm	1 – 400 µs	Reversible	Stress induction Nutritional value
Moderate	0.5 – 3 kV/cm	1 – 400 µs	Irreversible (plant and animal tissue)	Drying Expression Texture Extractability
High	15 – 40 kV/cm	1 – 400 µs	Irreversible (microbial cell)	Microbial safety

The main research in the field of food technology is focuse on preservation of electrical conductive food products by microbial cell destruction (Sale & Hamilton, 1967; Qin et al., 1994; Zhang et al., 1994; Ho & Mittal, 2000; Heinz, Alvarez, Angersbach & Knorr, 2002; Toepfl et al., 2005), as an alternative non-thermal method to heat pasteurization. Many reports in the last few decades demonstrated PEF as an effective inactivation technique for spoilage and pathogenic microorganisms. Even though viruses are not affected by PEF treatment (Lelieveld et al., 2007), limited inactivation of bacterial spores could have been induced after combined application of PEF and thermal treatment (Raso, Calderón, Góngora, Barbosa-Cánovas & G., 1998; Meneses, Reineke & Knorr, 2012).

Improvement of mass transfer processes (drying, extraction, expression, infusion), modification of enzymatic activities and functional properties of food ingredients have been suggested after application of mild to low electric field treatment (Bouzrara & Vorobiev, 2000; Ade-Omowaye, Rastogi, Angersbach & Knorr, 2002; Tedjo, Taiwo,

Eshtiaghi & Knorr, 2002; Fincan, DeVito & Dejmek, 2004; Lebovka, Praporscic, Ghnimi & Vorobiev, 2005). Furthermore, reversible membrane permeabilization provides a potential for targeted influence of biological systems, inducing stress reactions while maintaining cell viability (Sabri, Pelissier & Teissie, 1996; Angersbach et al., 2000; Gómez Galindo, Dejmek, Lundgren, Rasmusson, Vicente & Moritz, 2009).

2.3.1. Stress induction

Application of low electric fields in food technology attracted large attention in the last decade, but still very little information is available regarding functionality and stress responses of plant tissues, as a consequence of cell permeabilization. Low to mild treatment intensities induce temporary pore development in the cell membrane and affect cell normal metabolic processes. Reversible permeabilization in the field of medicine, biophysics and biotechnology is well documented as an efficient method for transfer of foreign materials such as drugs, genes, DNA etc. into the cell, as well as for obtaining good electrostimulative effects of plant intracellular components (Chang, Chassy, Saunders & Sower, 1992; Zimmermann & Niel, 1996; Teissie et al., 2005; Kanduser & Miklavcic, 2008).

One of the initial applications of PEF treatment (on the borderline between reversible and irreversible membrane permeabilization) in the field of food science and technology was studied by Dörnenburg and Knorr (1993). The influence of low intensity PEF treatment on production and recovery of secondary metabolites from cultured plant tissues (*Chenopodium rubrum* and *Morinda citrifolia*) in correlation with cell vitality was investigated. The effect of PEF treatment was observed through sustainable release of anthraquinones and from *Morinda citrifolia* (10 pulses of 1600 V/cm) and amaranthin from *Chenopodium rubrum* (3 pulses of 500 V/cm).

The process of reversible membrane permeabilization and extent of the pulse induced changes in the structural properties of the cell system have been investigated by Angesbach, Heinz and Knorr (2000). The formation of conductive channels (assigned as classical membrane breakdown phenomena) occurred across the cell membrane when critical transmembrane potential of approximately 0.7 to 2.2 V (for different cell systems: potato tissue, apple tissue, fish tissue and suspension cultured potato cells) was reached. It was observed that the electric field strength higher than critical value of 400 – 800 V/cm should be applied to the tissue with a cell size of 50 – 120 μm for a significant membrane breakdown. Furthermore, the reversibility of the structural change was found to be secondary effect of the applied external electric field. The pore zones resealed within very

short time after permeabilization and cell membrane recovered its electrically insulating properties (Angersbach et al., 2000).

PEF-induced release of intracellular pigments and additional production of secondary metabolites in plant systems (maize and soybeans) was studied by Guderjan, Toepfl, Angersbach and Knorr (2005). After application of 20 - 120 exponential decay pulses (duration 280 μs) with field strengths in a range of 600 - 1300 V/cm, increased phytosterol content in maize germs (32.4 %) and larger yield of isoflavonoids in soybeans (20 - 21 %) in comparison to untreated samples were reported. Interestingly, such a large increase of selected metabolites could not be asserted by treatment at 7300 V/cm.

Some studies have shown that reversible membrane permeabilization induces generation of oxygen species within plant cells and changes in cell metabolites accumulation (Sabri et al., 1996; Gómez Galindo et al., 2009; Pakhomova, Khorokhorina, Bowman, Rodaite-Riševiciene, Saulis, Xiao et al., 2012). Induced permeabilization of plant cells (black Mexican sweet maize cells) exhibited an oxidative jump that was not directly correlated to the cell viability (Sabri et al., 1996). Resealing of the pores was observed 5 minutes after permeabilization, while oxidative species generation was present over a longer period of time. Due to the non linear relationship between viability and production of oxidative species, it was suggested that stress reactions of intact plant cells were not limited to the electrically affected part of the cell membrane. The authors explained observed phenomenon induced with permeabilization through the well known defense response of cultured plant cells to abiotic stress (Figure 2-6). A model that describes the effect of pathogen and elicitor is shown on the left side of the figure, where GTA-binding protein coupled to the elicitor receptor induces Ca_2^+ channel opening. Increase of Ca_2^+ in the intracellular surrounding activates a protein kinase (pathway 1). Hypothetical consequence of permeabilization induced by electric field pulses is demonstrated as an influx of Ca_2^+ into the cell, leading to an activation of the same protein kinase (pathway 2). A membrane bound oxidase is being stimulated by protein kinase that leads to synthesis of O_2 in the cell wall (Mehdy, 1994; Sabri et al., 1996).

Reactive oxygen species have been suggested to affect many cellular processes involved in plant response to external elicitor, through being a part of endogenous signal components required for synthesis of secondary metabolites (Sabri et al., 1996; Gómez Galindo, Wadsö, Vicente & Dejmek, 2008; Gómez Galindo et al., 2009). To characterize stress response after reversible permeabilization of potato tissue, metabolite profiling was used, providing insight in tissue response to stress induced by external stimuli (Gómez

Galindo et al., 2009). Changes in hexose pool and decrease in chlorogenic acid content of potato tissue was observed 24 hours after induced electroporation with a single rectangular pulse (duration of 1 ms) at electric field strength in the range of 200 - 400 V/cm. Reversible pore formation and osmoregulation were suggested as relevant events caused by PEF stressor that contributed to observed changes.

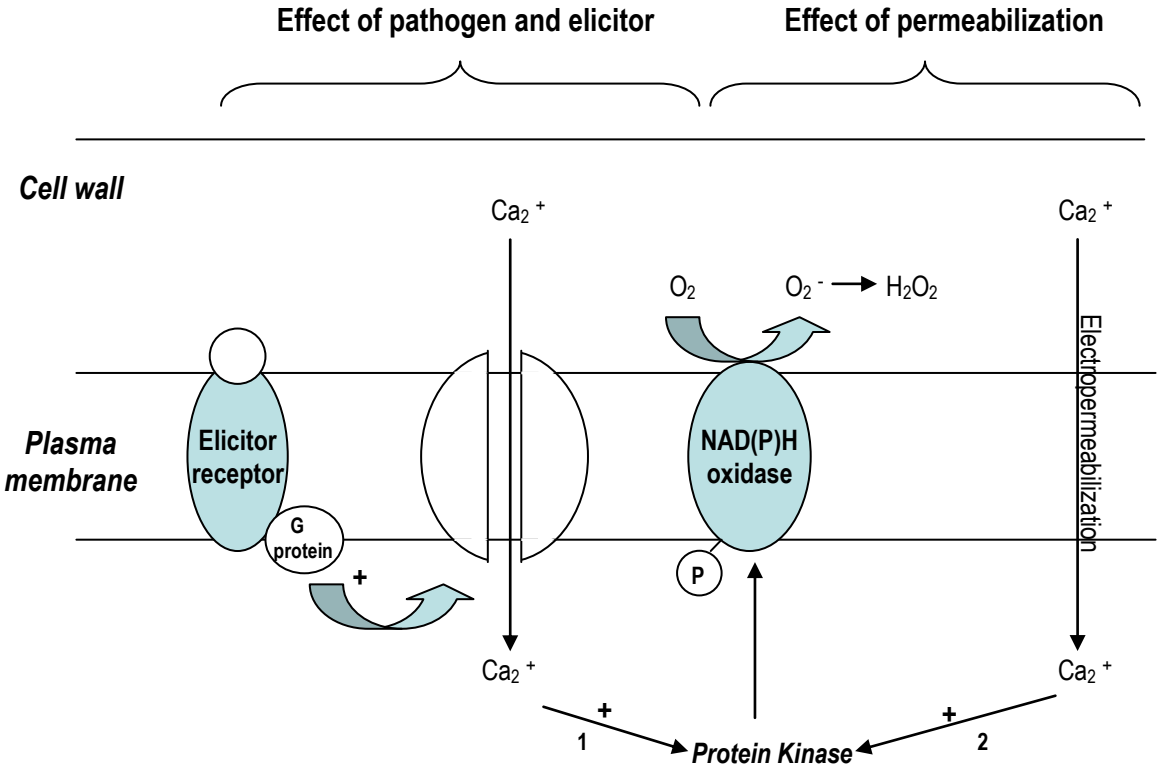


Figure 2-6: Proposed model describing the effect of elicitors or permeabilization induced by electric field pulses on generation of activated oxygen species in plant cells (modified after Sabri et al., 1996).

Metabolic responses of germinating barley seeds were studied after application of fifty 1 ms rectangular pulses with varying electric field strengths from 275 to 1200 V/cm (Dymek, Dejmek, Panarese, Vicente, Wadsö, Finnie et al., 2012). After application of an electric field strength of 1200 V/cm, radicle elongation was affected without significant changes in the gross metabolic activity of the seeds. Since no significant effect on protein pattern was detected, decreased radicle elongation was suggested to be a consequence of PEF effect on root development and α-amilase level. The authors proposed reduced availability of sugars released from starch degradation by α-amilase and oxidative stress as reasons for impaired root elongation.

Unlike decreased root development of barley seeds (Dymek et al., 2012), a growth stimulating effect of *Arabidopsis thaliana* seedlings was observed after PEF treatment with shorter pulse duration (Eing, Bonnet, Pacher, Puchta & Frey, 2009). The majority of

plants exhibited natural growth with much larger leaf area than non treated samples after the application of 100 rectangular pulses with 10 ns duration, at an electric field strength of 5 kV/cm (energy input of 100 J/kg). Similar observation was made after 10 rectangular pulses with 100 ns duration at the same electric field intensity and the same total energy input. After the application of larger treatment intensities (10 and 20 kV/cm; 40 pulses of 25 ns; energy inputs of 400 and 1600 J/kg, respectively) natural growth of seedlings was observed with plant size comparable to untreated samples. However, a treatment at even larger electric field strength (50 kV/cm) inhibited any growth when a pulse duration was 100 ns (10 pulses were applied resulting in a total energy input of 10 000 J/kg). Interestingly, 100 pulses of 10 ns duration, at the same field strength and energy input, exhibited no lethal effect on the seedlings, but some phenotypic changes in leaf growth symmetry were observed. After a wide range of PEF treatment intensities with different combinations of pulse protocol, positive and negative growth stimulations were observed.

The effect of electric field pulses on the growth of cell suspension protoplasts (*Solanum dulcamara* L.) was investigated after 3 successive pulses (duration of each pulse: 10 – 50 μs) in the electric field range of 250 to 1250 V/cm, (Chand, Ochatt, Rech, Power & Davey, 1988). Increased morphogenesis and cell wall regeneration were observed within PEF treated tissue, which occurred after a shorter period of time than in untreated culture.

The enhanced DNA synthesis in isolated *Prunus* and *Solanum* protoplasts have been demonstrated due to exposure to pulsed electric field treatment at 250 V/cm (pulse duration 87 μs) and 750 V/cm (pulse duration 29 μs), respectively (Rech, Ochatt, Chand, Davey, Mulligan & Power, 1988).

Protein synthesis was found to be stimulated in electroporated plant protoplasts of *Dactus carotta* L., *Nicotiana tabacum* L. and *Beta vulgaris* L. after a single rectangular pulse of 400 μs at 1700, 1200 and 1000 V/cm, respectively (Joersbo & Brunstedt, 1990). The effect of pulse duration in relation to electric field strength on maximal stimulation of protein synthesis was studied as well on *Beta vulgaris* L. protoplasts, and it was found that treatment at 1400 V/cm and 100 μs achieved similar results as treatment at 800 V/cm and 999 μs. In other words, when prolonging time duration of a pulse, lower field strength was necessary to achieve similar effect. Furthermore, the cultivation time after electroporation with maximal protein synthesis was found to be dependant on the applied field strength. Application of PEF treatment at 1000 – 1200 V/cm (pulse duration 100 μs) induced maximal stimulation of protein synthesis after 4 to 5 days of cultivation, while

the same duration of an electric pulse at 2000 V/cm resulted in more rapid increase, which reached maximal value 2 days after electroporation.

Current research activities reveal that biological systems can be affected by PEF treatment, which offers new possibilities to targeted modifications of functional food properties. When applying low to mild electric field treatments, PEF technology may be used as external stimulus for induction of stress reactions in plant systems promoting the production of secondary metabolites as a plant response on strained conditions.

2.3.2. Improvement of mass transfer processes

Processing techniques in food and pharmaceutical industry such as conventional thermal, mechanical and enzymatic treatments are used to increase mass transfer processes of plant and animal tissue (Knorr & Angersbach, 1998). Transfer of important intracellular components and liquids depends on conventional processing concepts, which include disintegration of cellular tissue in order to enhance the yield of valuable ingredients. These techniques may induce loss of nutritionally and physiologically valuable substances due to thermal degradation or enzyme activity (natural occurring or added). Furthermore, application of different organic solvents is undesirable in food and pharmaceutical industry, since residual substances might be present in final product, which requires their removal to fulfill safety criteria. In the case of PEF application, as a substitute or support to aforementioned techniques, disintegration of the cellular tissue and irreversible pore formation has a direct impact on diffusion properties and could improve the recovery of intracellular substances while retaining initial quality of the product.

Extractability of beetroot pigment betalain and different ionic species from fresh red beetroot tubers (*Beta vulgaris*) was investigated (Fincan et al., 2004). 90 % of total red colouring was released into the isotonic solution (following 1 h aqueous extraction) after applying 270 rectangular pulses of 10 μs duration at a field strength of 1 kV/cm. The amount of extracted pigment was found to be directly proportional to the release of ionic species, thus authors postulated that no differential permeabilization of any of the intracellular compartments occurred. Furthermore, Eshtiaghi and Knorr (2002) showed that PEF pretreated beets can easily be pressed and higher yield of sucrose obtained. That the pressed pulp from PEF pretreated samples in comparison to the conventional thermal process contained less residual sugar, and additionally shorter time for extraction process was required. In particular, field strength in the range from 1.2 to 2.5 kV/cm and pulse number from 1 to 200 was reported to have a key influence on the tissue disintegration.

The release of intracellular pigments (anthocyanins) from wine grapes was studied after application of PEF treatment (50 pulses at 3 kV/cm), which resulted in three-fold increase of total anthocyanin content (Tedjo, Eshtiaghi & Knorr, 2002). Improvement of phenolic extraction from grape skin and enhanced expression of juice from grapes due to PEF-induced disintegration (field strength in the range of 1 to 10 kV/cm) has been reported from several working groups (Eshtiaghi & Knorr, 2000; Praporscic, Lebovka, Vorobiev & Mietton-Peuchot, 2007; Corrales, Toepfl, Butz, Knorr & Tauscher, 2008; Lopez, Puertolas, Condon, Alvarez & Raso, 2008; Boussetta, Lebovka, Vorobiev, Adenier, Bedel-Cloutour & Lanoiselle, 2009; Puertolas, Lopez, Saldana, Alvarez & Raso, 2010). Further yield enhancements of juice from apple mash (Barsotti et al., 1999b; Schilling, Alber, Toepfl, Neidhart, Knorr, Schieber et al., 2007; Jaeger et al., 2012), from apple cossettes (Bazhal & Vorobiev, 2000), from carrot mash (Knorr, Geulen, Grahl & Sitzmann, 1994; Jaeger et al., 2012), from red beet (Bouzrara & Vorobiev, 2000), as well as sugar beet (Eshtiaghi & Knorr, 2002), were reported after PEF application of mild treatment intensities.

PEF assisted processes for enhanced and gentle recovery of oil yield were developed (Guderjan, Toepfl, Angersbach & Knorr, 2005). After applying PEF (5 kV/cm and 60 pulses; 7 kV/cm and 120 pulses; duration of each pulse 30 µs) as a pre-treatment method before mechanical pressing or solvent extraction of rapeseed (*Brassica napus*), increased oil yield and additionally higher concentration of tocopherols, polyphenols and phytosterols were measured in obtained oil (Guderjan, Elez-Martínez & Knorr, 2007).

Not only release of intracellular substances and liquids may be assisted with PEF processing, but uptake of different substances, such as the marinating or curing of fish and meat products may also be enhanced due to application of PEF technology (Toepfl, Heinz & Knorr, 2006).

Pulsed electric field treatment can also improve efficiency of dehydration processes of different fruits and vegetables. Published data showed that electric field treatment at low to mild treatment intensities (0.22 – 1.6 kV/cm, 5 pulses with duration from 322 to 405 µs) increases the diffusion coefficient and thereby reduces process duration (Rastogi, Eshtiaghi & Knorr, 1999). Higher drying rates, as well as higher mass and heat transfer coefficients were detected after PEF application (2.4 kV/cm, 10 pulses, 300 µs) in comparison to conventional pre-treatments (Ade-Omowaye, Rastogi, Angersbach & Knorr, 2001b). Reduced drying time and larger yield of coconut milk was obtained when PEF treatment (2.5 kV/cm, 20 pulses, 575 µs) was employed as a

processing step prior to coconut dehydration (Ade-Omowaye, Angersbach, Eshtiaghi & Knorr, 2001a). Impact of low to moderate intensity PEF treatments (0.5 – 2.5 kV/cm), applied prior to osmotic dehydration, on β-carotene and vitamin C content in bell peppers was further investigated (Ade-Omowaye et al., 2002). Larger vitamin C content and lower reduction of β-carotene content was determined after 20 pulses at 2.5 kV/cm (400 μs duration of each pulse), than in osmosed bell pepper at 55 °C.

Cell structure largely depends on the integrity of the cell. Since permeabilization causes loss of the cell membrane semipermeability, which subsequently results in loss of turgor pressure within the cell, textural properties and structure of treated tissue may be altered after PEF application. Tissue softening based on PEF permeabilization can be used to reduce the energy required for cutting or grinding plant material (Fincan & Dejmek, 2003; Janositz, 2005; Toepfl et al., 2005). Together with reduction of cutting energy, changed properties of the cut surface and low fat intake of French fries was reported after PEF pretreated potato tissue (Janositz, 2005). Decrease of compressive strength was determined in carrots (Rastogi et al., 1999) and textural changes followed by tissue softening of carrots, potatoes and apples were reported after combined PEF with mild heat treatment (Lebovka, Praporscic & Vorobiev, 2004).

2.3.3. Impact on enzyme activity

Naturally occurring plant enzymes are highly specialized globular proteins, responsible for regulation of internal biological processes in the living cell. They catalyze chemical reactions and their activity may be affected by several factors such as pH change, temperature change, substrate concentration, and presence of activators or inhibitors. The reactivity is dependant on enzyme structure, more specific on the configuration of the active site. The influence of PEF treatment on enzymes is often discussed in literature. However, possible impact of processing factors (electric field strength, pulse duration and shape, frequency, treatment temperature) and product parameters (pH change, concentration of substrate, inhibitors, activators) on enzyme activity is till date not completely elucidated. Due to different experimental setup and wide range of processing parameters used, it is very difficult to compare sensitivity of enzymes exposed to PEF treatment. Some research groups reported decreased enzyme activity after PEF treatment (Ho, Mittal & Cross, 1997; Giner, Gimeno, Espachs, Elez-Martínez, Barbosa-Cánovas & Martin-Belloso, 2000; Castro, Barbosa-Cánovas & Swanson, 2001; Giner, Gimeno, Barbosa-Cánovas & Martin-Belloso, 2001), while others have found that PEF treatment did not significantly effect selected enzymes (Barsotti,

Dumay, Mu, Fernandez Diaz & Cheftel, 2001; Van Loey, Verachtert & Hendrickx, 2002; Moritz, 2008). Furthermore, an increase in the enzymatic activities of lysozyme and pepsin were observed after PEF treatment of enzyme solutions (Ho et al., 1997).

PEF sensitivity depending on the enzyme type was reported and some authors indicated that the main variables affecting enzyme activity are field strength, pulse duration, number of pulses and pulse shape (Ho et al., 1997; Vega-Mercado, Martin-Belloso, Qin, Chang, Góngora-Nieto, Barbosa-Cánovas et al., 1997; Yang, Li & Zhang, 2004). It has been suggested that changes in the conformation of the active site were responsible for converting enzyme into more or less active form (Ho et al., 1997; Bendicho, Barbosa-Cánovas & Martin-Belloso, 2003). Due to energy dissipation during PEF treatment an increase in product temperature may occur, which could be also attributed to change in enzyme activity (Yang et al., 2004). However, understanding of PEF impact on enzymes and food component proteins need further research to gather background information. Most of the studies were investigating impact of large treatment intensities in order to achieve inactivation of undesirable enzymes which cause food spoilage. In many cases treatment intensities were larger than required for microorganism inactivation, and to authors' best knowledge no data were found concerning impact of low PEF treatment on enzyme activity during postharvest storage of fruits.

2.4. Stress in nature - The biological stress concept

Diverse environmental factors affect plants in various ways which can be either beneficial or non-beneficial to plant productivity. Seasonal changes, plant injury, insufficient or excessive light or mineral nutrients, disease or interaction with other organisms affect plant metabolic system and may lead to stress (Levitt, 1980; Larcher, 1987; Beck & Lüttge, 1990). Flexibility of plant metabolism allows the development of complex molecular chemical responses to strained conditions. Depending on the stress intensity and duration plants may avoid, tolerate or adapt to external factors. As soon as physiological resistance is surpassed, the plant organism can not further tolerate stress, which may cause greater damage to plant metabolism and consequently cell death. This behaviour can be considered as defence mechanism, evolved in plants as a response to extreme circumstances (Dixon & Paiva, 1995; Namdeo, 2007). The biological stress concept is considered as deviation from plant normal physiological capacity caused by any external factor, which can be of biotic and abiotic nature (Figure 2-7).

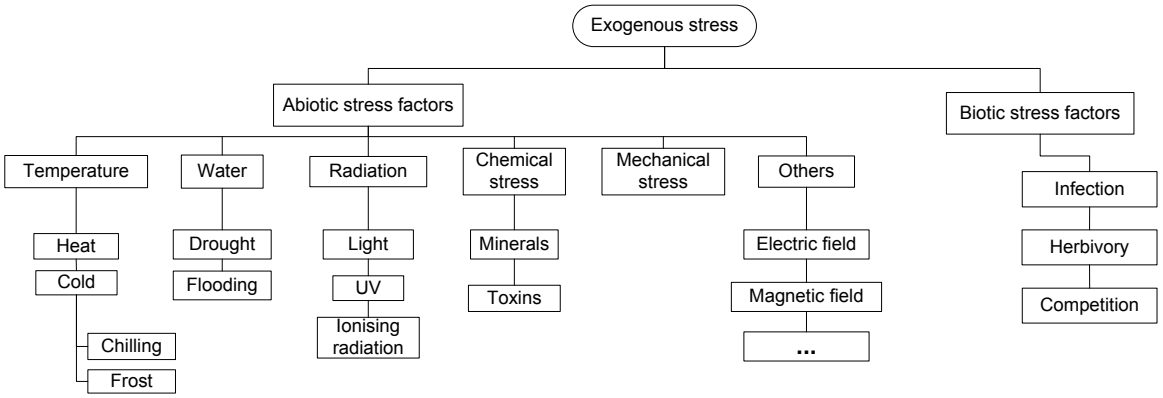


Figure 2-7: Biotic and abiotic external factors (modified after Beck, Schulze & Müller-Hohenstein, 2005).

When the stress is imposed to a biological system the initial reaction of responsiveness is a change in the plant metabolism that occurs within time scale from seconds to days (Gómez Galindo, Sjöholm, Rasmusson, Widell & Kaack, 2007). Induced stress can cause positive (eustress) and negative effects (distress), depending on its intensity, duration and fitness of the plant. Furthermore, variations in sensitivity to stress of different plant species, variety within species, organs of the plants and plants of the same variety grown in different environments, were observed (Basra & Basra, 1997; Munns, 2002).

The biological stress concept developed by Larcher (1987), based on the concepts of Seyle (1936) and Levitt (1980), illustrates plant response through phases which are shewed in Figure 2-8. The early stage of stress event is accompanied by destabilization of functional and structural properties of plant systems, which has been interpreted as an *alarm phase*. There are several outcomes which demonstrate that the plant stress reaction depends on the stress intensity and fitness of the plant. When the stress intensity overcomes a threshold value, the cell integrity collapses before a defence mechanism can be established and acute damage takes place. If the stress is not overcoming a critical value, but exhibits plant tolerance, initial destabilization of functions (in which catabolism predominates over anabolism) can lead to stabilization and repair process can be initiated. Protein synthesis and *de novo* synthesis of protective substances are being quickly induced and *recovery phase* takes place. If the stress is tolerated, plant systems may continue their normal functionality or may increase their *resistance* while maintaining a higher productivity level. After improved stability, normalization can take place, which is resulting in plant adjustment to strained conditions. However, if the biological system can not support a higher productivity level or an additional stressor is imposed to the plant

system, the state of *exhaustion* may take place. When the level of stress or multiple stresses can not be tolerated, chronic damage occurs which may lead to death. Described phase concept of the stress syndrome is showing sequences of events and trends. However, normalization can take place at any time, as well as repeated depletion phase or exhaustion if an additional stressor is imposed (Beck & Lüttge, 1990; Larcher, 2003).

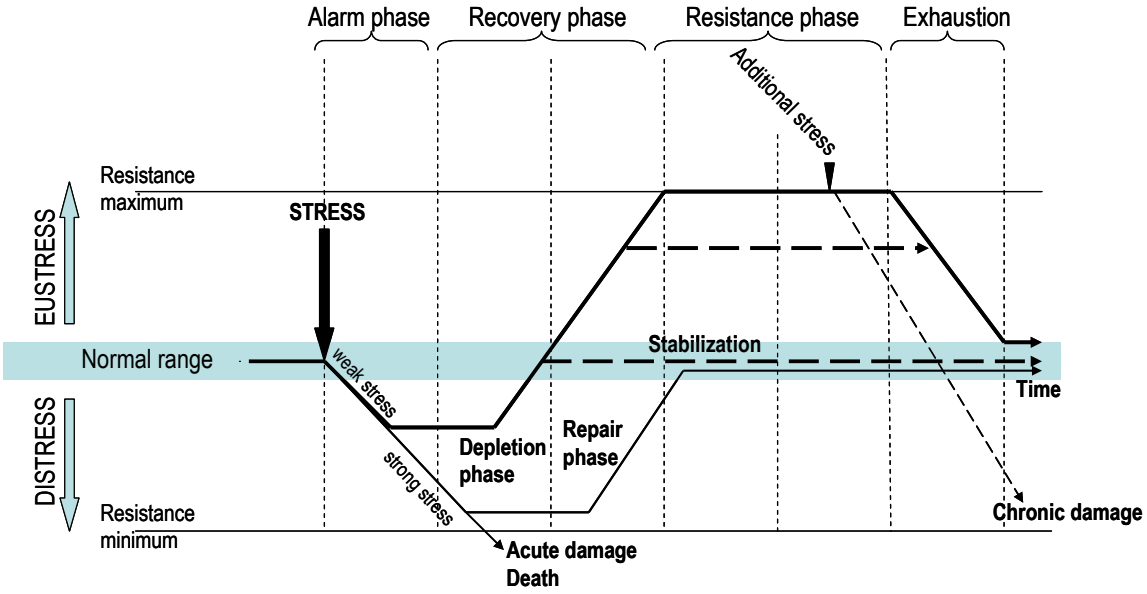


Figure 2-8: Biological stress concept (based on the concepts by Seyle, 1936; Stocker, 1947; modified after Beck & Lüttge, 1990; Larcher, 2003).

External factors trigger a wide range of possible plant responses, which induce changes on different levels: macroscopic, cellular and genetic level. The signal trigger of biotic stress is explained through degradation products that pathogens produce in the cell walls (of host plant or their own), which in most cases trigger a response through phytoalexins synthesis (Beck et al., 2005). Exposure of plants to environmental abiotic stress often results in increased production of free radicals (e.g. reactive oxygen species, ROS and reactive nitrogen species, RNS). Different abiotic factors, such as UV light, heat and cold stress, heavy metals, oxidant forming herbicides, air pollutants (increased amount of ozone or sulphur dioxide), can promote the formation of free radicals (Basra & Basra, 1997). Even though ROS and RNS are regularly formed as a result of normal biological functions; excess of oxidative stress may lead to intoxication and damage to cell organelles, which is caused by an imbalance between prooxidants and antioxidants in the system. Furthermore, ROS may contribute to plant defense response by serving as extracellular signals or might provide superoxide and hydrogen peroxide needed for

lignification and stabilization of the cell wall. ROS have also been suggested as trigger signal required for induced synthesis of polyphenolic compounds (Baker & Orlandi, 1995; Grassmann, Hippeli & Elstner, 2002; Edreva, 2005; Beck et al., 2007).

Physiological effects of abiotic stresses, such as drought, frost and salt lead to a partial dehydration of the cell and destabilization of the membrane structure. Changed ion concentration and water availability (by evaporation or freezing) changes in turn the charges at the surface of membranes and consequently transmembrane potential, thus causes destabilization of biomembranes. Furthermore, multiple stresses may cause multiple stress responses. When plant is exposed to high sun radiation during subfreezing temperature, the energy can not be utilized due to frost dehydration. The photosynthetic apparatus is therefore energized. Such increased energy dissipation via radicals might cause oxidative stress, which can be observed by the pigment destruction (Beck et al., 2005). Multiple responses are not simple reactions, but are involving the joint activity of two or more strategies to avoid and tolerate imposed stress. It is often very difficult to clearly differentiate impact from one another (Mittler, 2006).

Cold stress (low temperatures above the freezing point) causes retardation of plant metabolism and rigidity of the cell membranes. The lipid bilayer is altered due to limited mobility, spanning the membrane and making it difficult to maintain a concentration gradient. Most sensitive metabolic processes effected by cold stress are membrane-bound processes such as ion homeostasis and photosynthesis (Beck et al., 2007). The exact trigger factor for low temperature stress has not jet been fully identified.

Plant organisms react on stress through changes in their metabolism and re-routing of metabolic pathways as discussed above. Phytochemical defence can be observed through cell wall thickening or callose deposition and neutralization of reactive oxygen species (Apostol, Heinstein & Low, 1989; Sandermann, Ernst, Heller & Langebartels, 1998; Mittler, Vanderauwera, Gollery & Van Breusegem, 2004). A large diversity of mechanisms can be triggered in order to produce or release secondary compounds into the surroundings or to start synthesis of proteins and phytochemicals that remain in the cell (Dixon & Paiva, 1995; Larcher, 2003; Chinnusamy, Zhu & Zhu, 2007; Pociecha, Plazek, Janowaik & Zwierzykowski, 2009b).

2.4.1. Secondary metabolites and their role in plant defense

Plant secondary metabolites were for a long period of time regarded as nonessential substances, since they do not have an apparent function in plant growth and development. Although the absence of secondary metabolites does not result in immediate

death, nevertheless as metabolic intermediates they are needed for plant existence. Secondary metabolites play a major role in plant adaption to strained environmental conditions and therefore are today considered to be relevant for plant survival (Larcher, 2003; Edreva, Velikova, Tsonev, Dagnon, Gürel, Aktas et al., 2008).

Polyphenols are secondary plant metabolites generally involved in defensive roles, such as antimicrobial, photoprotective, structure stabilizing and signalling. A large diversity of chemical structures and interactions emphasizes the ability of polyphenols to serve as stress protective substances. They comprise a wide variety of molecules that have a polyphenol structure. According to the number of phenol rings and structural elements that bind rings to one another, polyphenols can be divided into several classes. The main classes of polyphenols are flavonoids (flavanols, anthocyanins and their oxidation products), phenolic acids (mainly caffeic acid), phenolic alcohols, stilbenes and lignans. Involved in stress response, phenolics act as screening agents to harmful ultraviolet irradiation and as potential scavengers of free radicals and other oxidative species (Grace & Logan, 2000). Early events of plant defense are manifested through many different stress-mediating pathways, whereas phenolic compounds, the largest group of plant secondary metabolites are formed through phenylpropanoid pathway (Figures 2-9 and 2-10).

Aromatic amino acids phenylalanine, tyrosine and triptofan are the primary metabolites (synthesized via shikimate pathway) which serve as precursors for many secondary metabolites. Phenylalanine occupies a particular position in the plant metabolism, as a starting material for the synthesis of a large number of aromatic compounds. All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the action of phenylalanine ammonia-lyase (PAL), the branch point enzyme between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism (Dixon & Paiva, 1995; Grace & Logan, 2000; Beck et al., 2005).

The initial steps for synthesis of all phenolic substances are similar and include deamination of phenylalanine to cinnamate, which is transferred to 4-coumarate by cinnamate 4-hydrolase (C4H), followed by synthesis of 4-coumarol-CoA by 4-coumarol-CoA Ligase (4CL) (Figure 2-10). Simple phenylpropanoids which include caffeic, p-coumaric, ferulic, and sinapic acids, rarely accumulate to high levels inside plant cells. They are usually conjugated and can accumulate either as stable sugars and/or organic acids or act as precursors to more complex structures such as flavonoids, tannins and lignin (Dixon & Paiva, 1995).

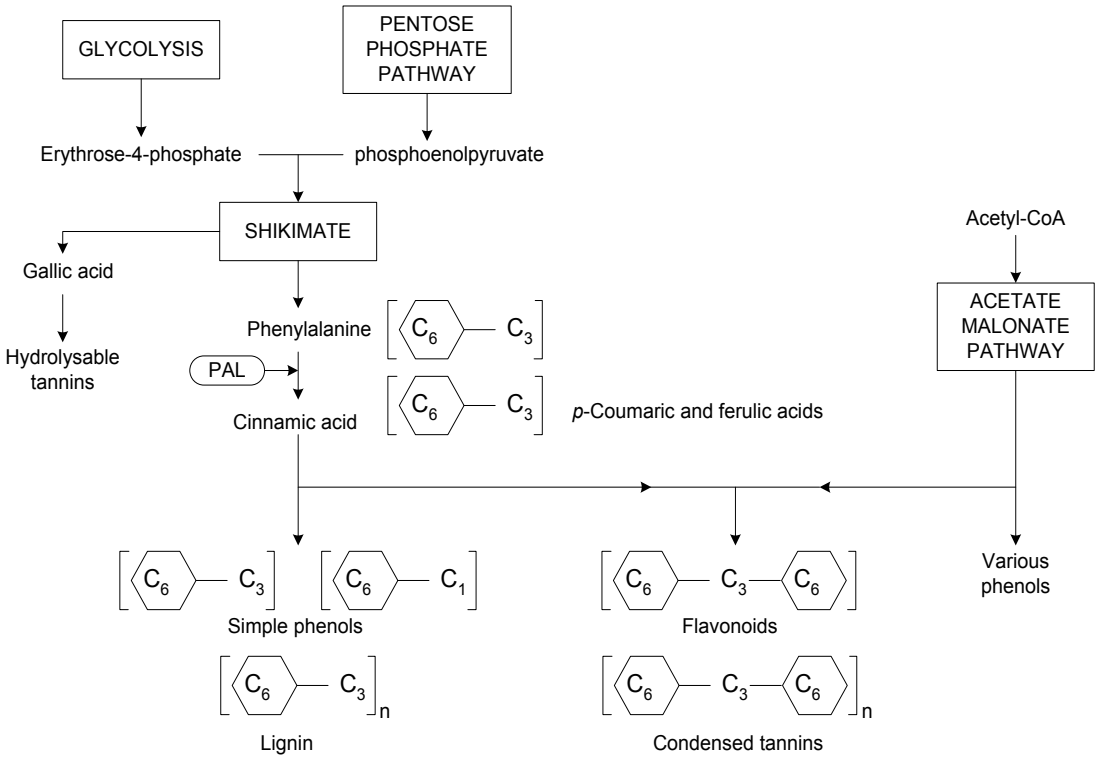


Figure 2-9: Relationship between phenol and phenylpropane biosynthesis in plants (modified after Beck et al., 2005).

The environmental conditions (primary UV radiation) regulate in most plants synthesis of different phenolic compounds. Interaction between plants and the environment have a very elaborate character due to complexity of their biological system. Branch pathways lead to the synthesis of different compounds. These compounds serve specific defense roles in plant tissue, such as cell wall repair and strengthening (e.g. lignin), signaling compounds (e.g. salicylic acid), antimicrobial activity etc. (Gómez-Vásquez, Day, Buschmann, Randless, Beeching & Cooper, 2004). Synthesized phenolic compounds may be further converted into more reactive species by polyphenol oxidases and peroxidases (Mayer & Harel, 1979), which will be discussed in detail in subchapter 2.4.2. Formed flavonoids are distributed in mesophyll (photosynthetic parenchyma cells, located between upper and lower epidermis layers), while anthocyanin accumulation takes place in the vacuoles of the epidermal cells (Beck et al., 2005). Tevini *et al.* (1991) reported that flavonoids accumulate in epidermis of rye seedlings already 4 hours after UV-B radiation.

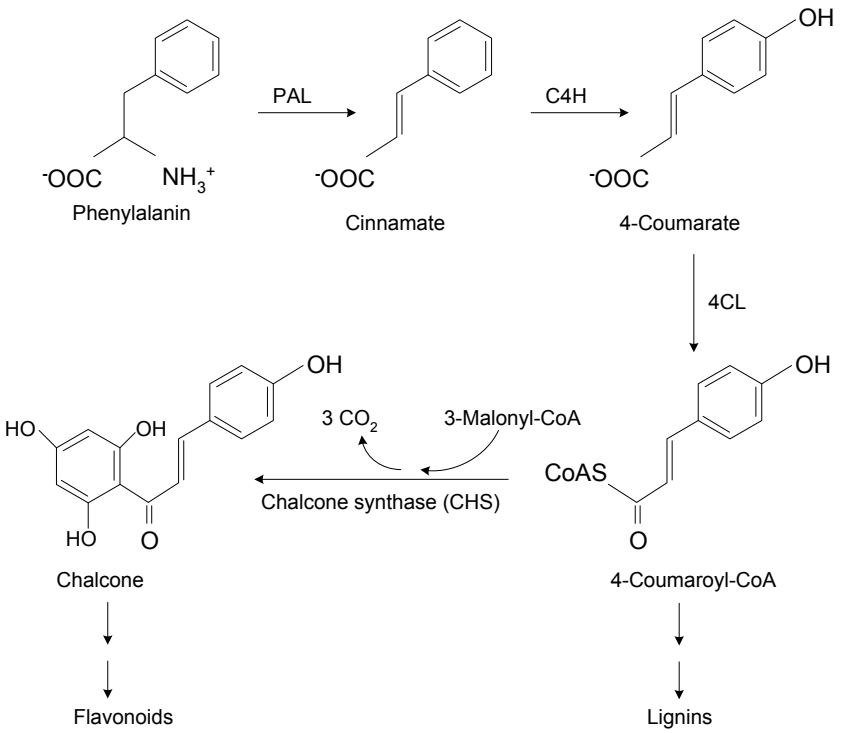


Figure 2-10: Synthesis of phenolic compounds via phenylpropanoid pathway (adopted from Hahlbrock, 1981).

Accumulation of bioactive compounds, pigments, flavours and other low molecular substances, which were recognized as effective antioxidant substances, contribute at certain levels to the nutritional value of food (Tapiero, Tew, Ba & Mathé, 2002; Albarracin, Stab, Casas, Sutachan, Samudio, Gonzalez et al., 2012). Therefore, many of these substances could be required in food, cosmetics and pharmaceutical industry. Examples of different types of stress induced phenylpropanoids are shown in Figure 2-11.

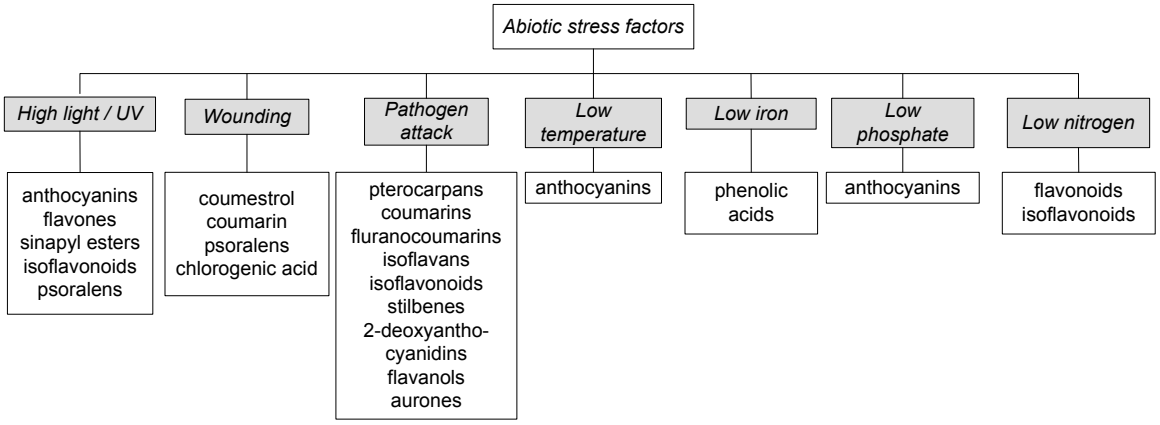


Figure 2-11: Phenylpropanoids induced by diverse environmental stressors (modified after Dixon & Paiva, 1995).

Highly reactive free radicals and oxygen species are present in all biological systems or may even be additionally produced during normal cell aerobic respiration in

organism. The main characteristic of antioxidants is their ability to quench free radicals and thus inhibit the oxidative mechanisms that lead to degenerative diseases. Antioxidant defence in human body may be of endogenous (enzymatic and nonenzymatic) or dietary origin (Harman, 1995). A number of clinical and epidemiological studies have demonstrated that dietary antioxidants help to maintain adequate antioxidant status in living organisms (Halliwell, Murcia, Chirico & Aruoma, 1995; Kohlmeier, Simonsen & Mohus, 1995; Steinmetz & Potter, 1996; Hininger, Chopra, Thurnham, Laporte, Richard, Favier et al., 1997; Ness & Powles, 1997; Conklin, 2000; Borek, 2004; Christensen, Naidu, Parent, Pintos, Abrahamowitz, Siemiatycki et al., 2012).

Polyphenols are the most abundant antioxidants in human diet. Due to high antioxidative capacity they have been recognized in prevention against human degenerative diseases including cancer, coronary heart diseases and neurodegenerative disorders such as Alzheimer's disease (Franke, Cooney, Henning & Custer, 2005; Kwon, Choi, Jeong, Kang, Kang, Lim et al., 2005; Riso, Visioli, Gardana, Grande, Brusamolino, Galvano et al., 2005; Villano, Fernandez-Pachon, Troncoso & Garcia-Parrilla, 2005; Pezzuto, 2008; Ghasemzadeh & Ghasemzadeh, 2011). Fruits and vegetables are commonly eaten and are therefore large contributors of polyphenol compounds in human diets. Despite their wide distribution in plant kingdom and human diet, the health benefits of dietary polyphenols have attracted larger attention only in recent years. Some of the main causes for delayed research are the variety and complexity of their chemical structure and interplay mechanisms contributing to their protective effects (D`Archivio, Filesi, Di Benedetto, Gargiulo, Giovannini & Masella, 2007).

2.4.2. Enzymes and their role in plant defense

Reaction rate and enzyme activity can be affected by various parameters such as temperature, chemical environment (e.g. pH), other molecules and the concentration of substrate and product. Increasing the temperature of the system results in increase of kinetic energy brought to the reacting molecules and the number of successful collisions between substrate and enzyme active site will increase. Consequently, enzyme activity will rise until the optimum temperature is reached and with further increase in temperature onwards it will start to decrease. An extreme change in temperature will cause enzyme denaturation. Changes in pH are affecting the shape or charge of enzyme active sites, but may also change the shape or charge of substrate. Thus, every enzyme demonstrates highest activity at optimal pH value. Above or below the optimum pH, enzyme activity decreases. Substrate concentration may affect enzyme activity to a certain level. By

increasing concentration of the substrate (enzyme concentration remains constant) the enzyme activity will be increased up to the maximal extent of enzyme capabilities. Since all active sites are occupied with substrate, from this point on the enzyme activity remains constant. Furthermore, presence of certain molecules might inhibit enzyme activity by occupying the active sites and preventing it from binding with a substrate (Nelson & Cox, 2005).

Enzymes are involved in the cell metabolism as defense enzymes to different abiotic stresses (Rivero et al., 2001; Beck et al., 2005). Being responsible for the specificity of metabolic reactions including DNA synthesis, changes in the enzyme activity and their *de novo* synthesis takes place as a result of gene expression for resistance (Bohnert, Nelson & Jensen, 1995; Ho et al., 1997; Van Loey et al., 2002; Beck et al., 2005).

Production of polyphenols requires synthesis and/or availability of respective enzymes. Phenylalanine ammonia-lyase (PAL) catalyses the first step of phenylpropanoid pathway and has been extensively studied due to its role in plant development and due to changes in PAL activity as a response to a wide variety of environmental stimuli (Jones, 1984; Lange, Lapierre & Sandermann, 1995; Basra & Basra, 1997; Rivero et al., 2001). Levels of PAL activity vary with the developmental stage of the cells and tissues and increased activity has been correlated to increased production of phenylpropanoid products (Jones, 1984; Ozeki & Komamine, 1985; Lois, Dietrich, Hahlbrock & Schulz, 1989). *De novo* synthesis of enzymes included in the phenylpropanoid pathway together with flavonoids production was established after UV radiation of cultured parsley cells (Hahlbrock & Scheel, 1989). Therefore, PAL together with polyphenols has been proposed to serve as a marker of induced plant resistance to different external factors.

Polyphenol oxidase (PPO), also frequently referred to as tyrosinase, phenolase or catecholoxidase, is an oxido-reductive enzyme that catalyses under the presence of atmospheric oxygen the hydroxylation of mono-phenols to di-phenols as well as the oxidation of di-phenols to di-quinones. Quinones are highly active and react with enzymes and other proteins, or may polymerize into black, brown or red pigments (Dixon & Paiva, 1995; Mayer, 2006; Rapeanu, Van Loey, Smout & Hendrickx, 2006). Since PPO is an intracellular enzyme probably stored in plastids and polyphenolic substances are stored in vacuoles or bound to polysaccharides in the cell wall (Basra & Basra, 1997; Mayer, 2006), enzymatic browning occurs only when enzyme and substrate come in contact after cell injury. Enzymatic browning is an unwanted process in fresh fruits and

vegetables, and therefore inhibition of PPO was studied by many scientists. PPO is a copper-containing enzyme and according to Janovitz-Klapp *et al.* (1990) can be inhibited by compounds that affect the active site for the phenolic substrate and compounds that interact with the copper in the enzyme. Furthermore, different phenolic substances can inhibit PPO due to their structural similarities (Mayer, 2006).

Plant peroxidase (POD) is as well as PPO an oxido-reductive enzyme, which catalyzes oxidation of diverse organic and inorganic substances through a reaction of the form: ROOR' + electron donor (2 e^-) + $2H^+$ → ROH + R'OH, with hydrogen peroxide as a substrate. Higher plants have a number of peroxidase isoenzymes, which roles in plant defense include phenol oxidation, lignification, cross-linking of polysaccharides, inhibition of pathogen growth or can generate active oxygen species as a part of oxidative burst (Gómez-Vásquez et al., 2004).

The activities of oxidizing enzymes, PPO and POD, are being altered in response to various types of biotic and abiotic stresses (Thipyapong, Hunt & Steffens, 1995; Kwak, Kim, Park & Lui, 1996; Rivero et al., 2001; Schnablová, Synková, Vicánková, Burketová, Eder & Cvikrová, 2006; Thipyapong et al., 2007). Thipyapong *et al.* (1995) reported 1.7 fold increase in PPO activity within 48 hours after wounding. Increase in PPO activity was accompanied by comparable increase in PPOs and PPO specific mRNA. Furthermore, authors suggested that only developmentally competent tissues were capable of responding to stress signal. Gómez-Vásquez *et al.* (2004) reported oxidative burst in elicited cells, which started already after 2-3 minutes and reached peak value between 5 and 35 minutes after elicitation. Changes in enzymatic activities followed, and 4 fold increase in POD activity was observed 48 h post-elicitation. PAL mRNA accumulation was observed with peak value 9 hours after the elicitation, which preceded induction of PAL activity that reached maximum 15 hours after elicitation.

Inhibition of PPO and POD activity was observed during plant response to thermal stress (Rivero et al., 2001). The authors reported activation of phenol biosynthesis observed through accumulation of soluble phenolics and highest PAL activity, simultaneously with inhibition of polyphenol oxidation observed through lowest PPO and POD activity.

2.5. Postharvest storage of fruits

Biochemical components of different fruits are influenced by environmental and genetic factors during plant development as well as after the harvest. For a long period of

time it was considered that biochemical transformations which occur during postharvest storage are consequence of disorganization and de-compartmentation of the cellular compounds. However, later on it has been recognized that postharvest biochemical transformations comprises both catabolic and anabolic reactions (Haard, 1984). Although the fruit is being detached from the plant (as the nutrient source), postharvest metabolism continues to occur (Kalt, Forney, Martin & Prior, 1999). Primary and secondary metabolites are being synthesized during postharvest storage simultaneously with other degradative changes as a consequence of plant normal postharvest metabolism. Degradative and synthetic metabolic changes that occur during postharvest storage of different fruits and vegetables are presented in Table 2-2.

Table 2-2: Postharvest metabolic changes in fruits and vegetables (adopted from Haard, 1984)

Degradative	*Synthetic*
Destruction of chloroplast	Anthocyanins and carotenoids synthesis
Breakdown of chlorophyll	Flavor volatiles synthesis
Starch hydrolysis	Starch synthesis
Organic acid catabolism	Lignin synthesis
Oxidation of substrate	Preservation of selective membranes
Inactivation by phenolic compounds	Interconversions of sugars
Pectin hydrolysis	Protein synthesis
Breakdown of biological membranes	Gene transcription
Cell wall softening	Formation of ethylene biosynthesis pathway

Life history of each fruit passes following stages: cell division, cell enlargement, maturation and senescence. Some fruits like apple, tomato, banana, avocado, peach, pear etc. undergo a rise in respiration associated with the ripening process which is termed *climacteric.* However, rise of respiration is not universal for all types of fruits. Hence grape, blueberry, orange, cherry, lime etc. exhibit relatively low changes in respiration and are classified as *non-climacteric* fruits. Respiratory rise may also occur during stress, however, a climacteric is co-occurring only with fruit ripening process, providing the energy necessary for cells to maintain structure and color and flavor development (Biale, 1950; Jones, Hulme & Wooltorton, 1964).

Fruits continue to respire after harvest, which plays a mayor role in postharvest life of fresh commodities. Respiration reflects overall metabolic activity of the tissue and includes the loss of substrate, the synthesis of new compounds and the release of thermal energy. In order to prevent deteriorative processes and other metabolic reactions associated with quality retention, the major part of postharvest technology is devoted to alterations of physical and chemical characteristics of fruits by manipulation of the external environment. Reducing respiration rate by storage at low temperatures, low

oxygen level and increased carbon dioxide level are some of most often used conditions to prolong storage life. Furthermore, different ethylene inhibitors or action blockers can be used together with controlled atmosphere storage, and selectively permeable packaging which uses fruit own respiration to maintain a modified atmosphere (Beaudry, 1999).

Other types of abiotic stresses may slow down or promote respiration, or may trigger a wide range of metabolic reactions leading to polyphenolic accumulation in plant tissue (Haard, 1984; Cisneros-Zevallos, 2003; Reyes & Cisneros-Zevallos, 2003; Saltveit, Choi & Tomas-Barberan, 2005). For some commodities, physical stress during postharvest storage can be utilized to prevent quality reduction or to promote changes that increase certain quality aspects. For that reason tomatoes and bananas are being harvested before desirable quality is reached. In optimized storage conditions synthesis of pigments and volatiles (amyl esters in bananas; lycopene in tomato), conversion of starch to sugar (sweetening of bananas) and chlorophyll loss (chlorophyll degrading enzymes) occur, bringing the commodities to optimal quality (Marriott & Palmer, 2009).

Internal factors are affected by external postharvest stress. Most aspects of biochemical changes in postharvest physiology related to secondary metabolites are not easy to be traced due to complexity of plant responses and variability of plant tissue. However, several groups reported the use of controlled stresses to enhance the accumulation of secondary metabolites (Cantos, García-Viguera, Pascual-Teresa & Tomás-Barberán, 2000; Cisneros-Zevallos, 2003; Padda & Picha, 2008; Ruiz-García & Gómez-Plaza, 2013).

Temperature stress has been reported to influence biosynthesis of phenylpropanoid compounds (Reyes & Cisneros-Zevallos, 2003; Hodges & Toivonen, 2008; Padda & Picha, 2008). After 4 weeks of low temperature storage, sweetpotatoe exposure to ambient temperature (~22 °C) for 3 days resulted in accelerated buildup of phenolic compounds (Padda & Picha, 2008). During storage of purple-flesh potato for 14 days at storage temperatures of 2, 10 and 20 °C, no significant changes of total anthocyanins and total polyphenolics were noticed (Reyes & Cisneros-Zevallos, 2003). However, wounding induced increase in PAL activity and phenolic accumulation in sliced potato tissue. Furthermore, larger PAL activity and larger increase in phenolic content was observed in potato flesh than in peel.

To determine the effect of storage temperature on anthocyanin, total polyphenol and ascorbic acid content and total antioxidant capacity, small fruit crops (strawberry, raspberry and blueberry) were stored at 0, 10, 20 and 30 °C for 8 days (Kalt et al., 1999).

The storage at or above ambient temperature was shown to affect polyphenolic metabolism, since larger concentrations of total polyphenolics and total anthocyanins were observed in fruits during storage at temperatures higher than 0 °C. The authors reported 1.7 fold increase in anthocyanin content of strawberries stored for 8 days at 0 °C, and 6.8 fold increase when stored for the same period at 30 °C. Furthermore, polyphenolic and anthocyanin concentrations were correlated with antioxidant capacity of tested fruits. The change in phenolic accumulation has been also reported when jicama roots were after two weeks storage at 10 °C transferred to higher storage temperatures (Cantwell, Peiser & Mercado-Silva, 2002).

Ultraviolet (UV) irradiation, as a postharvest treatment of different fruit commodities, has been shown to act as abiotic elicitor which triggers the resistance mechanism in plant systems, leading to an increase of stress-response compounds (Schreiner & Huyskens-Keil, 2006). Increased anthocyanin levels in apples, strawberries and mango were detected after UV irradiation (Dong, Mitra, Kootstra, Lister & Lancaster, 1995; Higashio, Hirokane, Sato, Tokuda & Uragami, 2005; González-Aguilar, Zavaleta-Gatica & Tiznado-Hernández, 2007). The increased anthocyanin concentration in apple skin was found to be dose dependant, and due to ten- to twenty-fold increase in enzymatic activities of PAL and chalcones isomerase, and an increase in PAL transcript, *de novo* synthesis was suggested to be triggered by UV irradiation (Dong et al., 1995). This effect was found in grapes as well. Controlled UV-C irradiation was used to increase polyphenol content in red table and wine grapes (Cantos et al., 2000; Cantos, Espín & Tomas-Barberan, 2002; Cantos, Espín & Fernandes, 2003; Takayanagi, Okuda, Mine & Yokotsuka, 2004; Crupi, Pichierri, Basile & Antonacci, 2013). Cantos et al. (2000) reported three-fold increase in resveratrol after postharvest treatment with UV-C light. The authors suggested that refrigerated storage together with UV irradiation of table grapes can be beneficial in terms of increasing the content of potentially health-promoting phenolics. The evolution of resveratrol during the traditional wine making process (including maceration step) was followed after UV-C irradiation of wine grapes (Cantos et al., 2002). The final wine obtained from UV-C irradiated grapes contained two-fold larger resveratrol content.

Different types of fresh produce were submitted to postharvest stress by wounding and changes in total polyphenolics, total anthocyanins, total carotenoids, ascorbic acid and antioxidant capacity were evaluated (Reyes, Villarreal & Cisneros-Zevallos, 2007). PAL activity increased six- to seventy three-fold after wounding of all types of tissue, whereas,

phenolic changes ranged from 26 % decrease to 191 % increase after 2 days storage at 15 °C (Table 2-3).

Table 2-3: Changes of total polyphenol content in various fresh produce due to postharvest stress by wounding (Reyes et al., 2007)

Fresh produce	***Carrot***	***Lettuce***	***Celery***	***Sweet potato***	***Parsnips***
TP increase	191 %	81 %	30 %	17 %	13 %
Fresh produce	***Zucchini***	***Potato***	***Red cabbage***		***Radish***
TP decrease	26 %	15 %	9 %		7 %
Fresh produce	***White cabbage*** No significant changes				

Wounding of iceberg lettuce leaves induced six- to twelve-fold increase in PAL activity within 24 h at 10 °C and three-fold increase in the phenolic content within 3 days after injury (Saltveit, 2000). Similar response has been reported in minimally processed lettuce leaves through increased phenolic content after wound induction of PAL (Gegl`Innocenti, Guidi, Pardossi & Tognoni, 2005). Furthermore, Saltveit *et al*. (2005) implied the action of a wound signal at the site of injury, through phospholipid-signaling pathway, which induced increased PAL activity and phenylpropanoid metabolism.

One of the main goals of postharvest technology is preservation of harvested commodities and reduction of metabolic reactions associated with quality deteriorations. However, controlled stress offers a valuable tool to enhance the health benefit properties of fresh commodities, or to enhance extractable nutraceutical yields. Many factors were found to affect plant commodities response to various induction processes. Differences in stress reactions between fruits and cultivars were detected (Haard, 1984; Kalt et al., 1999; Hodges & Toivonen, 2008). Fruit maturity was found to influence its response to abiotic stress as well, thus emphasizing the fact that stress tolerance changes with stage of the plant life cycle (Hodges & Toivonen, 2008; Romero, Caballero, Sanchez-Ballesta, Escribano & Merodio, 2009). Furthermore, multiple stresses may cause a different response than each by itself, or the stressed tissue may gain cross-protection against other stresses (Saltveit, 2000; Cisneros-Zevallos, 2003; Saltveit et al., 2005). Therefore, to achieve controlled stress induction, many variables should be considered.

2.6. Plant cell and tissue cultures

Higher plants are a valuable source of many ingredients with high nutritional value and are an important part of human diet. They are also a source of bioactive constituents or phyto-pharmaceuticals used in pharmaceutical industry (Namdeo, 2007). Many of them are difficult to cultivate, since growth relies on environmental conditions (climate, soil

composition etc.), and therefore great efforts are made to produce valuable substances independently from plants. Cultivation of plant cells in cultures represents a useful biotechnological tool to improve accessibility and production of desirable metabolites (Dörnenburg & Knorr, 1995; Vasconsuelo & Boland, 2007).

In vitro grown cells (biosynthetically totipotent) can be cultivated from nearly every part of the plant (Rao & Ravishankar, 2002). Complete genetic information is retained in cultured cells and therefore they are suitable for studying tissue specific biosynthetic pathways. Various techniques are used to grow cells and tissue cultures under aseptic conditions on a solid (callus culture) or liquid nutrient medium (cell suspension culture) (Constable & Shyluk, 1994). Callus segments represent all forms of cells and tissues which are regenerated by already differentiated cells, being initiated from cut sterilized surface of plant tissue. The explants are placed on solid growth media and in the course of two to six weeks callus tissue will appear on the explants (Mustafa, de Winter, van Iren & Verpoorte, 2011). Callus material can be subsequently inoculated in liquid medium and during constant agitation in suitable growth medium, cell suspension cultures are obtained. The cells are in suspension culture completely surrounded with medium, which maintains defined chemical and physical conditions required for desirable nutrient transport. In such a controlled environment, generation of the cells and cell components occurs more rapidly than in callus culture, which makes it more susceptible for large scale applications (Rao & Ravishankar, 2002). Cell growth in suspension cultures passes through adaptation phase to new environmental conditions and stabilization in the new regime, which may take 6 to 9 months (Mustafa et al., 2011). Cells are being further subcultivated in fresh medium in regular intervals. Growth parameters are being measured in order to attain reproducible growth cycles of cell culture batches with optimal cell viability, biomass level, protein, RNAs and secondary metabolite production.

During the growth cycle, cells pass several development phases (Stepan-Sarkissian & Grey, 1990). After inoculation into a new nutrient medium, a short incubation time is necessary for adaptation to new environmental conditions. During that time (*lag phase*) the cells will regain the ability to divide and the culture will grow slowly and prepare for the next phase (Figure 2-12). In succession, a period of fast cell proliferation follows (*log* or *exponential phase*), where the cells utilize the nutrients from the growth medium, until factors in the culture environment (volume and nutrients) become limiting. At that point cell division declines (*linear* and *progressive deceleration phase*) and eventually ceases.

Cell wall material is synthesized during the linear phase from available carbohydrate and while passing into the progressive deceleration phase and entering the *stationary phase* the cells metabolize all available carbohydrates (Stepan-Sarkissian & Grey, 1990). In the stationary phase cell proliferation is reduced or ceases entirely and cell dry weight declines. The energy in the cells may be utilized by other metabolic pathways, e.g. for production of secondary metabolites (Chawla, 2002; Mustafa et al., 2011). The stability of cultured cells in the stationary phase varies between the species, and the cells may remain viable for days by utilizing intracellular reserves (Chawla, 2002).

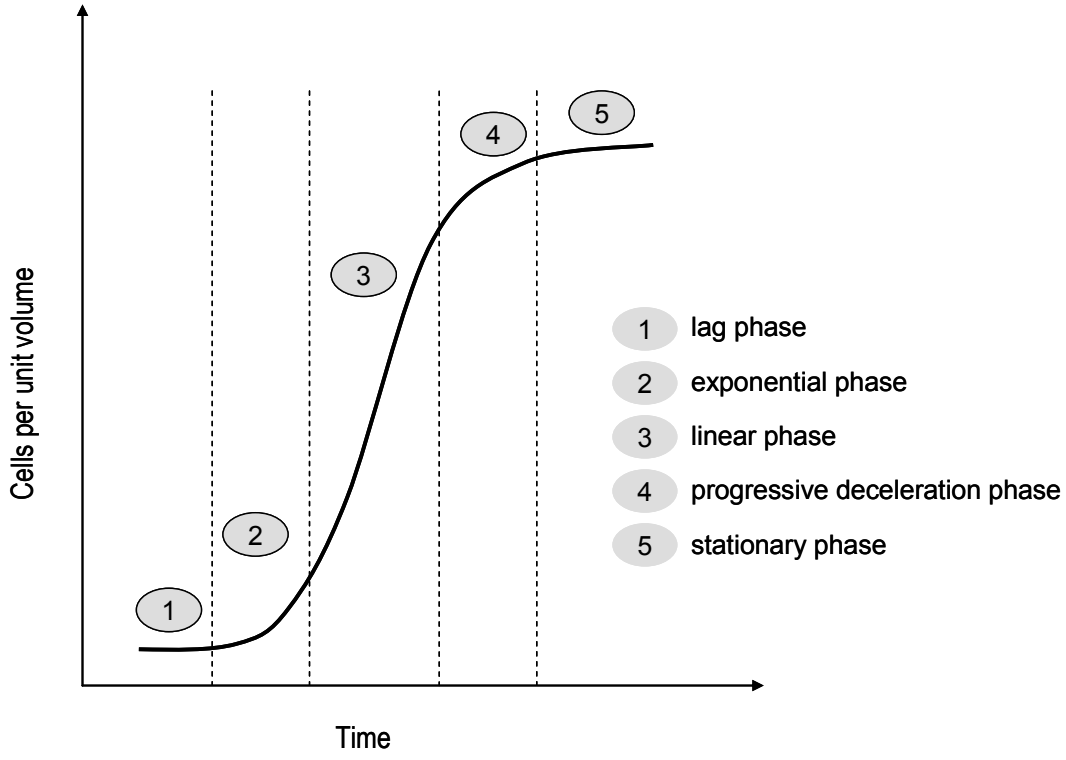


Figure 2-12: Model curve representing growth phases in plant cell suspension cultures.

The cell culture and its metabolic processes are being stabilized after several subcultivation cycles. However, the developmental state of the cells in suspension cultures changes during subcultivation for a longer period of time, which represent one of the challenges concerning long term maintenance of suitable cell lines.

Cultivation of plant cell and tissue cultures in controlled environment offers a wide range of research possibilities of cellular and molecular processes, since they maintain parent plants metabolic activities and offer a simplified model system.

2.6.1. Plant cell culture as source of secondary metabolites

The ability of higher plants to synthesize secondary metabolites is transferred into cell cultures, which offers an alternative way for production of this highly valuable substances (Dörnenburg & Knorr, 1995; 1997a; Rao & Ravishankar, 2002). Secondary metabolites are used as food additives, pigments, fragrances, pesticides and drugs, and about half pharmaceuticals in use today are derived from natural products (Sasson, 1992; Namdeo, 2007). In the past two decades, production of plant metabolites from cell suspension cultures has attracted large interest within the field of biotechnology. Being independent on geographical and seasonal variations, having fast life cycle (one to four weeks), *in vitro* grown cultures offer targeted synthesis of desired products. For certain compounds of interest (anthocyanins, berberines, betacyanins, ginseng, shikonin etc.), cell culture cultivation became a feasible process, allowing sustainable production of secondary metabolites (Verpoorte, van der Heijden, Hoge & ten Hoopen, 1994; Wilson & Roberts, 2012). However several constraints for regular commercial application on industrial scale are present, including instability of cell lines over longer period of time in large bioreactors, variable yields of metabolite accumulation, and insufficient cognition of biosynthetic routes and enzymology (Wilson & Roberts, 2012).

Improvement of metabolite yields and controlling variability in product accumulation can be achieved through strain improvement (selection and screening of parent plant and/or gene transformation and metabolic engineering), change of medium and growth conditions, and through specialized elicitation techniques. Similar to plants in nature (see subchapter 2.4), cell cultures respond to different biotic and abiotic elicitors which activate secondary pathways and stimulate secondary metabolites as a response to stress (Rao & Ravishankar, 2002; Vasconsuelo & Boland, 2007). Most adequate time to impose biotic or abiotic stress was proposed to be during the exponential phase of growth, when enzyme activity holds highest level (Vasconsuelo & Boland, 2007). Although intensive research on the production of secondary metabolites due to different stresses has been made, the exact mechanism of elicitation is poorly understood. Changes in osmotic balance, inhibition and/or activation of intracellular pathways, pH change, production of ROS and other factors affecting membrane integrity have been proposed to trigger metabolite accumulation (Bolwell, Buti, Davies & Zimmerlin, 1995; Gelli, Higgins & Blumwald, 1997; Pugin, Frachisse, Tavernier, Bligny, Gout, Douce et al., 1997; Yang, Yu, January & January, 1997; Droillard, Thibivilliers, Cazale, Barbier-Brygoo & Lauriere, 2000). Due to the lack of understanding of trigger pathways of secondary

metabolite synthesis, many biotechnological strategies have been hypothesized and explained by the empirical nature of accumulated compounds.

The production of these compounds depends greatly on the physiological and developmental stage of the plant as well as upon intensity and duration of the imposed stress (Namdeo, 2007; Vasconsuelo & Boland, 2007). The effective dosage of a certain elicitor which produces maximal accumulation of secondary metabolites is characteristic for each plant species (Dörnenburg & Knorr, 1993; Vasconsuelo & Boland, 2007).

In the last two decades attempts to provoke accumulation of secondary metabolites with application of novel technologies such as pulsed electric field (see subchapter 2.3.1), high pressure (HP) and ultrasound (US) treatment, have been made (Dörnenburg & Knorr, 1993; 1997b; Dörnenburg & Knorr, 1998; Wu & Lin, 2002; Cai, Riedel, Thaw Saw, Kütük, Mewis, Jäger et al., 2011; Cai, Riedel, Thaw Saw, Mewis, Reineke, Knorr et al., 2011; Gueven & Knorr, 2011). Dörnenburg and Knorr (1993) investigated recovery of secondary metabolites from cultured plant tissues (*Chenopodium rubrum* and *Morinda citrifolia*) after low intensity PEF and HP treatments in correlation with cell vitality. Increased enzyme activity followed by an increased polyphenol concentration in *Vitis vinifera* and *Solanum tuberosum* cell suspension cultures was reported after HP treatment (Dörnenburg & Knorr, 1997b; 1998). Gaining valuable substances from cultured cells requires an extraction process, whereas permeabilization of the cell membrane could elevate the availability of desired compounds. Therefore, processing techniques that are able to attain permeabilization of the cell membrane may not only improve the yield of desired compounds, but may also allow a release of secondary metabolites from the cell.

3. Materials and Methods

3.1. Raw materials

Apple variety Golden Delicious (*Malus domestica* cv. Golden delicious) was obtained after 3 months postharvest ultra-low-oxygen-atmosphere storage from a local fruit and vegetable supplier (Berlin, Germany). The raw material was stored at 4 °C within a week of delivery before further processing.

Cultivated berries (blueberry, *Vaccinium corymbosum* and red currant, *Ribes rubrum*) were obtained from a local supermarket from different commercial cultivars grown in Germany, France and Chile (harvest season 2006). The raw material was processed immediately or stored at 4 °C for 48 h before further processing.

Red grape variety (*Vitis vinifera*) cultivar Spät Burgunder, vintage 2005, was obtained directly after harvest from the Sächsische Winzergenosenschaft Meissen e.G. (Meissen, Germany). The raw material has been stored at 4 °C for 1 to 4 days before further processing.

Red grape variety (*Vitis vinifera*) cultivar Dornfelder, vintage 2006, obtained directly from the wine-growers (Weinbauverband Sachsen, Germany) were manually harvested and grape clusters were stored at 4 °C for 1 to 6 days before further processing.

3.2. Plant cell cultures

Malus domestica suspension culture was established from callus culture *Malus domestica* Borkh. cv. Cox Orange, family *Rosaceae*, obtained from Leibnitz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany). Cultivation was performed on LS basal medium (Linsmaier & Skoog Medium, Duchefa B.V. Netherlands). Subcultivation of plant cell suspension took place in cycles of seven days. 5 g cells (fresh weight) were inoculated under sterile conditions in 200 ml Erlenmeyer flasks containing 60 ml LS medium. Suspension cultures were maintained at 25 ± 0.2 °C in the dark on an orbital shaker at 100 rpm. Growth cycle characteristics of cell suspension were observed during 10 days cultivation. Changes in fresh and dry weight (determined according to §64 LFBG, previous §35 LMBG), pH (Knick Digital-pH-Meter), conductivity (conductivity meter, WTW Cond 3110), enzyme activity (subchapter 3.3.3), total polyphenol concentration (subchapter 3.3.1) and cell viability (subchapter 3.3.4) were investigated. The cells were for each analysis harvested on day 0,

2, 3, 4, 5, 6, 7, 8, 9 and 10 after inoculation, using a vacuum pump (AEG AMEB 90SY 4R3) for one minute.

Vitis vinifera L. suspension culture was established from callus culture *Vitis vinifera c.v. Muscat de Frontignan*, family *Vitaceae*, obtained from Agriculture et Agroalimentaire Canada (Québec, Canada). Cultivation was performed on B5VIT medium (Gamborg B5 medium, Duchefa B.V. Netherlands), and cells were subcultured in periods of fourteenth days. For each new growth cycle, 5 g cells (fresh weight) were inoculated under sterile conditions in 500 ml Erlenmeyer flasks containing 100 ml of B5VIT medium. To assure homogeneity of the cell culture, only representative cells with deep red colour, from clear culture medium and without visible aggregates, were chosen for further inoculation. The cells were kept at 25 ± 0.2 °C under permanent illumination (fluorescent lamp of approx. 3000 lux) on an orbital shaker at 100 rpm. Growth cycle characteristics were investigated through changes in fresh and dry weight (determined according to §64 LFBG, previous §35 LMBG), pH (Knick Digital-pH-Meter), conductivity (conductivity meter, WTW Cond 3110), enzyme activity (subchapter 3.3.3), total polyphenol concentration (subchapter 3.3.1), anthocyanin concentration (subchapter 3.3.2) and cell viability (subchapter 3.3.4). The cells were for each analysis harvested using a vacuum filtration pump for one minute, on day 2, 4, 6, 8, 9, 10, 11, 12, 13 and 14 after inoculation.

3.3. Analytical methods

3.3.1. Determination of Total Polyphenol (TP) concentration

3.3.1.1. TP extraction

***Malus domestica*, apple variety Golden Delicious**

The apples were washed, cut in quarters and the apple core was taken out. Apple tissue was homogenized with an Ultra Turrax T 25 (IKA- Labortechnik, Janke & Kunke, Staufen, Germany) for 60 seconds and 5 g of homogenized mash was suspended in 7 ml (5 g) of 96 % undenatured ethanol (Merck, Darmstadt, Germany) containing 1 M hydrochloric acid (Merck, Darmstadt, Germany). After 60 minutes of extraction under constant agitation (100 rpm), solution was filtered through Whatman No. 1 paper and used for TP determination (subchapter 3.3.1.2).

To obtain fresh apple juice a customary juicer (Design Juicer advanced, Gastroback) was used. Randomly chosen apple quarters (without core) were placed in the juicer. The juice

was filtered through Whatman No. 1 paper and directly used for TP determination (subchapter 3.3.1.2). Extraction of remaining apple residue (5 g fresh weight) was performed in 7 ml (5 g) of 96 % undenatured ethanol containing 1 M hydrochloric acid, for 60 minutes under constant agitation (100 rpm). The solution was filtered through Whatman No. 1 paper and used for TP determination according to Folin-Ciocalteau assay (subchapter 3.3.1.2). Each extraction was performed in triplicate.

***Vaccinium corymbosum*, blueberries and *Ribes rubrum*, red currant**

For the extraction of total polyphenolics, 50 g of berries were manually mashed to separate juice and pulp from the skin through a sieve with pore diameter of 2 mm for blueberries and 1 mm for red currant. Separation of pulp from the juice was done by centrifugation (Sorvall SS 34) at 10 °C and 7800 *g* for 10 minutes. TP concentration was determined from collected supernatant fraction according to Folin-Ciocalteau assay (subchapter 3.3.1.2). Each extraction was performed in triplicate.

***Vitis vinifera*, red grape variety**

Total polyphenol concentration was determined in grape juice and grape residue extracts obtained after pressing (subchapters 3.4.2.3 and 3.4.2.4). Grape residue was weighed and extracted with deionized water, to avoid disintegration of cellular material with strong organic solvents. Residue and extracting agent were mixed at a ratio of 1:4 and placed on rotary shaker (100 rpm) for 2 hours at ambient temperature, protected from light. Fresh grape juice and residue extracts were filtered with Whatman paper No. 1 to remove insoluble solids and TP concentration was determined according to Folin-Ciocalteau assay (subchapter 3.3.1.2). Each extraction was performed in triplicate.

Plant cell suspension cultures

To obtain total polyphenol extracts, the method described by Bligh and Dyer, (1959) modified by Galinski, (1986) was used. The harvested plant cells were frozen at -20 °C and lyophilized (Leybold- Heraeus® GT2). 10 mg and 15 mg of freeze dried *Vitis vinifera* and *Malus domestica* cells, respectively, were suspended in 500 µL of Bligh and Dyer solution (monophasic mixture of methanol:chloroform:water = 10:5:4). The solution was shaken for 30 minutes at room temperature in micro tube shaker (Eppendorf mixer 5432). Addition of 130 µl chloroform (Merck, Darmstadt, Germany) and 130 µl bidest water followed and suspension was placed on micro tube shaker for another 15 minutes before centrifugation (Heraeus biofuge pico, Osterode, Germany) at 9800 *g* for 5 minutes. Each extraction was performed in triplicate. Total polyphenol concentration was determined from methanol-water phase according to Folin-Ciocalteau (subchapter 3.3.1.2).

3.3.1.2. Folin-Ciocalteau assay

The total polyphenol concentration was measured according to Folin-Ciocalteau assay (Singleton & Rossi, 1965) modified by Waterhouse (2001). Sample solution or blank were filled up with distilled water to 1.6 ml total volume depending on the required dilution. The reaction was initiated by addition of 100 µl of Folin-Ciocalteu´s phenol reagent (2 N, Sigma-Aldrich, Steinheim, Germany). After a reaction time of 30 seconds to 8 minutes, 300 µl sodium carbonate solution (20%, Sigma-Aldrich, Steinheim, Germany) was added. The blank sample contained the same mixture solution without the extract. Samples were kept for 30 minutes at 40 °C before the absorbance was measured at 765 nm using a Shimadzu (UV-240) spectrophotometer. A calibration curve with gallic acid (Sigma-Aldrich, Steinheim, Germany) as standard was used and total polyphenol concentration was expressed as gallic acid equivalents (GAE). Each measurement was performed at least in duplicate.

Changes of TP concentration during stress reaction times were expressed as a percent of increase/decrease in comparison to untreated sample and calculated by the formula:

$$\left(\frac{TP_{PEF} - TP_{untreated}}{TP_{untreated}} \right) * 100$$

Equation 3-1

3.3.2. Determination of anthocyanin concentration

Vitis vinifera cell suspension was filtered using a vacuum pump (AEG AMEB 90SY 4R3) for one minute. 100 mg of harvested cells were extracted at 4 °C for 24 h using 4.9 ml ethanol (Merck, Darmstadt, Germany) containing 15 % of hydrochloric acid (Merck, Darmstadt, Germany). The suspension was mixed and centrifuged (Megafuge 1.0 R, Heraeus) at 4000 *g* for 10 minutes. After phase separation pellets were re-extracted and supernatant fractions collected. The absorbance was measured in spectrophotometer (Uvikon 922, Kontron instruments, Groß-Zimmern, Germany) at 535 nm wavelength. Extraction medium was used as a reference and concentration of anthocyanins was calculated by given equation:

$$c = \frac{A}{d * \varepsilon}$$

Equation 3-2

where c represents the concentration of anthocyanins in mol/L, A the extinction, d the film thickness in cm and ε the coefficient of extinction (98.2 ml * μmol^{-1} * cm^{-1}). Content of anthocyanins was presented as mg/g dry matter. Each determination was performed in triplicate.

3.3.3. Determination of enzyme activity

3.3.3.1. Enzyme extraction

Extraction of polyphenoloxidase (PPO) from *Malus domestica*, apple variety Golden Delicious was carried out on ice. 4 g of small cut pieces of apples (without core) were homogenised in 10 ml of sodium phosphate buffer (pH 6.5) containing 1% Polyvinylpyrrolidon (Sigma-Aldrich, Steinheim, Germany) and 0.25 % Triton X_{114} (Merck, Darmstadt, Germany) using an Ultra Turrax T 25 (IKA- Labortechnik, Janke & Kunke, Staufen, Germany) for 1 min. Separation was carried out by centrifugation (Sorvall RC-5B) at 16500 *g* for 30 min at 4 °C. Each extraction was performed in triplicate. Enzyme activity was subsequently measured in the supernatant (subchapter 3.3.3.2).

Polyphenoloxidase (PPO) extraction from *Vitis vinifera,* red grape variety Dornfelder was carried out on ice. Grape berries were cut in half around the equator with a scalpel and seeds were manually removed. 5 g of grape berry pulp and skin were homogenized with an Ultra Turrax T 25 (IKA- Labortechnik, Janke & Kunke, Staufen, Germany) while kept on ice for approximately two minutes, in buffer containing:

- 0.1 M Tri-potassium phosphate (Merck, Darmstadt, Germany),
- 0.002 M Ethylene Di-amino tetra acetic acid (EDTA, Merck, Darmstadt, Germany),
- 0.001 M Phenylmethylsulfonyl fluoride (PMSF, Merck, Darmstadt, Germany),
- 1 % Polyvinylpolypyrrolydon (PVPP, Sigma-Aldrich, Steinheim, Germany);

adjusted with 0.1 M hydrochloric acid (Merck, Darmstadt, Germany) to pH 7.0. The phases were separated by centrifugation (Sorvall RC-5B) at 12000 *g* and 4 °C for 15 minutes. Each extraction was performed at least in duplicate. The supernatant was kept at -20 °C until enzyme activity assay was performed (subchapter 3.3.3.2).

Polyphenoloxidase (PPO) and peroxidase (POD) extraction from *Vitis vinifera* cell culture was performed in one gram of harvested cells each, using a vacuum filtration pump (AEG AMEB 90SY 4R3) for one minute. The cells were placed in pre-cooled test tubes and suspended in 3 ml of phosphate buffer (100 mmol/L, pH 6.5), followed by homogenization for 30 seconds with an Ultra Turrax T 25 (IKA- Labortechnik, Janke &

Kunke, Staufen, Germany) while kept on ice. After 30 minutes extraction time the resulting suspension was centrifuged at 25000 *g* for 10 minutes at 4 °C (Sorvall RC-5B, Refrigerated Superspeed Centrifuge, Heraeus®). Each extraction was performed in triplicate. The supernatant was kept on ice (for less than 2 h) until further detection of enzyme activity was performed (subchapters 3.3.3.2 and 3.3.3.3).

Extraction of phenylalanine-ammonium-lyase (PAL) from *Malus domestica* cell culture was carried out in two grams of harvested *Malus domestica* cells, using a vacuum filtration pump (AEG AMEB 90SY 4R3) for one minute. The cells were suspended in 3 ml boric acid-borax-buffer (0.05 M, pH 8.8) and homogenized 3 times for 30 seconds (pause of 30 seconds between) on ice, with an Ultra Turrax T 25 (IKA- Labortechnik, Janke & Kunke, Staufen, Germany). The homogenate was centrifuged for 15 minutes at 25000 *g* (Sorvall RC-5B, Bad Homburg, Germany). The supernatant was again centrifuged for 5 minutes at 9500 *g* (Heraeus biofuge pico, Osterode, Germany). Each extraction was performed at least in duplicate. PAL activity was determined in the final supernatant, which was kept at -20 °C for 45 h until measurement (subchapter 3.3.3.4).

3.3.3.2. Polyphenoloxidase (PPO) activity assay

Detection of Polyphenoloxidase (PPO) activity was performed according to the method of Siriphanich and Kader (1985). The PPO activity was quantified from 100 µl of obtained enzyme extract from plant cell cultures and 200 µl of extract obtained from tested fruits. Enzyme extracts were added to 10 mM catechol solution (Merck, Darmstadt, Germany) in 0.05 M phosphate buffer, (pH 6.5 for plant cell cultures, pH 6.3 for apples and pH 7 for grapes) to final volume of 3 ml. Instead of enzyme extracts the blank sample contained same aliquot of related extraction solution. PPO activity was determined by plotting the increase in absorbance at wavelength of 420 nm, during 60 seconds to 5 minutes at 25 °C with a recording spectrophotometer (Hitachi U-3000). The enzyme activity was calculated from the linear portion of the curve and presented as the change in absorbance per minute and gram fresh weight. Each measurement was performed in triplicate.

3.3.3.3. Peroxidase (POD) activity assay

Determination of peroxidase activity from plant cells extracts were conducted with pyrogallol as a substrate (Stellmach, 1988). 50 µl of enzyme extract were added to 1950 µl of 0.05 M phosphate buffer (pH 6.5), containing 0.005 M hydrogen peroxide (Merck, Darmstadt, Germany) and 0.01 M pyrogallol (Sigma-Aldrich, Steinheim,

Germany). The blank sample contained the same mixture solution without the enzyme extract. Increase in absorbance was determined for 60 seconds at 420 nm and 25 °C with a recording spectrophotometer (Hitachi U-3000) and presented as the change in absorbance per minute and gram fresh weight. POD activity assay was performed in triplicate.

3.3.3.4. Phenylalanine-ammonium-lyase (PAL) activity assay

Measurement of PAL activity was carried out at 36 °C. All used solutions were preheated to 36 °C before being added to enzyme extracts. For determination, 500 µl of extract was added to 1900 µl boric acid-borax-buffer (0.05 M, pH 8.8) and 600 µl substrate (0.1 M L-phenylalanine (Sigma-Aldrich, Steinheim, Germany) in 0.05 M boric acid-borax-buffer, pH 8.8). The solution was transferred in UV-cuvettes and the extinction was measured in the Jenway 6505 UV/VIS-Spectrophotometer (Barloworld Scientific, Dunmow/ Essex, UK) at 290 nm after 15 and 75 minutes reaction time against the blank (Seitz, Seitz & Alfermann, 1985). For each sample a blank value was determined by adding 500 µl extract in 2500 µl buffer solution. PAL activity assay was performed in triplicate and enzyme activity was determined by the production of cinnamate during 60 minutes. PAL activity was calculated from the following equation:

$$E_a = \frac{\Delta A}{t} * \frac{V}{d * \varepsilon_{290}}$$

Equation 3-3

where E_a represents enzyme activity, ΔA the difference in absorption, t the reaction time, V the total volume of reaction solution (3 ml), d cuvette thickness (1 cm) and ε_{290} is the extinction coefficient (cinnamic acid 10 ml *cm^{-1} *mol^{-1}).

3.3.4. Determination of plant cell culture viability

Since only vital cells can contribute to a stress response from the external factor, a method for measurement of cell viability and their metabolic state was used. Chosen enzymatic test measures the ability of viable cells to reduce 2,3,5-triphenyltetrazolinum chloride (TTC) to red water insoluble triphenylformazin which is accumulated in mitochondria of intact cells and can be spectrophotometrically determined. For the analysis 100 mg of harvested plant cells (*Malus domestica* and *Vitis vinifera*) were suspended in 1 ml 2,3,5-triphenyl-tetrazolinumchlorid solution (0.3 % in 66 mmol/L sodium phosphate buffer, pH 7.5). Buffer solution was used instead of TTC as reference. The mixture was incubated for 24 h at room temperature in the dark and due to reductase

activity TTC was reduced to the triphenylformazin. The reaction was stopped by addition of 5 ml ethanol 95 % (v/v). The suspension was heated to 60 °C and kept for 15 minutes and thoroughly mixed to extract red coloured triphenylformazin. Phases were separated by centrifugation (Megafuge 1.0 R, Heraeus) for 10 minutes at 4000 *g*. 5 ml ethanol (95 %) was added to the pellet and the extraction procedure was repeated. The supernatant fractions were collected for absorbance readings using a spectrophotometer (Uvikon 922, Kontron instruments, Groß-Zimmern, Germany) at 485 nm wavelength. The absorbance compared to reference was presented per gram dry matter. Each measurement was performed in triplicate.

3.3.5. Determination of membrane permeabilization

The impact of PEF treatment on the cell membrane permeabilization of biological tissue material was done by a method developed by Angersbach *et al*. (1999). Impedance measurement equipment (Biotronix GmbH, A. Angersbach, Henningsdorf, Germany) was used for determination of membrane permeabilization in fruit tissue and in plant cell cultures. The CDI was calculated using the Equation 2-8 (see subchapter 2.2.3).

For determination of CDI in plant cell cultures, 0.4 g of vacuum harvested cells (filtration pump, AEG AMEB 90SY 4R3) were suspended in 1 ml 0.5 M mannitol (Sigma-Aldrich, Steinheim, Germany) and transferred into the measuring cell (polyethylene tube closed from both sides with stainless steal cylindrically shaped electrodes and electrode distance of 10 mm). To determine CDI in fruits, cylindrical shapes of 10 mm diameter and 10 mm length were cut out of the fruit tissue with a sharp knife and placed into a measuring cell to occupy the total area between electrodes.

3.4. PEF protocol

3.4.1. PEF pilot plant equipment

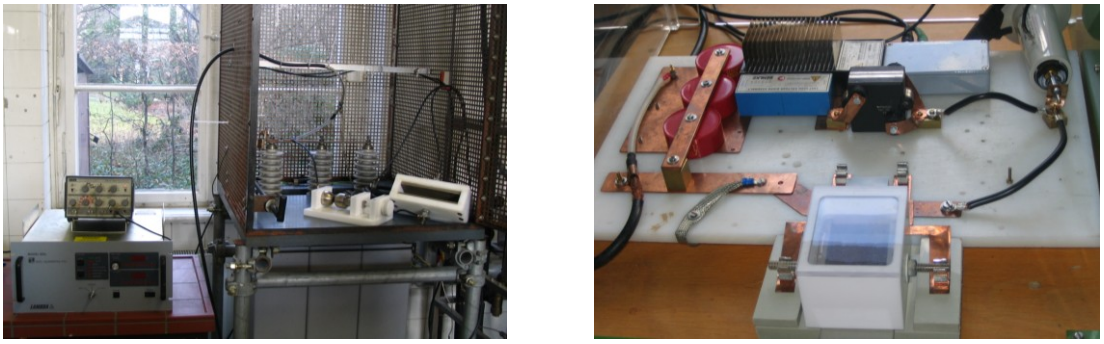
During the course of this study PEF processing was carried out on different batch lab scale systems designed and constructed for various biological raw materials at the Department of Food Biotechnology and Food Process Engineering, Technische Universität Berlin (Toepfl, 2006). In order to reach postulated treatment parameters and raw material requirements, following pulse modulators and treatment chambers were used:

Pulse modulator (PM 1)

Power supply ALE802 (Lambda-Emi, Neptune, USA) with a 40 kV maximum voltage and 8 kW maximum power was used to charge a capacitor bank with capacitance of 3 μF (Figure 3-1A). Discharging of capacitors over a spark gap provided monopolar exponential decay pulses, controlled by the TGP110 10 MHz Pulse Generator from Thurlby Thandar Instruments (TTi). Pulse wave form was monitored with TDS 220 (Sony Tektronix, Beaverton, US) oscilloscope.

Pulse micro modulator (PM 2)

The electrical energy was delivered by a power supply FUG HCK 800M-20000 (FUG, Rosenheim, Germany) with maximum voltage of 20 kV and maximum power of 0.8 kW to a capacitors with a storage capacity of 19,1 nF (Figure 3-1B). Monopolar exponential decay pulses were generated by switching unit which consisted from: power supply A400, 5 V, 2 A (EMS power, Basingstoke, U.K.), high voltage switch HTS 160-500 SRC 16 kV, 5 kA, 2 kHz (Behlke, Kronberg, Germany), free wheeling diode FDA 150-200, 20 kV, 1.5 A (Behlke, Kronberg, Germany) and frequency generator (AFG 320 Sony Tektronix, Beaverton, US). Monitoring of pulse parameters was performed by a high voltage and current probe, coupled to a TDS 220 (Sony Tektronix, Beaverton, US) oscilloscope. To control PEF treatment parameters a PC computer was used.



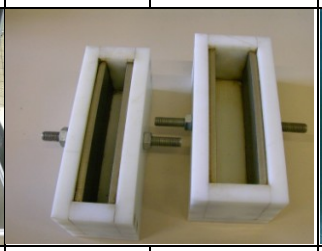
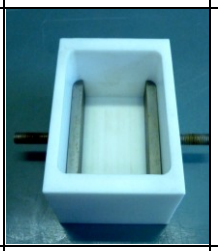
(A) (B)

Figure 3-1: High intensity electric field apparatus (A) PM 1 and (B) PM 2.

Treatment chambers

Pulse modulators (PM 1 and PM 2) were used in combination with different treatment chambers suitable for biological materials chosen for this study. Pulsed electric field treatment took place in parallel plate batch treatment chambers (stainless steel electrodes, food grade) with different filling capacities and electrode distance, as shown in Table 3-1. Temperature fluctuations within described PEF systems used in the course of this study were negligible.

Table 3-1: Treatment chamber characteristics

Treatment chamber	TC1a	TC1b	TC2	TC3	TC4	TC5
TC						
Volume (ml)	12700	10670	700	420	100	1
Electrode surface shape	Rectangle (P=a x b)	Rectangle (P=a x b)	Rectangle (P=a x b)	Rectangle (P=a x b)	Rectangle (P=a x b)	Circle ($P=r^2 \pi$)
Electrode size (cm)	a=49.5 b=32	a=49.5 b=32	a=20 b=7	a=20 b=7	a=8 b= 2	r = 0.5
Electrode distance (cm)	8	6	5	3	4.5	1

* P= surface area; a and b=rectangle sides; r=radius of a circle.

3.4.2. Treatment conditions and experimental set up

The experimental set up used for determination of stress reactions during postharvest storage induced by PEF treatment was designed and customized for the different biological material analyzed in this study. A systematic approach for fruits and related plant cell cultures was developed. PEF treatment was introduced as an additional processing step during postharvest storage of apples, blueberries and red currant, and as pre-treatment of grapes during traditional winemaking process. To investigate the effect of PEF on plant cell cultures, grown in controlled environment, PEF treatment was introduced in different phases of culture growth cycle. General concept and processing steps are shown in Figure 3-2.

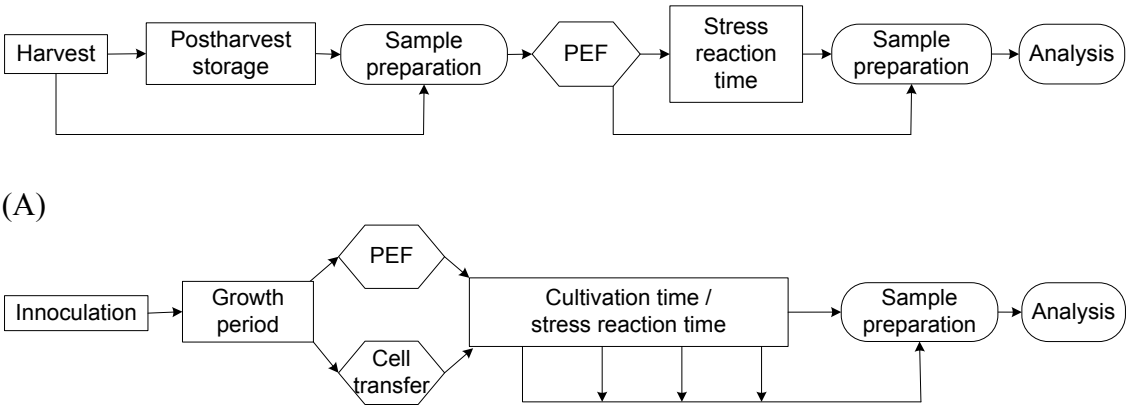


Figure 3-2: Schematic diagram of PEF induced postharvest stress treatment of (A) fruit tissue and (B) plant cell cultures.

3.4.2.1. Processing of apple variety Golden Delicious

Apples of the variety Golden Delicious were used to investigate impact of PEF treatments with different intensities. Randomly chosen apples were washed, cut in quarters and the apple core was taken out. There is a high variability of phenolic content in each fruit. To obtain a homogeneous sample for every treatment condition/stress reaction time and corresponding reference sample lot, from each chosen apple one quarter was assigned to a reference lot, while each next quarter was assigned to the lot treated with pulsed electric fields (Figure 3-3). At least 2 kg of apples were processed for each PEF treatment intensity applied (Table 3-2) to randomly chosen fruits from the same batch of apples.

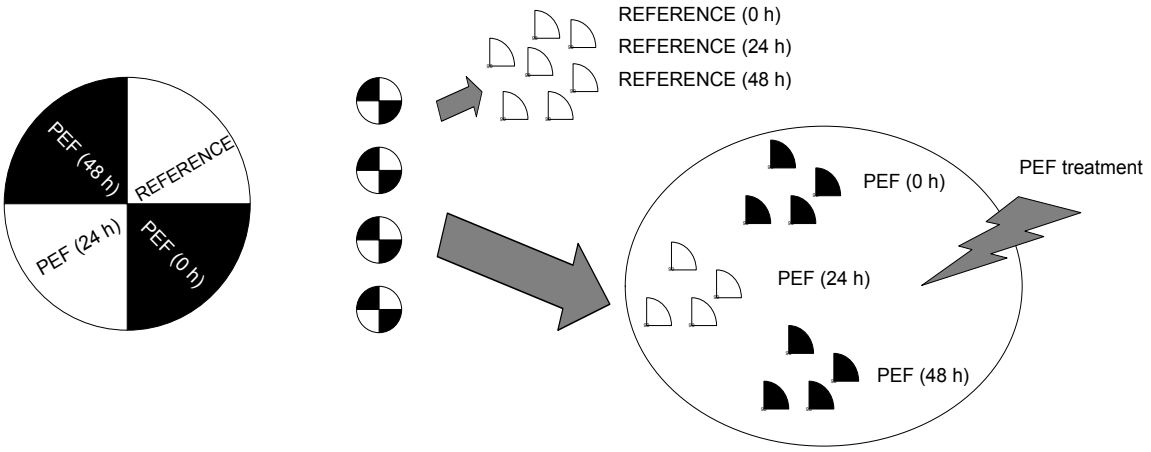


Figure 3-3: Schematic view of sample preparation to obtain homogenous lot of apples.

The apples were placed into the PEF-treatment chamber, which was filled with water as an electrically transmitting media and treated with pulsed electric fields at chosen parameters (Table 3-2). To obtain equivalent experimental conditions, the reference samples of the same lot were analogously handled without exposure to PEF. Each PEF treatment intensity with corresponding reference has been performed in triplicate. Following PEF exposure, samples were collected and immediately analyzed (which corresponds to a stress reaction time of 0 h), or kept in subsequent storage at 16 ± 1 °C for chosen stress reaction period (24 and 48 h) before being analyzed.

Apples have been treated to determine impact of low intensity PEF on cell membrane permeabilization (subchapter 3.3.5) and total polyphenol concentration from whole fruit (without apple core and seeds) and subsequently from apple juice and residue extracts (subchapter 3.3.1).

Table 3-2: PEF treatment parameters and equipment set-up used for stress induction in apples

Sample	Electric field strength (V/cm)	Output voltage (V)	Pulse number / Pulse frequency (Hz)	Total energy input (kJ/kg)	PM* TC**	Pulse duration (µs)	Energy per pulse (J/kg)
Apples (Golden Delicious)	300	2400	20 / 1	0.014	PM1 TC1a	400	8.64
	700	5600	20 / 1	0.074	PM1 TC1a	400	47.04
	1200	9600	20 / 1	0.217	PM1 TC1a	400	138.24
	4000	12000	20 / 1	10.286	PM1 TC3	100	216

PM* Pulse modulator used for PEF treatment (Figure 3-1)
TC** Treatment chamber used for PEF treatment (Table 3-1)

3.4.2.2. Processing of blueberries and red currant

The raw material was randomly divided in lots of 50 g of berries, which were placed into the treatment chamber and filled with water (electrically transmitting media) to cover electrodes. The PEF treatment has been performed with different treatment intensities, as shown in Table 3-3.

Table 3-3: PEF treatment parameters and equipment set-up used for stress induction in berries

Sample	Electric field strength (V/cm)	Output voltage (V)	Pulse number / Pulse frequency (Hz)	Total energy input (kJ/kg)	PM* TC**	Pulse duration (µs)	Energy per pulse (J/kg)
Blueberry & Red currant	300	900	4 / 1	0.012	PM1 TC3	100	1.215
			7 / 1	0.020			
	500	4000	4 / 1	0.0075	PM1 TC1a	400	24
			7 / 1	0.0131			
	1000	5000	4 / 1	0.214	PM1 TC2	175	37.5
			7 / 1	0.375			
	2000	10000	4 / 1	0.875	PM1 TC2	175	150
			7 / 1	1.5			
	4000	12000	7 / 1	3.6	PM1 TC3	100	216
			20 / 1	10.285			

PM* Pulse modulator used for PEF treatment (Figure 3-1)
TC** Treatment chamber used for PEF treatment (Table 3-1)

After PEF treatment berries were gently dried with paper towel and immediately analyzed (which corresponds to 0 h stress reaction time), or kept in subsequent storage at

4 °C and at room temperature (20 ± 2 °C) for chosen stress reaction period of 24 and 48 h before being analyzed. The reference lots were chosen from the same batch of raw material and were analogously handled, without induction of PEF treatment. Each treatment (with corresponding reference) has been performed in triplicate. Cell membrane permeabilization and total polyphenol concentration were determined as described in subchapters 3.3.5 and 3.3.1, respectively.

3.4.2.3. Processing of red grape variety Spät Burgunder

To obtain a homogenous sample 12.75 ± 0.1 kg of randomly chosen grape clusters were washed and placed in the PEF-treatment chamber. Grape clusters were manually squeezed to float in their own juice which was utilized as an electrically transmitting media. The PEF treatment has been performed with different treatment intensities (Table 3-4) and was introduced as a pre-treatment method within traditional wine making process. Subsequently, grape clusters were transferred to a 10 L wine press (Vierka, Bad Königshofen, Germany) and pressed at ambient temperature till the juice flow had stopped (total duration of 2 hours). Fresh pressed grape juice was collected and immediately analyzed, or kept at 16 °C in plastic wine containers for a period of 8 weeks. During this time fermentation occurred. Total polyphenol concentration was measured in grape juice and residue extracts immediately after pressing and in young wine after 4, 6 and 8 weeks of storage, according to the Folin-Ciocalteau assay (subchapter 3.3.1). To establish the degree of cell membrane permeabilization, impedance measurement was carried out (subchapter 3.3.5) on whole grape berries prior to PEF treatment, 3 min after the treatment and measurements have been continued every minute for the next 2.5 hours.

Table 3-4: PEF treatment parameters and equipment set-up used for stress induction in red grape variety Spät Burgunder

Sample	Electric field strength (V/cm)	Output voltage (V)	Pulse number / Pulse frequency (Hz)	Total energy input (kJ/kg)	PM* TC**	Pulse duration (µs)	Energy per pulse (J/kg)
Grapes (Spaet Burgunder)	300	2400	50 / 2	0.0340	PM1 TC1a	400	8.64
	500	4000	50 / 2	0.1042	PM1 TC1a	400	26.46
	1000	8000	50 / 2	0.3780	PM1 TC1a	400	96
	2400	19200	50 / 2	2.1770	PM1 TC1a	400	552.96

PM* Pulse modulator used for PEF treatment (Figure 3-1)
TC** Treatment chamber used for PEF treatment (Table 3-1)

Since wine grapes during postharvest storage demonstrate dehydration and fast metabolic changes in grape berries, reference lots were processed on the same day with PEF treated lots. Each treatment and corresponding reference has been processed at least in duplicate.

3.4.2.4. Processing of red grape variety Dornfelder

The raw material was taken out of the cold room 2 to 5 h before further processing to reach ambient temperature. Randomly chosen fresh grape clusters were washed and divided in six lots of 5 kg each. Three lots (sample a, b and c) were placed in the treatment chamber, filled with water as electrically transmitting media and subjected to PEF treatment (Table 3-5), while other three lots (sample d, e and f) were used as reference samples.

Table 3-5: PEF treatment parameters and equipment set-up used for stress induction in red grape variety Dornfelder

Sample	Electric field strength (V/cm)	Output voltage (V)	Pulse number / Pulse frequency (Hz)	Total energy input (kJ/kg)	PM* TC**	Pulse duration (µs)	Energy per pulse (J/kg)
Grapes (Dorn - felder)	300	2400	10 / 1	0.0068	PM1 TC1a	400	8.64
	700	5600	10 / 1	0.037	PM1 TC1a	400	47.04

PM* Pulse modulator used for PEF treatment (Figure 3-1)
TC** Treatment chamber used for PEF treatment (Table 3-1)

Grape clusters (sample a, b and c) subjected to the same PEF treatment intensity were mixed together and pressed immediately or stored at 4 °C and at room temperature (16 ± 1 °C) for stress reaction times of 24 and 48 h before being pressed. The pressing was performed with a lab scale hand press (Hafico HP 2, Schwanke Tinkturenpressen, Neuss, Germany) at ambient temperature, with a pressure of 8 bar, obtaining at hydraulic pressure of 250 bar. Manual pressing was discontinued after the juice flow had stopped. Three lots representing control sample d, e and f were analogously handled, without induction of PEF treatment. Total polyphenol concentration was measured in grape juice and residue extracts obtained after pressing according to the Folin-Ciocalteau assay (subchapter 3.3.1), while polyphenoloxidase was extracted from the whole grape berries and determined according to PPO activity assay (subchapter 3.3.3).

3.4.2.5. Processing of plant cell cultures (*Malus domestica* and *Vitis vinifera*)

The cells were subcultivated (subchapter 3.2) for periodic growth cycles of 7 days for *Malus domestica* (apple), and 14 days for *Vitis vinifera* (red grapes) suspension culture. To minimize the impact of raw material variability, the cell material of 15 - 20 Erlenmeyer flasks were combined in one pool, mixed carefully under sterile conditions and medium was dripped off under atmospheric pressure. New flasks were inoculated with 8 and 5 g of *Malus domestica* and *Vitis vinifera* cell mass, respectively. Cell culture suspensions were sterile transferred into the PEF-treatment chamber TC4 (Table 3-1) at day 3 and 5 after inoculation for *Malus domestica* and at day 7 after inoculation for *Vitis vinifera*. The early log phase and the exponential phase of growth have been chosen for stress induction by PEF treatment due to cultivation properties of cultured cells. PEF treatment (Table 3-6) was carried out in a closed treatment chamber under sterile conditions, subsequently cell culture suspensions were transfered to Erlenmeyer flasks.

Table 3-6: PEF treatment parameters and equipment set-up used for stress induction in plant cell cultures (*Malus domestica* and *Vitis vinifera*)

Cell culture	Cultivation day	Electric field strength (V/cm)	Output voltage (V)	Pulse number / Pulse frequency (Hz)	Total energy input (kJ/kg)	PM* TC**	Pulse duration (µs)	Energy per pulse (J/kg)
Malus domestica	5	200	1700	100 / 2	0.0099	PM2 TC4	6	0.010
		400	2700	100 / 2	0.0413	PM2 TC4	6	0.025
	3	200	1400	25 / 1	0.0041	PM2 TC4	6	0.011
		200	1400	50 / 1	0.0082	PM2 TC4	6	0.011
Vitis vinifera	7	200	1300	20 / 1	0.0049	PM2 TC4	6	0.016
		500	2700	20 / 1	0.02142	PM2 TC4	6	0.070
		800	4400	20 / 1	0.05689	PM2 TC4	6	0.184
		1200	6600	20 / 1	0.1279	PM2 TC4	6	0.416
		2400	12000	20 / 1	66.461	PM1 TC4	330	216

PM* Pulse modulator used for PEF treatment (Figure 3-1)
TC** Treatment chamber used for PEF treatment (Table 3-1)

An additional set of PEF treatments was performed for the determination of *Malus domestica* cell membrane permeabilization in two different treatment media (LS-medium and mannitol), as shown in Table 3-7.

Table 3-7: PEF treatment parameters and equipment set-up used for determination of cell membrane permeabilization in *Malus domestica* cell cultures

Electric field strength (V/cm)	540						
Output voltage (V)	700						
Pulse duration (µs)	1.5–6 #						
Energy per pulse (J)	0.0047						
PM* and TC**	PM2 and TC5						
Pulse number/Pulse frequency (Hz)	100/2	200/2	400/2	800/2	1600/2	3200/2	6400/2
Total energy input (kJ/kg)	0.47	0.94	1.88	3.76	7.52	15.04	30.08

Electric field strength (V/cm)	1240						
Output voltage (V)	1600						
Pulse duration (µs)	1.5–6 #						
Energy per pulse (J)	0.025						
PM* and TC**	PM2 and TC5						
Pulse number/Pulse frequency (Hz)	20/2	40/2	80/2	160/2	320/2	640/2	1280/2
Total energy input (kJ/kg)	0.49	0.98	1.96	3.92	7.84	15.68	31.36

Electric field strength (V/cm)	2540						
Output voltage (V)	3300						
Pulse duration (µs)	1.5–6 #						
Energy per pulse (J)	0.104						
PM* and TC**	PM2 and TC5						
Pulse number/Pulse frequency (Hz)	5/2	10/2	20/2	40/2	80/2	160/2	320/2
Total energy input (kJ/kg)	0.52	1.04	2.08	4.16	8.32	16.64	33.28

Electric field strength (V/cm)	7040					
Output voltage (V)	9900					
Pulse duration (µs)	1.5–6 #					
Energy per pulse (J)	0.81					
PM* and TC**	PM2 and TC5					
Pulse number/Pulse frequency (Hz)	5/5	10/5	20/5	40/5	80/5	160/5
Total energy input (kJ/kg)	4	8	16	32	64	128

PM* Pulse modulator used for PEF treatment (Figure 3-1)
TC** Treatment chamber used for PEF treatment (Table 3-1)
Depending on the load voltage and electric conductivity of treated media

Following PEF treatment, cell suspension cultures were maintained in growth/treatment media for the stress reaction period under controlled cultivation conditions before being further analysed. *Malus domestica* suspension cultures were maintained for chosen stress reaction times of 0, 9, 24 and 48 h in the dark, while *Vitis vinifera* were kept for 0, 24, 48, 72 and 96 h under permanent illumination (fluorescent lamp of approx. 3000 lux); both on an orbital shaker at 100 rpm and 25 ± 0.2 °C. Referent cell culture suspensions were handled correspondingly without

induction of PEF treatment. Each experiment with *Malus domestica* cell culture was performed in duplicate, and with *Vitis vinifera* cell culture in triplicate.

The cells were harvested for each analysis after the chosen stress reaction time using a vacuum pump (AEG AMEB 90SY 4R3) for one minute, followed by measurements of fresh and dry weight (determined according to §64 LFBG, previous §35 LMBG), pH (Knick Digital-pH-Meter), conductivity (conductivity meter, WTW Cond 3110), degree of cell membrane permeabilization (subchapter 3.3.5), total polyphenol concentration (subchapter 3.3.1), enzyme activity (subchapter 3.3.3) and cell viability (subchapter 3.3.4).

3.5. Statistical analysis

Statistical analysis of data was carried out through an analysis of variance (ANOVA) using Microcal Origin 7.0 Software. Results were expressed as mean with standard deviation. The statistical significance was considered at the level of $\alpha=0.05$ established for differences among mean values.

4. Results and Discussion

4.1. PEF impact on apples

To determine the impact of PEF treatment on apple variety Golden Delicious, permeabilization of the cell membrane was measured after chosen treatment intensities in order to gain better insight in disintegration degree of the cellular apple tissue. Polyphenols, secondary plant metabolites involved in stress response to different abiotic stressors, were selected as indicators to stress reactions triggered by PEF. Total polyphenol (TP) concentration in whole apples (without seeds and core) and separately in apple juice and residue extracts were studied. Furthermore, to determine possible stress related changes, PEF treated and corresponding untreated apples were kept for chosen stress reaction period after imposed stress.

4.1.1. Degree of membrane permeabilization

The impact of PEF treatment at different electric field strengths and 20 pulses on the degree of cell membrane permeabilization was measured and expressed as CDI. The cell disintegration index determined in untreated and PEF treated apple tissue immediately after treatment (which corresponds to 0 h) and after period of 24 and 48 h, stored at 16 °C, is shown in Figure 4-1.

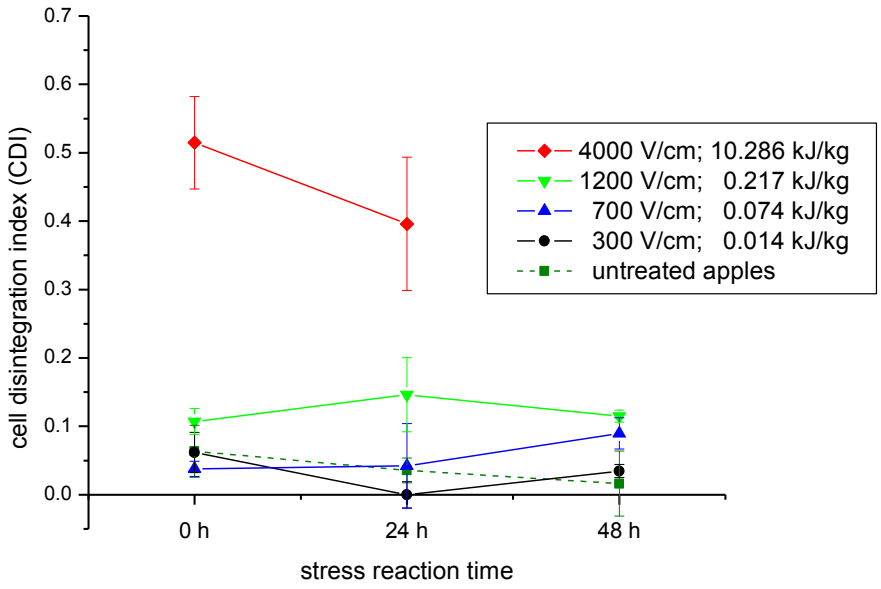


Figure 4-1: Impact of PEF treatment on the extent of membrane permeabilization in *Malus domestica*, apple cultivar Golden Delicious, measured as cell disintegration index (CDI), after 0, 24 and 48 h stress reaction times.

Disintegration of the cellular material measured immediately after application of 20 pulses with 300 and 700 V/cm was not significant different than in untreated apples. After 24 h, CDI value of apples treated with 300 V/cm was even lower than in corresponding untreated samples. These results indicate that such a slight increase in CDI value (up to 0.06, which denotes membrane permeabilization of 6 % of the cells) could be neglected since it occurred most probably due to sharp cut of apple tissue, a necessary step in sample preparation before CDI measurements could have been performed (see subchapter 3.3.5). Greater differences to untreated samples were neither observed 48 hours after the treatment indicating reversible permeabilization of apple tissue.

Larger impact was noticed after 20 pulses at 1200 V/cm, where a CDI value of 0.11 was measured immediately after treatment. An insignificant increase after 24 h storage at 16 °C to 0.15 was observed, which might indicate the extension of pores, or release of electro conductive substances in extracellular surroundings. The permeabilization of the cell membrane involves creation of a number of metastable pores which remain open after removal of applied PEF treatment if the threshold value is exceeded (Zimmermann et al., 1974; Weaver, 1995). Since a CDI value of 0.11 measured 48 h after the treatment was observed, irreversible permeabilization of 11 % of the cells can be expected. Immediately after application of 20 pulses at 4000 V/cm, much larger disintegration of apple tissue was noticed and reached a value of 0.52. After 24 h storage, a decrease in CDI value to 0.40 was observed, which most probably occurred due to liquid loss through highly permeabilized tissue. Loss of liquids and severe deteriorations were observed in apples after 48 h storage as well, due to which samples were discarded and no measurements were performed after this storage time.

Level of cell disintegration expressed in Figure 4-1 is related to PEF treatment intensity. Higher PEF efficiency for tissue disintegration was observed after application of higher electric field intensity, consequently larger energy input, which is in accordance with observations reported by several authors (Toepfl, 2006; Vorobiev & Lebovka, 2008; Janositz & Knorr, 2010; Jaeger, 2011).

4.1.2. Impact of stress by PEF on TP concentration in apples

The impact of 20 pulses at different levels of electric field strength on TP concentration in whole apples during 48 h after the treatment was investigated. Immediately after PEF treatment with 300 V/cm significantly larger TP concentration in apples was observed, in comparison to untreated samples (Figure 4-2A). After 24 h stress reaction time TP concentration of untreated apples increased, whereas decrease was

observed in PEF treated samples, due to which TP concentration of PEF treated apples approached value of untreated control. After 48 h rise in TP concentration of PEF treated apples was observed and significantly exceeded values of untreated apples. Interestingly, with such a large TP concentration measured in PEF treated samples immediately after treatment (with respect to approximately 30 - 60 minutes of sample preparation time), large degree of cell membrane permeabilization would be expected and therefore easier release of intracellular compounds. However, the observed degree of membrane permeabilization after PEF treatment with 300 V/cm did not differ from untreated control (Figure 4-1) and therefore another mechanism was most likely responsible for larger TP concentration in PEF treated apples.

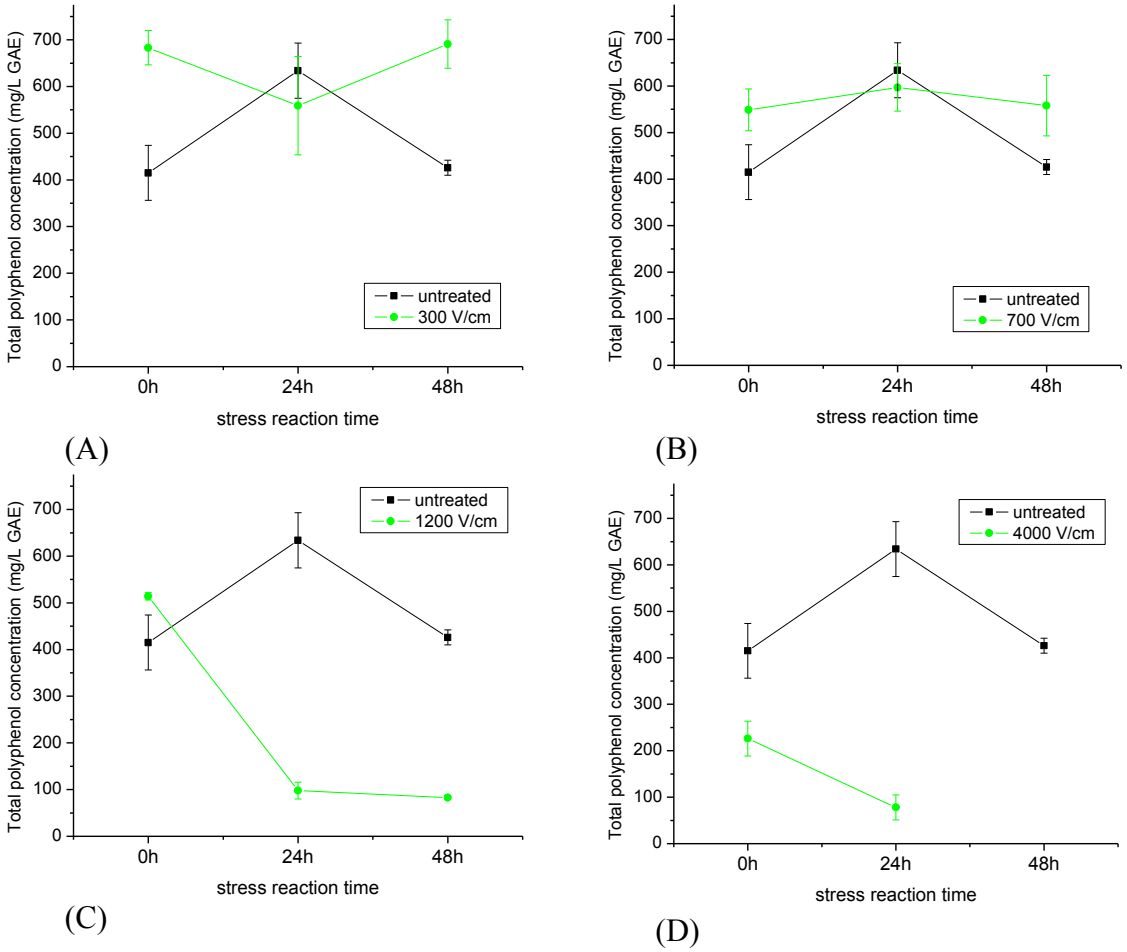


Figure 4-2: Total polyphenol concentration of untreated and PEF treated apples at: (A) 300 V/cm, 20 pulses, 0.014 kJ/kg; (B) 700 V/cm, 20 pulses, 0.074 kJ/kg; (C) 1200 V/cm, 20 pulses, 0.217 kJ/kg; (D) 4000 V/cm, 20 pulses, 10.286 kJ/kg; measured after 0, 24 and 48 h stress reaction times.

Similar observations were made after PEF treatment with 700 V/cm (Figure 4-2B). Larger TP concentration in PEF treated apples was observed immediately after treatment followed by stabilization after 24 h and again after 48 h TP concentration of PEF treated apples exceeded values of untreated control samples.

Higher TP concentration was observed immediately after treatment with 1200 V/cm as well (Figure 4-2C). However, during stress reaction period of 24 and 48 h, TP concentration in PEF treated apples significantly decreased and observed values were significantly lower than in corresponding untreated apples. Permeabilization of cellular tissue after 1200 V/cm was larger than after 300 V/cm (Figure 4-1). However, TP concentration immediately after 1200 V/cm reached 514 mg/L GAE and after 300 V/cm reached 683 mg/L GAE. It seems that PEF treatment at 1200 V/cm initially destabilized cell membrane and normal cell postharvest metabolism, which caused damage that increased over storage period. Physiological effect of irreversible damage was observed through loss of phenolic substances after stress reaction period, which could be attributed to the deleterious effect of enzymes such as PPO and/or POD, since enzymatic oxidation of polyphenols occurs only after cell injury.

Immediately after application of 4000 V/cm, TP concentration in PEF treated apples was significantly lower than untreated samples and declined after 24 h (Figure 4-2D). Large disintegration of cellular material after PEF treatment at 4000 V/cm (Figure 4-1) resulted in loss of phenolic substances most probably due to oxidation and leakage through damaged cell membrane. Due to strong deterioration and excessive browning of apple tissue observed after 48 h storage, samples were discarded and no further measurements were performed. Easier release of intracellular substances after PEF pre-treatment application which include disintegration of cellular tissue has been often reported (Knorr & Angersbach, 1998; Fincan et al., 2004; Lebovka et al., 2005; Jaeger et al., 2012). However, loss of phenolic substances observed within this study occurred due to experimental design suitable for stress induction. Different experimental design should be developed in order to acquire suitable application concept.

4.1.3. Changes of TP concentration in apples due to PEF intensity

Since stress has been considered as a deviation from the optimal plant conditions of life, followed by reversible or permanent changes in plant metabolism at all levels (Larcher, 2003), observed changes in polyphenol concentration caused by PEF treatment can be expressed as percentage of polyphenol increase/decrease in respect to untreated samples, revealing divergences caused only by PEF as external stressor.

Increase in TP concentration of 65 and 32 % was observed immediately after PEF treatments with 300 and 700 V/cm, respectively (Figure 4-3). Largest increase was observed after lowest treatment intensity used, when no disintegration of cellular material was noticed (Figure 4-1). Larger TP concentration immediately after treatment is

attributed to plant effort to sustain externally imposed stress through accumulation of protective substances. Since polyphenol concentration reached values of untreated samples after 24 h stress reaction time and repeated increase 48 h after PEF treatments (300 and 700 V/cm) was observed, destabilization of metabolic functions and plant adjustment to strained conditions through *de novo* synthesis and utilization of phenolic compounds are indicated. Increased synthesis of polyphenolic compounds has been often described in literature through resistance phase of biological stress concept (Beck & Lüttge, 1990; Dixon & Paiva, 1995; Larcher, 2003), and therefore it can be assumed that apple postharvest metabolism suffered changes that allowed recovery and adaptation to imposed stress.

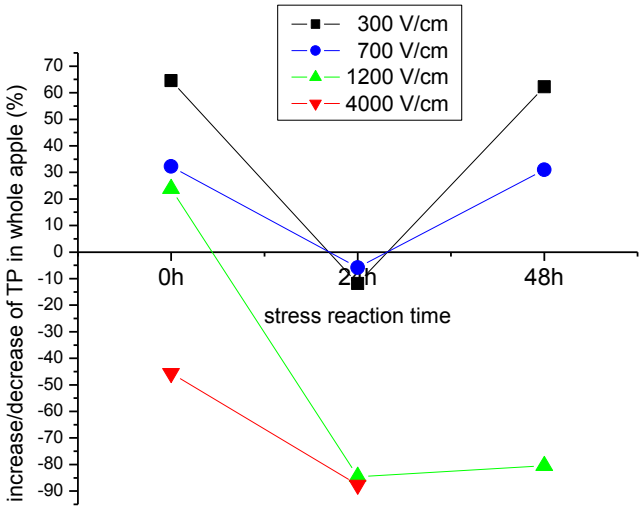


Figure 4-3: Changes of total polyphenol concentration (TP) in apples due to PEF treatment of different intensities during stress reaction period, stored at 16 °C.

When apples were treated with 1200 V/cm increased TP concentration observed immediately after treatment indicated plant effort to sustain enforced stress (Figure 4-3). However, a large decrease observed 24 and 48 h after the treatment indicated catabolism predomination, which finally lead to exhaustion. Stress induced by PEF at 1200 V/cm was too large to be tolerated and negative effect concerning TP concentration, leading to acute damage, was observed.

Larger impact on cell membrane structure after the application of 4000 V/cm and loss of polyphenol substances revealed that this treatment intensity was too excessive to generate stress response. 46 % decrease of TP concentration immediately after treatment and 88 % after 24 h was observed (Figure 4-3). Loss of polyphenolics occurred most probably due to leakage through damaged membrane, since large disintegration of cellular

tissue was detected (Figure 4-1). Furthermore, due to permeabilization of the cell membrane free passage of substrates and products into and out of the cell is permitted (Felix, Brodelius & Mosbach, 1981), which may lead to different chemical reactions and result in deterioration of polyphenolic compounds.

PEF treatment has been often utilized to improve mass transfer processes and increase TP concentration in juices extracted from different raw material (Toepfl, 2006). Therefore, additional trials were setup and PEF treated and corresponding untreated apples were dejuiced, followed by determination of TP concentration in juice and residue extracts.

4.1.4. Impact of stress by PEF on TP concentration of apple juice and residue extracts

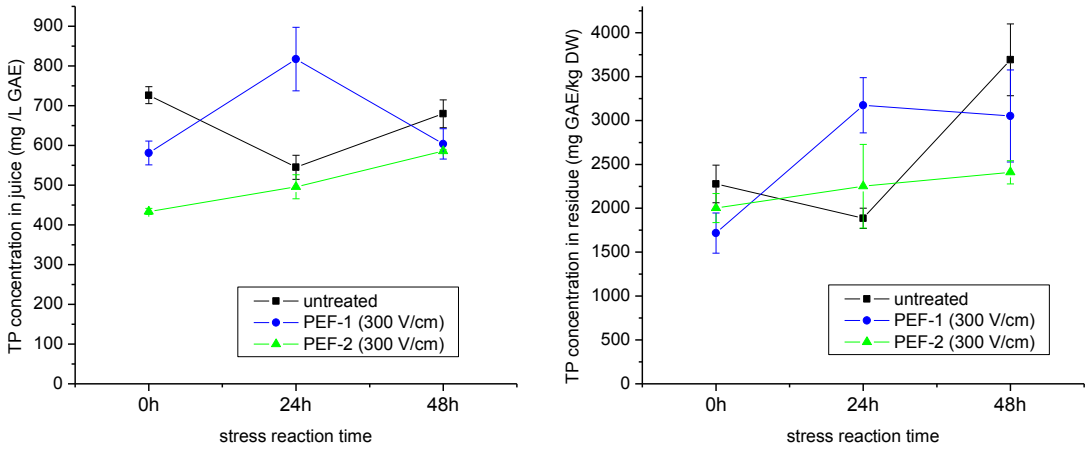
To better understand plant response to electric field strength of low intensities, an additional lot of apples (variety Golden Delicious) has been newly subjected to PEF treatments. To investigate repeatability of plant answer on controlled PEF stress conditions, apples from the same lot were divided in four batches and each batch was used to perform replications with one treatment intensity (20 pulses at 300, 700, 1200 or 4000 V/cm). Apple quarters (subchapter 3.4.2.1) were subjected to PEF and kept during stress reaction period after imposed treatment before juice was separated from apple residue and TP concentration determined.

4.1.4.1. Treatment with 300 V/cm

The impact of 20 pulses at 300 V/cm (resulting in total energy input of 0.014 kJ/kg) on TP concentration in apple juice and residue extracts is shown in Figure 4-4. Two replications of PEF treatments (PEF-1 and PEF-2) were performed in direct comparison to untreated control in order to trace variability of plant answer to same stress conditions (treatment intensity, stress reaction time).

Total polyphenol concentration in fresh prepared apple juice and residue extracts, immediately after PEF treatment of apple quarters, was lower than in corresponding untreated samples. Increase in TP concentration was observed in juice obtained 24 h after the treatment (Figure 4-4A). However, after PEF-1 TP concentration exceeded values of untreated samples, while after same treatment intensity in corresponding batch (PEF-2) TP concentration remained below values of untreated control. After 48 h stress reaction period, TP concentration in juice obtained from both replications was lower than in untreated control. Significant difference in TP concentration of PEF-1 and PEF-2

replicates can be noticed, indicating different response of apple tissue after 300 V/cm. A similar trend in corresponding apple residue extracts of untreated and PEF treated samples was observed (Figure 4-4B). Initial lower TP concentration observed immediately after treatment increased after 24 h and again after 48 h TP concentration of residue extracts was lower than in untreated control samples.



(A) (B)

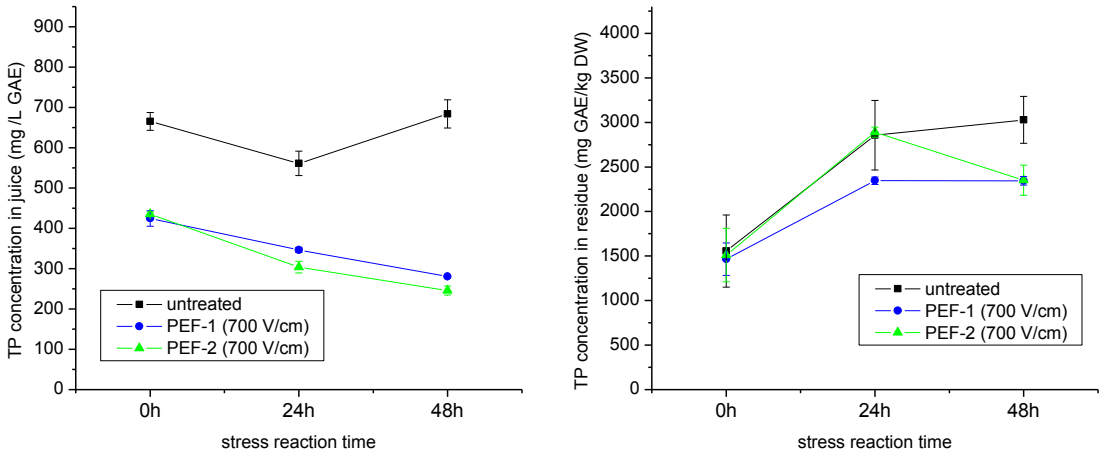
Figure 4-4: Total polyphenol (TP) concentration of untreated apples and apples subjected to PEF treatment of 300 V/cm and 20 pulses after stress reaction times of 0, 24 and 48 h (replications PEF-1 and PEF-2), measured in: (A) fresh prepared apple juice; and (B) corresponding apple residue extracts.

Large variability in cell shape and size (from 70 µm of cells under skin, to approximately 250 µm towards the centre of the flesh) has been described in apple tissue (McAtee, Hallett, Johnston & Schaffer, 2009). Since PEF was reported to be cell size and orientation dependant (Heinz et al., 2002; Chalermchat, Fincan & Dejmek, 2004), induction of reversible or irreversible permeabilization in multicellular tissue will depend on variations in cell structure of treated sample. Thereafter, two replications of PEF treatment at 300 V/cm must have induced different stress impact to multicellular tissue and provoked distinguishable response in terms of polyphenol accumulation.

4.1.4.2. Treatment with 700 V/cm

Total polyphenol concentration in apple juice obtained from apples treated with 20 pulses at 700 V/cm (total energy input of 0.074 kJ/kg) was significantly lower than in untreated samples (Figure 4-5A). Furthermore, same trend was observed between two replications (PEF-1 and PEF-2). Due to cell permeabilization larger TP concentration would be expected in juice obtained immediately after treatment and eventual losses caused by oxidation and enzymatic degradation after stress reaction period. However, insignificant difference of measured CDI value after 700 V/cm in comparison to untreated apples was observed (Figure 4-1) and lower TP concentration measured immediately after

treatment indicate occurrence of other metabolic processes. Triggering phytochemical defense with polyphenol utilization as potential scavengers of free radicals and other oxidative species may be responsible for lower TP concentrations in PEF treated samples and not permeabilization itself. Several groups have shown that reversible membrane permeabilization induced generation of intracellular and extracellular reactive oxygen species and changes in cell metabolites accumulation (Sabri et al., 1996; Gómez Galindo et al., 2009; Pakhomova et al., 2012), supporting this hypothesis.



(A) (B)

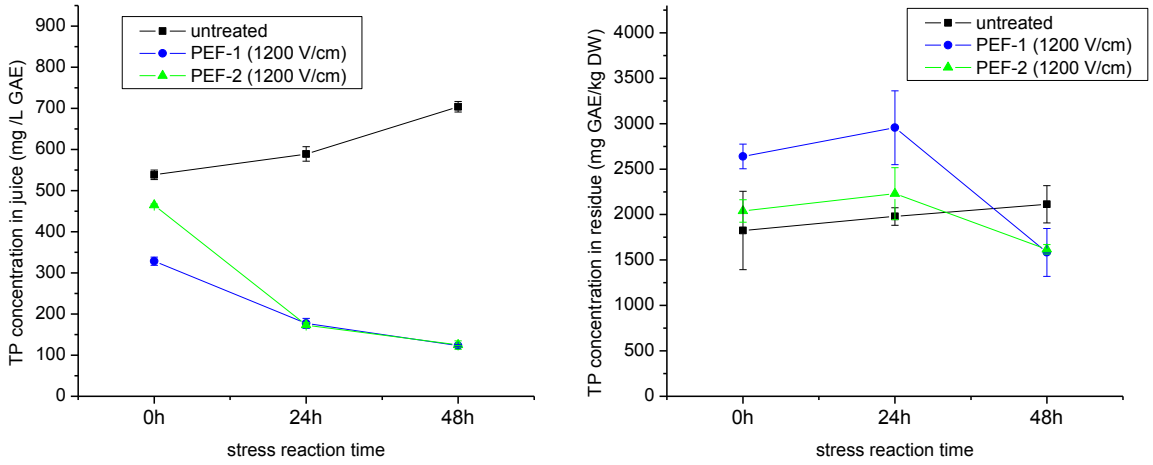
Figure 4-5: Total polyphenol (TP) concentration of untreated apples and apples subjected to PEF treatment of 700 V/cm and 20 pulses after stress reaction times of 0, 24 and 48 h (replications PEF-1 and PEF-2), measured in: (A) fresh prepared apple juice; and (B) corresponding apple residue extracts.

Total polyphenol concentration in corresponding apple residue did not differ from untreated samples obtained immediately after PEF treatment (Figure 4-5B). However, 24 h after PEF-1 and 48 h after PEF-2 lower TP concentration than in untreated control was observed. Again different response was observed in two replications after 24 h stress reaction time, measured in apple residue extracts.

Larger cells are located towards the centre of a fruit and a multilayer of small, thick-walled cells lies beneath the peel with reduced cell to cell contact (Glenn & Poovaiah, 1987). Since apple skin contains only limited fraction of juice and remains in residue after solid-liquid separation, it can be assumed that difference in TP concentrations of juice and residue extracts (as a response to PEF treatment) lies in cell size. Chalermchat et al. (2004) confirmed that lower electric field strength was necessary to permeabilize larger cells from the inner regions of apple, while smaller cells at the outer regions required larger electric field strengths to achieve same degree of permeabilization. Consequently, larger cells suffered larger degree of stress imposed by the same treatment intensity.

4.1.4.3. Treatment with 1200 V/cm

When larger treatment intensity was used (1200 V/cm; 20 pulses; total energy input 0.217 kJ/kg) lower TP concentration was observed in fresh pressed juice immediately after treatment and descend was observed during 48 h stress reaction period (Figure 4-6A). Destabilization of cellular functions by PEF treatment at 1200 V/cm occurred. In multicellular apple tissue certain number of larger cells suffered irreversible tissue damage (Figure 4-1), while other part which consists of smaller cells could have exhibited stress response. Regarding multilayer of smaller cells located bellow apple peal, larger TP concentration was observed in residue extracts immediately after PEF treatment and after 24 h stress reaction time (Figure 4-6B).



(A) (B)

Figure 4-6: Total polyphenol (TP) concentration of untreated apples and apples subjected to PEF treatment of 1200 V/cm and 20 pulses after stress reaction times of 0, 24 and 48 h (replications PEF-1 and PEF-2), measured in: (A) fresh prepared apple juice; and (B) corresponding apple residue extracts.

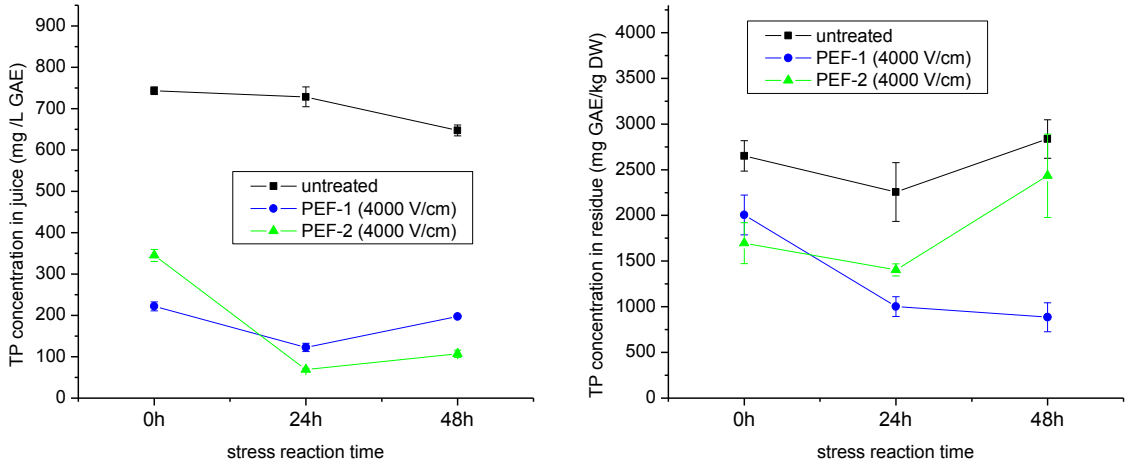
Lower TP concentration in juice from PEF treated apples decreased over stress reaction period (Figure 4-6) most probably due leakage of intracellular substances caused by membrane damage and consequently enzymatic oxidation of polyphenols. However, smaller cells must have suffered lower degree of perturbation caused by same treatment intensity and accumulation of polyphenolic substances in residue was observed.

4.1.4.4. Treatment with 4000 V/cm

After application of 20 pulses at 4000 V/cm (total energy input of 10.286 kJ/kg) lower TP concentration in juice and corresponding residue extracts was observed immediately after treatment and after stress reaction period (Figure 4-7).

Mechanical destruction of apple tissue in a customary juicer induces release of liquid from extracellular space together with the intracellular liquid from the vacuoles.

Oxidation processes due to decompartmentalization and enzyme substrate contact occur immediately after dejuicing of untreated and PEF treated apples, presumably to the same extent. However, when disintegration of cellular tissue occurs before juice is separated, uncontrolled oxidation processes may take place. Due to large degree of membrane permeabilization after 4000 V/cm and obtained disintegration index of 0.51 (Figure 4-1), release of oxidative enzymes and interaction with polyphenolic substances must have occurred, which resulted in significantly lower TP concentration of juice and residue extracts obtained from PEF treated apples (Figure 4-7).



(A) (B)

Figure 4-7: Total polyphenol (TP) concentration of untreated apples and apples subjected to PEF treatment of 4000 V/cm and 20 pulses after stress reaction times of 0, 24 and 48 h (replications PEF-1 and PEF-2), measured in: (A) fresh prepared apple juice; and (B) corresponding apple residue extracts.

4.1.5. Changes of TP concentration in juice and residue extracts due to PEF intensity

To compare different treatment intensities and impact of PEF as external stressor, changes of TP concentration expressed as percentage of polyphenolic increase/decrease in respect to untreated samples are represented in Figure 4-8. Variations in TP concentrations between replications (PEF-1 and PEF-2) are reduced when larger electric field strengths (700, 1200 and 4000 V/cm) were applied. Thereafter, values in Figure 4-8 represent mean value of PEF-1 and PEF-2 replications for corresponding field strengths applied. Due to large variation in plant response after 300 V/cm, data points for two replications are shown separately (black triangles connected with dotted line), and mean value (black squares connected with straight line) representing trend of plant response.

Increase in TP concentration was observed after 300 V/cm in juice and corresponding residue, during 24 h after the treatment (Figure 4-8). 50 % larger TP

concentration was observed in juice and 68 % in residue extracts 24 h after PEF-1 treatment at 300 V/cm. Although significant differences in reached TP concentrations 24 h after 300 V/cm in PEF-1 and PEF-2 was observed, similar trend can be noticed, suggesting *de novo synthesis* of protective substances.

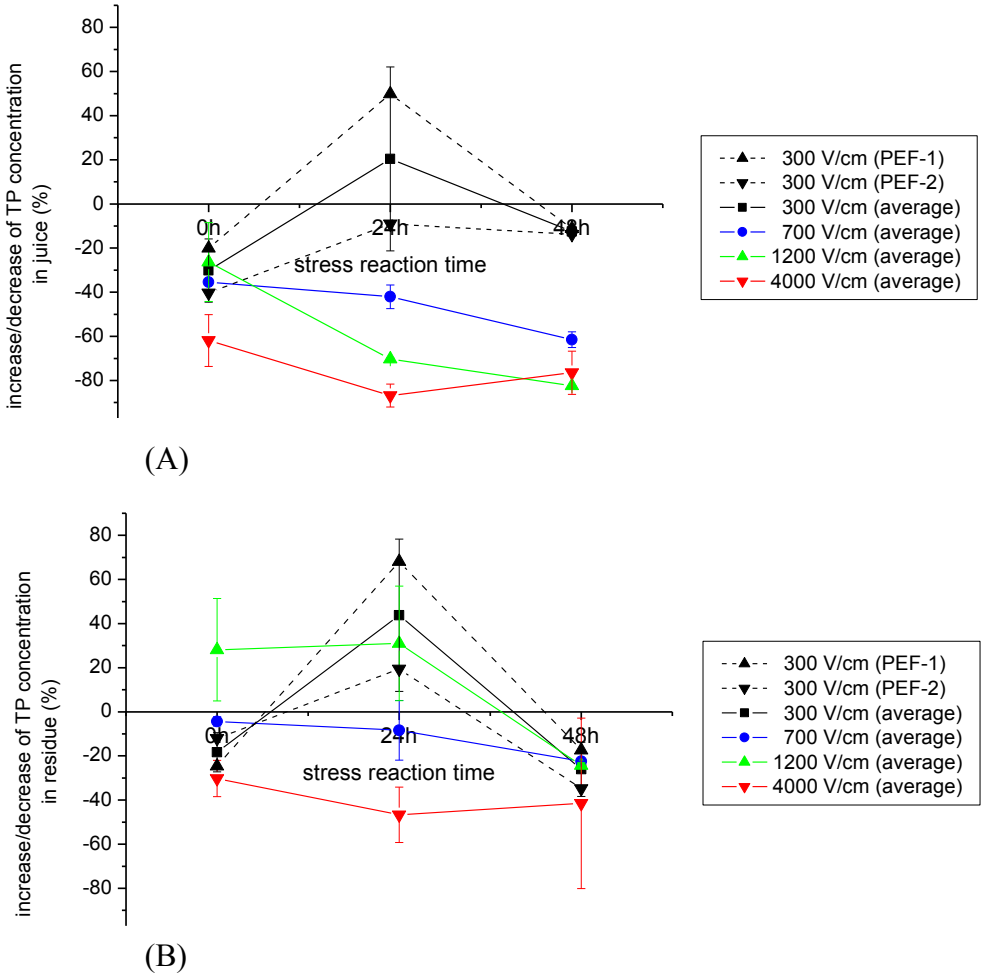


Figure 4-8: Changes of total polyphenol (TP) concentration due to PEF treatment, during stress reaction period in: (A) fresh prepared apple juice; and (B) corresponding apple residue extracts. PEF-1 and PEF-2 represent replications of PEF treatment at 300 V/cm.

After application of 700 V/cm, lower TP concentration was observed with slight decrease over time, indicating occurrence of plant adjustment to strained conditions through depletion phase, which is observed in juice to a larger extent than in residue extracts. When larger treatment intensity was applied, 26 % lower TP concentration in juice detected immediately after treatment with 1200 V/cm decreased to 82 % lower concentration after 48 h (Figure 4-8A), indicating exhaustion of large cell fraction. However, larger TP concentration was observed in corresponding residue (Figure 4-8B), which can be explained through specific impact of PEF treatment depending on the cell size. Smaller cells in apple skin suffered lower degree of stress (possible reversible permeabilization), while larger cells from apple flesh suffered larger degree of stress and

irreversible disruption of cell membranes. Much larger treatment intensity (4000 V/cm) provoked exhaustion and chronic damage of multicellular tissue. 87 % and 47 % lower TP concentration in juice and residue extracts occurred, respectively, 24 h after PEF treatment.

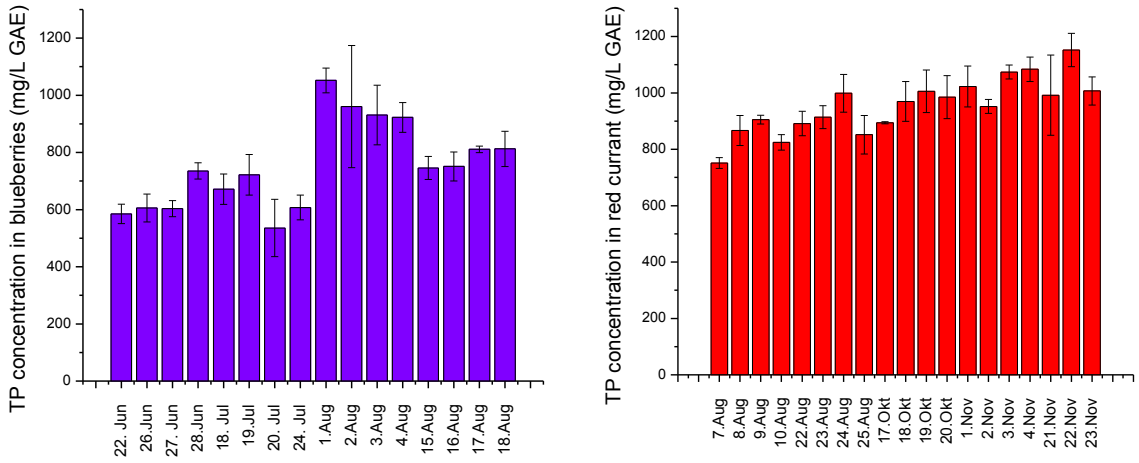
Different responses of plant organism to changed environmental conditions which differ from normal conditions of life have been explained in literature through physical principle of matrix elasticity (Schulze, Beck & Müller-Hohenstein, 2005). If the stress intensity is low, tolerance and elasticity of a material (in this case plant) will withstand enforced stress and adapt its metabolic activity to the change without experiencing permanent damage. However, if the stress reaches its maximum of what material (plant) is able to withstand, elastic limit is reached and irreversible damage will occur. This principle was observed by increasing PEF intensity, confirming stress response through dose dependency. Largest increase in TP concentration was observed after lowest treatment intensity used (300 V/cm), when no significant disintegration of cellular material was observed (Figure 4-1). Varying number of intact cells increased resistance while inducing higher productivity level. By increasing treatment intensity larger stress impact was induced, which resulted in plant adjustment after 700 V/cm, exhaustion of large cells fraction after 1200 V/cm and chronic damage after 4000 V/cm (Figure 4-8).

Since polyphenolic substances are recognized as effective antioxidant and anti-inflammatory substances with preventive roles against certain cancers and cardiovascular diseases (Cisneros-Zevallos, 2003), their enhancement in plant tissue may enhance the nutritional value of food. Thereafter increase in TP concentration in stressed fruit has been considered within this thesis as positive effect (eustress) which offers a potential to increase health benefit properties of stressed products.

Due to different cell size and natural protective function in flesh and skin, induction of stress response by PEF treatment onto multicellular systems needs to be optimized in order to achieve repeatable and controlled plant response. To further investigate plant resistance on PEF treatment as external abiotic stress factor, cell suspension cultures with almost homogenous cell size distribution and controlled growth conditions were investigated (see subchapter 4.5). Furthermore, selected enzyme activity, cell viability and other factors were measured, in order to get better insight in stress mechanism caused by PEF treatment.

4.2. PEF impact on berries

Due to the fact that different cell types exhibit diverse electroporation behaviour when exposed to PEF treatment (Weaver, 2000), two of the main berry fruits with a diverse range of micronutrients have been chosen as additional "polyphenolic factories" to investigate the effect of PEF as stressor. Blueberries (*Vaccinium corymbosum*) and red currant (*Ribes rubrum*) were obtained from local supermarket, from different commercial cultivars over one growth season. Environmental growth conditions can impact levels of total polyphenolic compounds (Cisneros-Zevallos, 2003), which was observed through large variability in TP concentration obtained from different batches of fruits (Figure 4-9). To limit the impact of raw material variability and assure same postharvest conditions, each batch was used for one set of experiments (replications of chosen treatment intensity with corresponding untreated control samples during stress reaction period).



(A) (B)

Figure 4-9: Total polyphenol (TP) concentration of: (A) Blueberry (*Vaccinium corymbosum*) juice; and (B) Red currant (*Ribes rubrum*) juice; obtained from local market over one growth season.

Berry fruits contain large amount of pectin which makes it difficult to obtain juice economically including liquid from vacuoles. Thus common juice winning procedures include enzymatic pretreatment supported by high temperatures. Since target of this study was not to win large volume of juice, but to investigate impact of PEF on polyphenolic substances from berry flesh, gentle pressing method was used, followed by centrifugation to separate solids. Total polyphenol concentration was measured in juice obtained from untreated and PEF treated berries during stress reaction period of 48 h, representing polyphenol content of berry flesh. To observe impact of temperature during stress reaction period, berries were kept at room temperature (RT) and in parallel at 4 °C, for chosen time after the treatment. The impact of coldness and PEF, two different postharvest abiotic

stressors and their interaction, on levels of polyphenolic compounds in berries was studied. Furthermore, the effect of pulse number while maintaining electric field strength has been investigated.

4.2.1. Degree of membrane permeabilization in blueberries

To determine range of PEF treatment intensities required for stress induction (with no detrimental disintegration of cell membranes in berry tissue) impedance measurements were carried out. Extent of membrane poration was measured within 60 minutes after exposure to PEF treatment (stored at room temperature) and compared to corresponding untreated blueberry tissue (Figure 4-10). Immediately after PEF treatment with 300, 500 and 1000 V/cm (with 4 and 7 pulses at each electric field strength applied) CDI values were lower than 0.02, indicating insignificant membrane permeabilization of less than 2 % of the cells. During measurement period of 60 minutes no significant changes from initial CDI value were observed. After application of 2000 V/cm, larger CDI value of approximately 0.06 was measured immediately after the treatment, with insignificant increase to 0.09 after 60 minutes. Increasing treatment intensity by increasing electric field strength to 4000 V/cm, the cell disintegration index of 0.20 has been obtained immediately after 7 pulses and of 0.38 after application of 20 pulses at corresponding electric field strength.

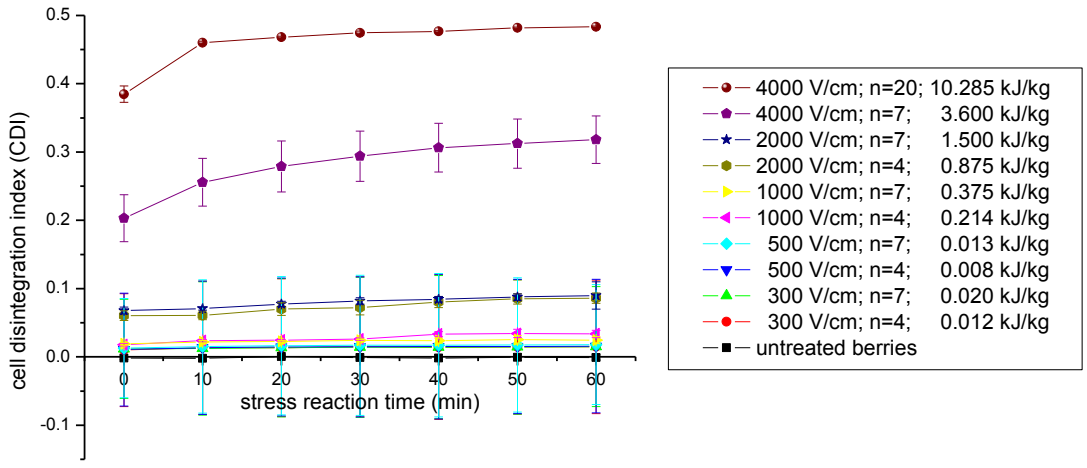


Figure 4-10: Impact of PEF treatment on the extent of membrane permeabilization in blueberries (*Vaccinium corymbosum*), measured as cell disintegration index (CDI), during 60 minutes stress reaction period.

Using this technique, CDI was determined in blueberry tissue, however, it was not suitable for red currant due to smaller fruit size and tissue damage induced during sample insertion into impedance measurement equipment. Since no significant changes in cell

membrane permeabilization were observed after PEF treatments with 300, 500 and 1000 V/cm, these treatment intensities were chosen to be suitable for stress induction in berries.

4.2.2. Impact of stress by coldness and PEF on blueberries

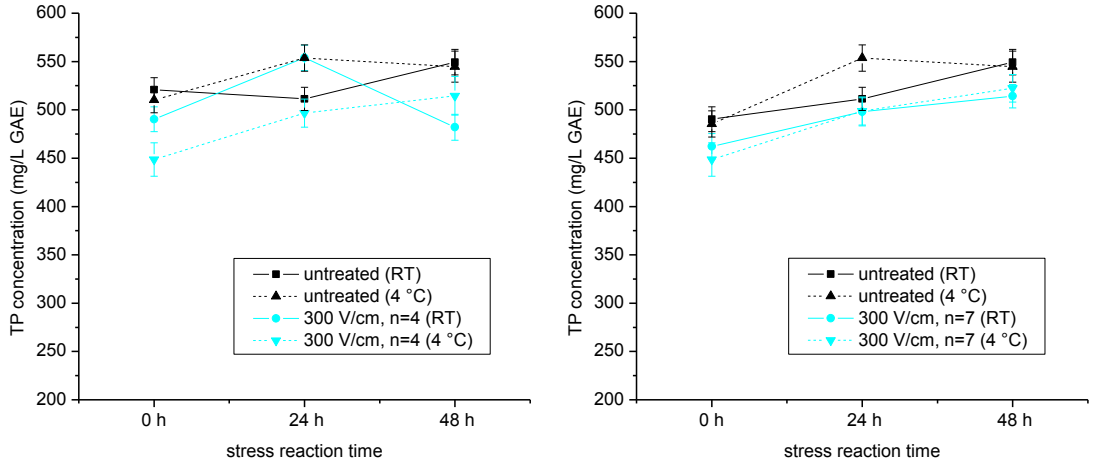
The impact of two different abiotic factors was studied in order to enhance nutraceutical content of fresh berries during postharvest storage. Since fluid structure of cellular membrane is reduced at cold temperature, causing rigidity of cell structure (Chinnusamy et al., 2007), it was assumed that PEF treatment may exhibit different response during cold postharvest storage temperature. To investigate impact of PEF, cold storage and PEF during cold storage, one batch of berry fruits was separated into four parts. First part of the batch has been subjected to PEF treatment and stored at room temperature (RT) for stress reaction period, second represents corresponding untreated control, third was stored at 4 °C after PEF treatment and forth represents corresponding untreated control stored at 4 °C. Due to raw material variability and differences in fruit fitness obtained from local market, each batch has been used for one treatment intensity, to assure representative control samples.

4.2.2.1. Treatment with 300 V/cm

Total polyphenol concentration of blueberry juice obtained immediately after 4 pulses at 300 V/cm was 6 % lower than in untreated samples (Figure 4-11A). After 24 h stress reaction time at room temperature polyphenol concentration of PEF stressed berries exceeded values of untreated samples for 8 %, indicating accumulation of polyphenolic substances. When untreated berries were kept at 4 °C for 24 h, similar observations were made and initial lower TP concentration exceeded values of untreated berries kept at RT. It seems that PEF treatment and coldness might be supportive for enhancement of nutraceutical content of blueberries after 24 h storage. However, when samples were stored at 4 °C after PEF treatment, initial 14 % lower TP concentration increased over 48 h stress reaction period, and did not exceed values of untreated controls.

By increasing pulse number at the same electric field strength applied (7 pulses at 300 V/cm), lower TP concentration during 48 h stress reaction period at both storage temperatures was observed (Figure 4-11B). It seems that similar response can be obtained after cumulative stress from coldness and 4 pulses at 300 V/cm (Figure 4-11A), and after 7 pulses at corresponding field strength (Figure 4-11B). This observation contributes to

the theory of cumulative stress (Schulze et al., 2005), indicating that coldness can be assigned as additional stress factor and increase stress intensity.



(A) (B)

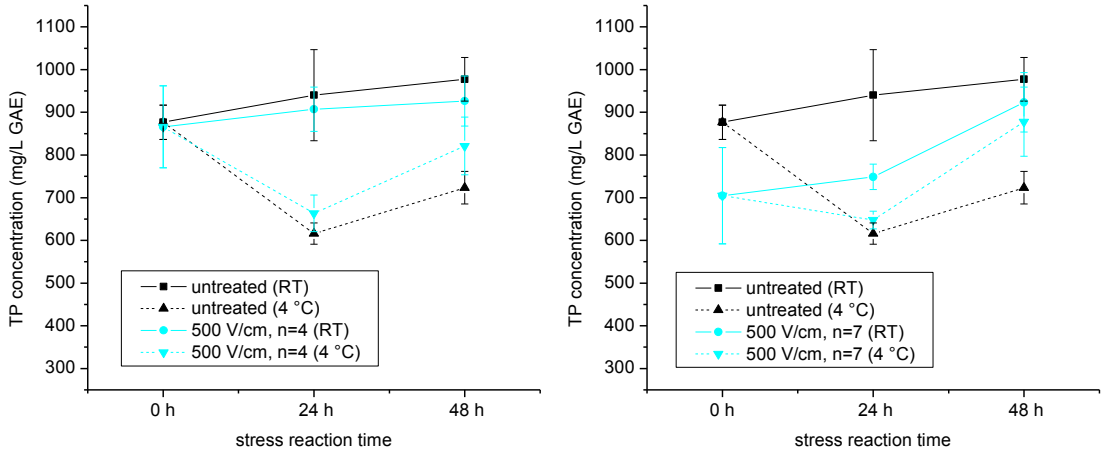
Figure 4-11: Total polyphenol (TP) concentration of blueberry juice obtained from untreated and PEF treated fruits with: (A) 300 V/cm, 4 pulses, total energy input of 0.012 kJ/kg; and (B) 300 V/cm, 7 pulses, total energy input of 0.020 kJ/kg; during stress reaction period at room temperature (RT) and at 4 °C.

4.2.2.2. Treatment with 500 V/cm

Different batch of berries obtained from local market was subjected to PEF treatment with 500 V/cm (4 and 7 pulses, respectively) and changes in TP concentrations were observed during stress reaction period (stored at RT and 4 °C). Since exact storage conditions and possible stress fruit has suffered before tested in lab facilities are not known, each batch needs to be separately observed. Total polyphenol concentrations of juice obtained from untreated berries stored at 4 °C for 24 and 48 h were significantly lower than in juice obtained from untreated berries stored at room temperature (Figure 4-12). In contrast to this batch of tested fruits, different response was observed in previously tested batch as a reaction to coldness (Figure 4-11). A variation in sensitivity to stress and several outcomes of plant stress response has been often associated to the plant fitness (Basra & Basra, 1997; Larcher, 2003; Beck et al., 2005). Furthermore, effect of temperature during storage was shown to depend on the developmental stage of the product. Prono-Widayt *et al.* (2003) reported that ripe pepino fruits were not affected by storage temperature, while stronger increase of β-carotene in premature and mature fruits was observed during storage at 18 °C, in comparison to storage at 5 °C. Thereafter, different response of tested blueberry batches can be attributed to different initial physiological condition of fruits.

Application of 4 pulses at 500 V/cm resulted in no different TP concentration measured immediately after treatment, when compared to corresponding reference (Figure

4-12A). When fruits were left for stress reaction period at room temperature, lower TP concentration was observed after 48 h in comparison to reference stored at RT for the same period of time. However, storage at 4 °C for 48 h after PEF resulted in 14 % larger TP concentration than in corresponding untreated berries (stored at 4 °C).



(A) (B)

Figure 4-12: Total polyphenol (TP) concentration of blueberry juice obtained from untreated and PEF treated fruits with: (A) 500 V/cm, 4 pulses, total energy input of 0.008 kJ/kg; and (B) 500 V/cm, 7 pulses, total energy input of 0.013 kJ/kg; during stress reaction period at room temperature (RT) and at 4 °C.

Aforementioned changes were even more pronounced when stress intensity was enhanced by applying 7 pulses at 500 V/cm. 20 % lower polyphenol concentration was observed immediately after PEF treatment (Figure 4-12B). This difference was kept after 24 h and slowly approached values of untreated berries after 48 h storage at room temperature. Initial lower TP concentration observed immediately after PEF treatment, exceeded values of untreated samples after 24 h at 4 °C, and rose to 21 % larger TP concentration than in corresponding reference after 48 h. Again coldness contributed to different plant response during stress reaction period and together with PEF treatment increased stress intensity.

4.2.2.3. Treatment with 1000 V/cm

Increasing electric field strength applied to provoke stress response to 1000 V/cm and 4 pulses, 13 % larger polyphenol concentration was observed immediately after treatment (Figure 4-13A). Increased concentration of polyphenolic compounds was kept after 24 h stress reaction time at room temperature, and reached difference of 26 % to corresponding reference after 48 h. When berries were kept at 4 °C during stress reaction period after PEF treatment, initial larger TP concentration approached values of untreated control after 24 h.

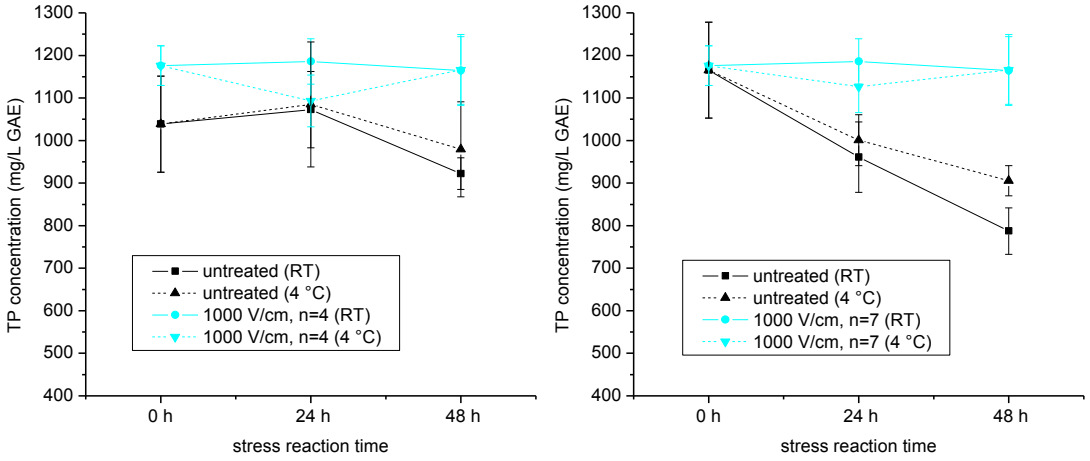


Figure 4-13: Total polyphenol (TP) concentration of blueberry juice obtained from untreated and PEF treated fruits with: (A) 1000 V/cm, 4 pulses, total energy input of 0.214 kJ/kg; and (B) 1000 V/cm, 7 pulses, total energy input of 0.375 kJ/kg; during stress reaction period at room temperature (RT) and at 4 °C.

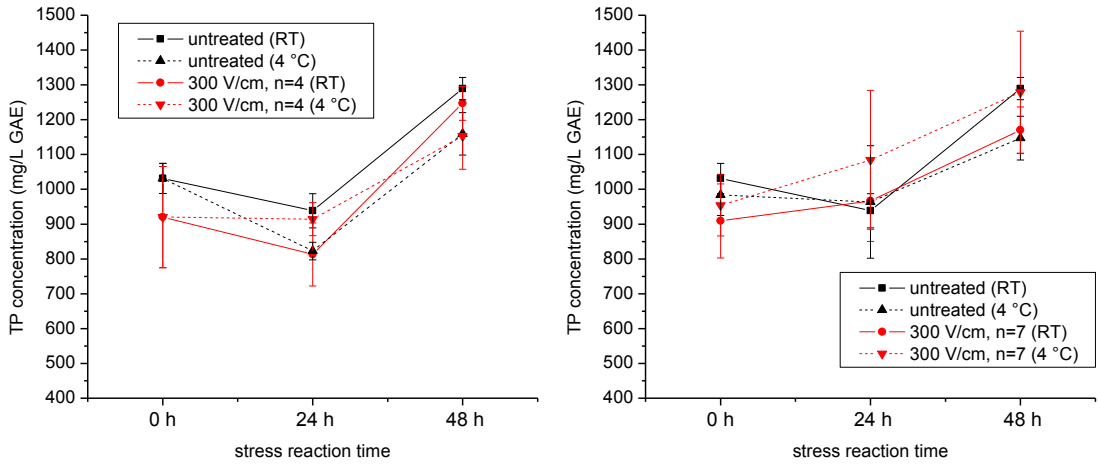
Additional enhancement of stress intensity by increasing number of pulses (7 pulses at 1000 V/cm) resulted in no change in TP concentration immediately after imposed treatment (Figure 4-13B). After 24 h stress reaction time at room temperature, TP concentration of PEF stressed berries was 23 % larger and reached its maximum after 48 h, when 48 % larger TP concentration was observed in stressed fruits. After storage at 4 °C during stress reaction period similar behavior was observed, however, 29 % larger polyphenol concentration (in comparison to corresponding reference stored at 4 °C) obtained after 48 h did not exceed maximum obtained during storage at room temperature.

Larger TP concentration during storage time has been obtained after imposed stresses for the tested batch of blueberries. Although CDI value of less than 0.02 has been observed after chosen treatment intensities (Figure 4-10), certain number of permeabilized cells might have exhibited membrane leakiness to polar compounds. Such an enhanced diffusion of intracellular substances could have resulted in larger TP concentration in PEF treated berries. However, since no change in TP concentration was observed immediately after 7 pulses at 1000 V/cm, it can be suggested that other mechanism was involved, which resulted in increased concentration of polyphenolic substances. Similar observations were reported due to temperature change during postharvest storage of different berry fruits (such as blueberries, raspberries and strawberries), where higher anthocyanin and phenolic content was observed in fruits stored at temperatures >15 °C, in comparison to cold storage at 0 - 6 °C (Kalt et al., 1999; Cordenunsi, Genovese, do Nascimento, Hassimotto, dos Santos & Lajolo, 2005). Even though different abiotic stressor was imposed, similar response in terms of phenolic accumulation was noticed.

4.2.3. Impact of stress by coldness and PEF on red currant

4.2.3.1. Treatment with 300 V/cm

Total polyphenol concentration in juice obtained from untreated red currant berries after 24 and 48 h storage at 4 °C was 10 to 12 % lower than after storage at RT (Figure 4-14A). Application of 4 pulses at 300 V/cm resulted in 11 % lower TP concentration immediately after treatment and was kept lower during stress reaction period at RT. However, when coldness was introduced as second stress factor during reaction period after PEF, initial 11 % lower TP concentration exceeded values of corresponding control samples after 24 h storage at 4 °C. This batch of examined red currant fruits exhibited similar behavior after imposed stressors (coldness, PEF treatment and their combination) through reduced TP concentration measured over stress reaction period.



(A) (B)

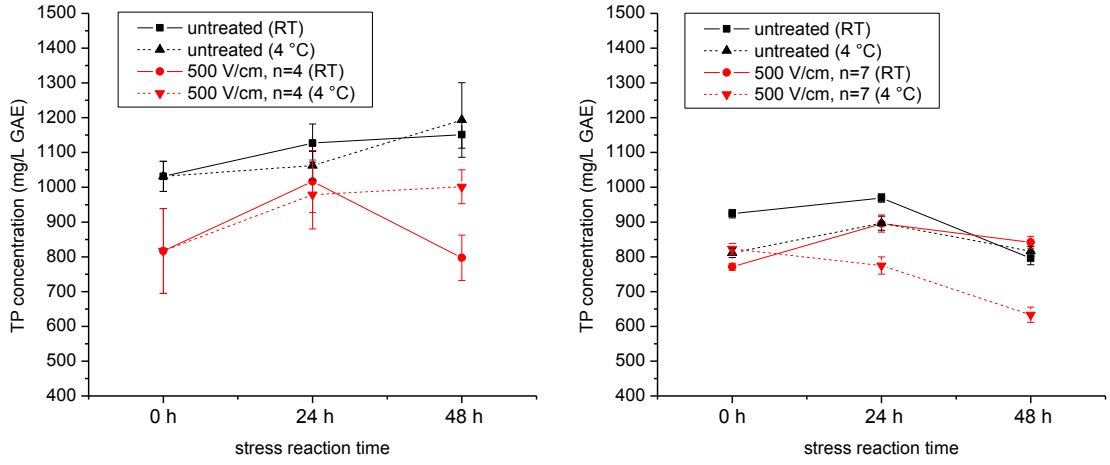
Figure 4-14: Total polyphenol (TP) concentration of red currant juice obtained from untreated and PEF treated fruits with: (A) 300 V/cm, 4 pulses, total energy input of 0.012 kJ/kg; and (B) 300 V/cm, 7 pulses, total energy input of 0.020 kJ/kg; during stress reaction period at room temperature (RT) and at 4 °C.

Impact of larger treatment intensity (7 pulses at 300 V/cm) and coldness on red currant fruits during stress reaction period is shown in Figure 4-14B. TP concentration of juice obtained from untreated fruits after 48 h storage at 4 °C was lower than in corresponding samples stored at RT. Similar response can be observed 48 h after 7 pulses at 300 V/cm, stored at RT. However, when fruits were kept at 4 °C during stress reaction period, 16 % larger TP concentration was observed 24 h after imposed PEF treatment. Again it can be noticed that coldness (during 48 h storage at 4 °C) exhibited similar response as PEF treatment (300 V/cm, 7 pulses, during 48 h storage at RT), whereas combination of aforementioned stressors induced different stress response.

4.2.3.2. Treatment with 500 V/cm

Two different batches of red currant berries were used to investigate impact of coldness and PEF treatment at 500 V/cm with 4 and 7 pulses, respectively. Different levels of TP concentration can be observed between batch-B1 (Figure 4-15A) and batch-B2 (Figure 4-15B) during stress reaction period at room temperature (RT) and in parallel at 4 °C (in figures addressed as untreated (RT) and untreated (4°C)). Similar observations were made between different batches of blueberries (subchapter 4.2.2). Environmental growth conditions and postharvest storage can impact total polyphenol content of fruits (Cisneros-Zevallos, 2003) and observed differences in subsequent postharvest storage can be attributed to different initial physiological fitness of fruits.

Impact of 4 pulses at 500 V/cm is shown in Figure 4-15A. TP concentration in juice obtained immediately after PEF treatment of red currant was 21 % lower than in corresponding reference. During stress reaction period TP concentration was kept lower at both storage temperatures, and after 48 h at RT this difference was even more pronounced and reached 31 % lower polyphenol concentration than in untreated samples.



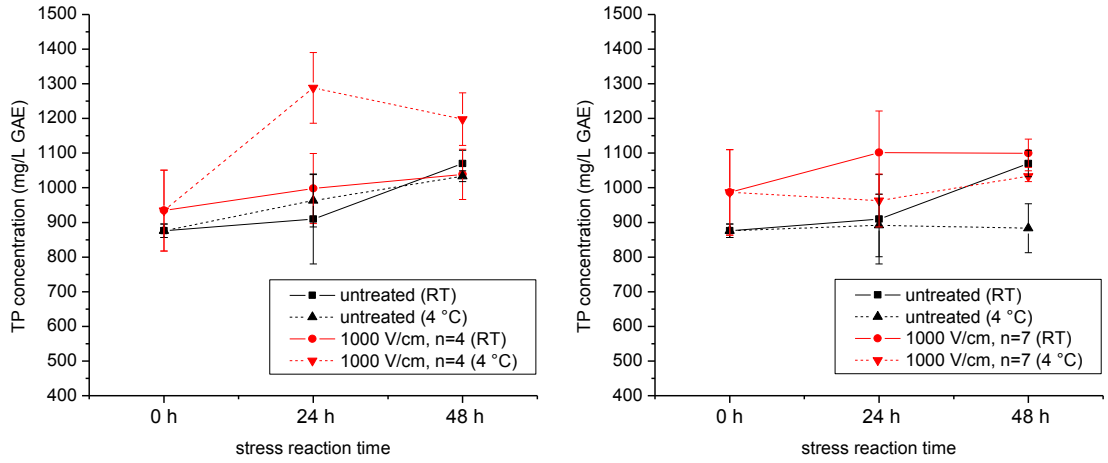
(A) (B)

Figure 4-15: Total polyphenol (TP) concentration of red currant juice obtained from untreated and PEF treated fruits with: (A) 500 V/cm, 4 pulses, total energy input of 0.008 kJ/kg; and (B) 500 V/cm, 7 pulses, total energy input of 0.013 kJ/kg; during stress reaction period at room temperature (RT) and at 4 °C.

Initially 17 % lower TP concentration obtained immediately after 7 pulses at 500 V/cm increased during stress reaction period at RT and approached values of untreated control after 48 h (Figure 4-15B). Similar response during stress reaction period was achieved after cold storage of untreated berries. When PEF treated samples were kept at 4 °C during stress reaction period, initially lower TP concentration was kept below values of untreated controls, indicating cumulative effect of PEF and coldness.

4.2.3.3. Treatment with 1000 V/cm

Concentration of total polyphenols in juice obtained immediately after 4 pulses at 1000 V/cm, imposed to red currant berries, was 7 % larger than in corresponding reference (Figure 4-16A). After 24 h stress reaction time at RT polyphenol concentration of PEF stressed berries remained elevated and approached values of untreated control after 48 h. When PEF treated berries were kept for 24 h at 4 °C, 34 % larger TP concentration has been observed in juice in comparison to untreated control stored at 4 °C. Cumulative effect of PEF and coldness resulted in largest accumulation of polyphenolic substances after 24 h.



(A) (B)

Figure 4-16: Total polyphenol (TP) concentration of red currant juice obtained from untreated and PEF treated fruits with: (A) 1000 V/cm, 4 pulses, total energy input of 0.214 kJ/kg; and (B) 1000 V/cm, 7 pulses, total energy input of 0.375 kJ/kg; during stress reaction period at room temperature (RT) and at 4 °C.

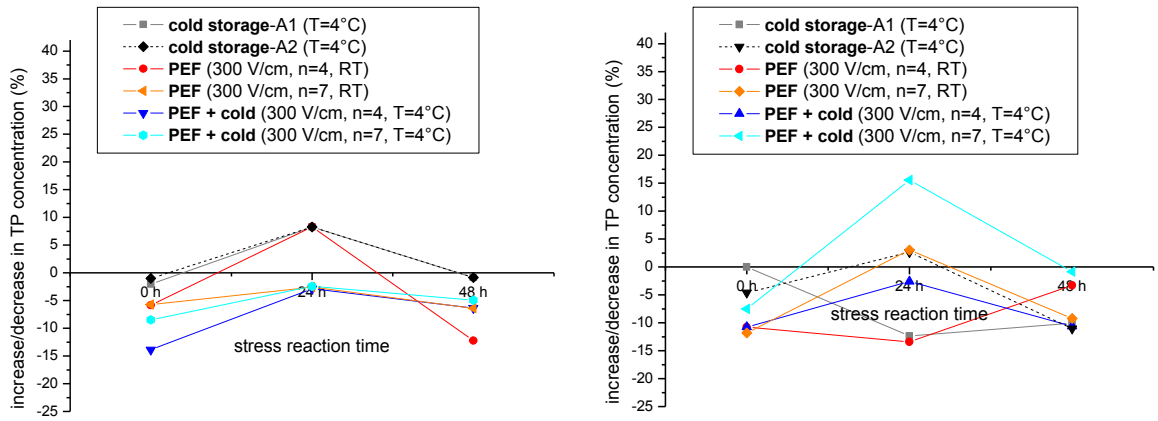
Immediately after 7 pulses at 1000 V/cm, 13 % larger TP concentration was observed (Figure 4-16B). After 24 h stress reaction time at RT difference increased to 21 % and approached values of untreated berries 48 h after the treatment. Similar observations were made after 4 pulses at corresponding field strength and storage conditions (Figure 4-16A). When PEF treated samples (7 pulses, 1000 V/cm) were kept at 4 °C for stress reaction period, 17 % larger TP concentration was observed after 48 h.

Level of TP concentration measured from untreated berries differed from batch to batch, which were associated with plant initial physiological state. In order to combine all aspects together and compare stress intensity within two types of tested berries, results are represented in the next subchapter through biological stress concept.

4.2.4. Stress induced changes of TP concentration in blueberries and red currant

To observe the impact of coldness, PEF treatment and interaction among these two abiotic stressors during storage time (expressed as stress reaction period), stress exposed fruits were compared to untreated fruits stored at room temperature. Since gentle pressing method followed by centrifugation was used to obtain juice, during which liquid is released from berry flesh, TP concentration of berry juice will be in further discussion considered as polyphenolic pool enclosed within berry.

Lower total polyphenol concentration could have been observed immediately after PEF treatment with 300 V/cm (Figure 4-17) displaying predomination of catabolic over anabolic processes in tested berries, indicating plant effort to sustain stress (alarm phase of biological stress concept). Increase in TP concentration after 24 h stress reaction time at RT was observed in blueberries treated with 4 pulses at 300 V/cm (Figure 4-17A). Due to increased level of polyphenolic substances it seems that repair processes were initiated through *de novo* synthesis of protective substances. Similar response can be observed after coldness as well, representing recovery phase of stressed blueberries. However, initial destabilization of functional properties may persist over stress reaction period which can be seen in stressed blueberries after 7 pulses at 300 V/cm, and after cumulative effect of coldness and PEF treatment. Changes in TP concentration through biological stress concept were shown to be dependant on PEF intensity, and coldness can be assigned as additional stress factor contributing to cumulative effect when applied together with PEF treatment.



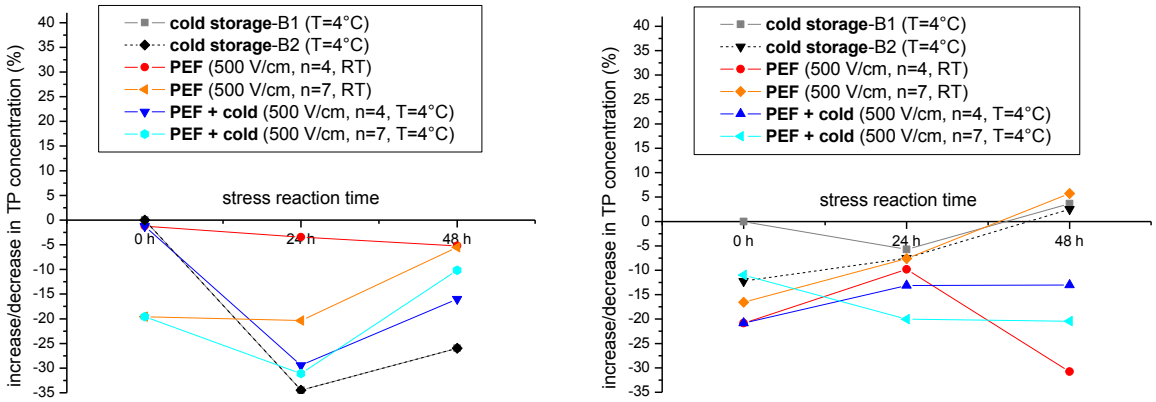
(A) (B)

Figure 4-17: Changes of total polyphenol (TP) concentration in: (A) blueberries and (B) red currant; due to cold storage at 4 °C (two batches A1 and A2), PEF treatment of different intensities (300 V/cm, n=4 and 7 pulses, respectively), and their cumulative effect (PEF+cold), during stress reaction period of 48 h.

During stress reaction period, two tested batches of red currant fruits (A1 and A2) exhibited different response after cold storage (Figure 4-17B). Production of protective substances was observed after 24 h cold storage at 4 °C in batch A1, while distress was observed through decreased TP concentration during stress reaction period in batch A2, indicating predomination of catabolic processes. Variation in response to the same stress is attributed to the initial plant fitness and several outcomes are possible (Larcher, 2003), which were observed through different TP concentration in tested batches.

When PEF treatment of 4 pulses at 300 V/cm was applied to red currant, initial destabilization through alarm phase remained present during stress reaction period and reached normalization after 48 h (Figure 4-17B). Introducing coldness as additional stress factor to the same PEF treatment intensity, increase in TP concentration can be observed after 24 h indicating start of repair phase. Increasing treatment intensity to 7 pulses at 300 V/cm, plant resistance through larger production of phenolic substances can be observed. After including coldness to PEF treatment at 300 V/cm and 7 pulses, recovery phase was even more pronounced and TP concentration reached maximum after 24 h. Additions of stressors (PEF and coldness) were synergistically causing larger impact on red currant and consequently different response occurred.

Functional properties of tested berries were destabilized by imposed PEF treatment with 500 V/cm and coldness as well (Figure 4-18).



(A) (B)

Figure 4-18: Changes of total polyphenol (TP) concentration in: (A) blueberries and (B) red currant; due to cold storage at 4 °C (two batches B1 and B2), PEF treatment of different intensities (500 V/cm, n=4 and 7 pulses, respectively), and their cumulative effect (PEF+cold), during stress reaction period of 48 h.

Immediately after 4 pulses at corresponding field strength no changes of TP concentration in blueberries were noticed, while slight decrease during stress reaction period at RT was observed (Figure 4-18A). Increasing stress intensity by adding coldness

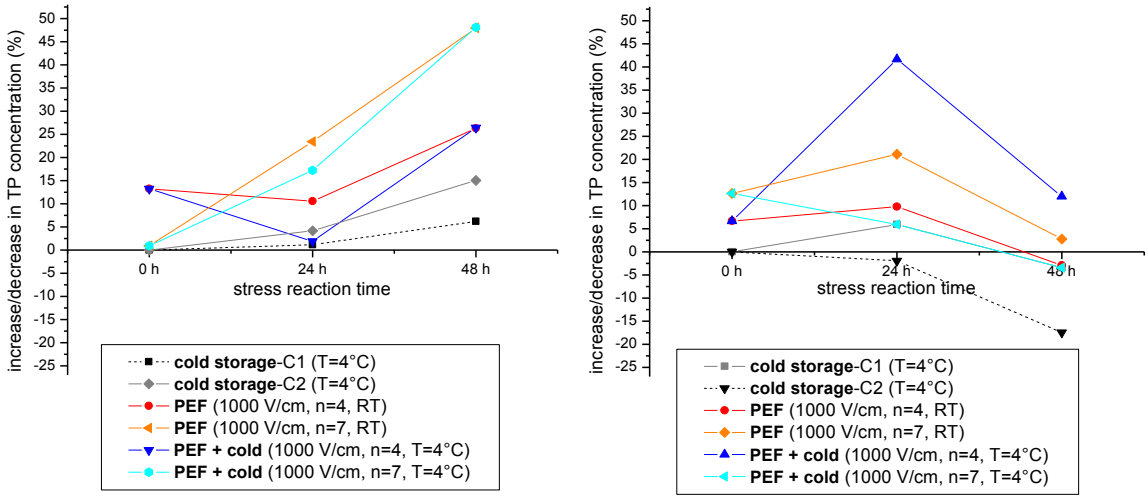
during stress reaction period after 4 pulses at 500 V/cm, alarm phase through strong depletion of phenolic substances can be observed after 24 h. Depletion during alarm phase was reached faster (immediately after treatment) when larger stress by 7 pulses at 500 V/cm was imposed. Addition of coldness to PEF (500 V/cm, 7 pulses) resulted in even larger depletion than PEF itself. Stress dose dependency can be recognized, as mentioned above, where larger number of pulses at corresponding field strength and cumulative effect of two stressors resulted in stronger impact on concentration of polyphenolic substances. Plant recovery and repair phase can be recognized between 24 and 48 h stress reaction time through increase in TP concentration.

Two tested batches of red currant (B1 and B2) exhibited again slightly different response after cold storage (Figure 4-18B). Stronger impact was observed in batch B2, where depletion of TP was noticed after 0 h, while in batch B1 after 24 h cold storage, representing alarm phase of the biological stress concept. However, normalization occurred after 48 h stress reaction period in both tested batches, when TP concentration reached values of untreated control.

Alarm phase can be observed in all treated red currant fruits immediately after PEF treatments with 500 V/cm (Figure 4-18B). Destabilization of red currant metabolic activities caused by PEF remained during tested period of 48 h, with the exception of fruits which were stored at RT after 7 pulses (at corresponding field strength), where TP content retrieved into normal range.

Destabilization of functional properties is accompanied by plant adaptation to unfavorable environment. Polyphenolic substances have been recognized as strong antioxidants and are involved in phytochemical defense through neutralization of reactive oxygen species (Mittler et al., 2004). Plant exposure to abiotic stress often results in increased production of free radicals, which formation has been promoted after PEF exposure (Sabri et al., 1996). Thereafter, depletion of phenolic substances in the early stage of stress event after PEF treatment at 500 V/cm may be attributed to cell utilization of protective substances in order to overcome stress and adapt to strained conditions. However, by increasing PEF treatment intensity to 1000 V/cm, no depletion has been observed in both tested berries (Figure 4-19). Larger TP concentration obtained in blueberries immediately after 4 pulses at 1000 V/cm, maintained higher productivity level in resistance phase during 24 h, and increased again after 48 h stress reaction time at RT (Figure 4-19A). By increasing number of pulses (7 pulses at 1000 V/cm), no change was observed immediately after treatment, however, faster increase followed over 48 h stress

reaction period (Figure 4-19A). When blueberries were stressed with the corresponding treatment intensity and kept for stress reaction period at 4 °C, similar behavior can be observed with slower increase in TP concentration. Due to different PEF intensity applied, fruits exhibited initial reaction of responsiveness within different time scale. Similar observations were reported after diverse abiotic stressors, where an instance or single occasion for a response event may vary from seconds to days (Basra & Basra, 1997). Recovery and fruit resistance were manifested through *de novo* synthesis of protective substances which was maintained in higher productivity level over longer period of time due to intensity of applied treatment. Competitiveness between production and utilization of protective substances results in increase or decrease of TP concentration in tested fruits at the chosen stress reaction time. Responsive reaction was shown to be dependant on PEF intensity, where increase in TP concentration was observed after 1000 V/cm (Figure 4-19) and decrease after 500 V/cm (Figure 4-18).



(A) (B)

Figure 4-19: Changes of total polyphenol (TP) concentration in: (A) blueberries and (B) red currant; due to cold storage at 4 °C (two batches C1 and C2), PEF treatment of different intensities (1000 V/cm, n=4 and 7 pulses, respectively), and their cumulative effect (PEF+cold), during stress reaction period of 48 h.

Similar to changes induced in blueberries, larger TP concentration was observed in red currant immediately after 1000 V/cm (Figure 4-19B). When PEF stressed red currant fruits were left at RT during 24 h, higher productivity level was maintained (resistance phase) and after 48 h TP content retrieved into normal range. The largest increase was observed after 24 h due to cumulative stress (4 pulses at 1000 V/cm and coldness), after which stabilization took place.

Levels of secondary metabolites were affected by postharvest storage temperature. Coldness exhibited similar response as specific PEF treatment imposed on the same batch of tested fruits. Low temperatures were reported to regulate cellular metabolism, either directly by reducing the rate of biochemical reactions or indirectly by gene reprogramming (Chinnusamy et al., 2007). Furthermore, increased production of free radicals, which formation has been reported after PEF exposure (Sabri et al., 1996) and due to cold stress (Basra & Basra, 1997), could have triggered similar responses. Lower concentrations of anthocyanins and other phenolics were observed in different berries at low storage temperatures (Miszczak, Forney & Prange, 1995; Kalt et al., 1999). In contrast, an increase of phenolic content during cold storage has been reported in sweet potatoes and apples, products that are not sensitive to chilling (Lattanzio, 2003; Padda & Picha, 2008). In tested blueberries and red currant fruits, both increase and decrease of TP concentration has been observed after subsequent cold storage. Different response occurred most probably due to different fruit fitness at the time they reached lab facilities. Furthermore, same plant variety grown in different environments, were observed to show different sensitivity to induced stress (Basra & Basra, 1997; Munns, 2002). Since growth environment and exact postharvest storage conditions of tested fruits are not known, the repeatability of observed changes should be considered. However, the results discussed here are presented in order to illustrate the complexity of the plant response to different abiotic factors tested within the performed trials. Specificity of stress response in complex fruit multicellular tissue represents limitation in underlying clear evidence of typical fruit behavior. Therefore, to avoid uncontrolled environmental growth and storage conditions and exclude other factors that were shown to affect stress response, cell suspension cultures (cultivated *in vitro* under controlled conditions) were further used within this study to observe impact of PEF as abiotic stressor (see subchapters 4.5 and 4.6).

To correlate the impact of PEF treatment with same intensity (300, 500 or 1000 V/cm) to different berries certain repetitions can be observed. After induced stress with 300 V/cm, smaller divergences in TP concentrations of tested berries can be observed, ranging from -14 % decrease to +15 % increase from normal range measured in corresponding reference (Figure 4-17). When berries were treated with 500 V/cm, larger depletion was observed, ranging from -31 % to +6 % (Figure 4-18). Treatment of 1000 V/cm induced larger divergences from reference, ranging from – 3 % to +48 % increase of TP concentration (Figure 4-19). Induced stress with 300 V/cm exhibited combination of positive (eustress) and negative effects (distress), whereas 500 V/cm

induced distress and 1000 V/cm eustress, in terms of polyphenol accumulation of treated fruits. Plant biochemical processes are very complex and simultaneous degradation and production of plant protective substances (secondary metabolites) in order to regulate their metabolic processes and overcome external disturbances, result in diversity of responses previously defined.

4.3. PEF as pretreatment method in wine making process

PEF applicability during traditional wine making process has been investigated in order to enhance nutritional value of expressed juice by increasing concentration of polyphenolic substances. To investigate the impact of different PEF treatment intensities on cell membrane permeabilization in grape tissue, impedance measurements have been carried out on whole berries. Total phenol concentration has been measured in juice and later on must (young wine), obtained after pressing of PEF treated grapes in comparison to corresponding untreated samples. Grape by-products obtained during the wine making process contain large amount of phenolic compounds that could be recovered as functional food ingredients (Bonilla, Mayen, Merida & Medina, 1999), and therefore polyphenol concentration in grape skin residue has been additionally determined.

4.3.1. Degree of membrane permeabilization

The disintegration level of grape tissue (*Vitis vinifera*, red cultivar Spät Burgunder) was measured within 150 minutes after PEF treatment (stored at RT) and compared to corresponding untreated samples (Figure 4-20).

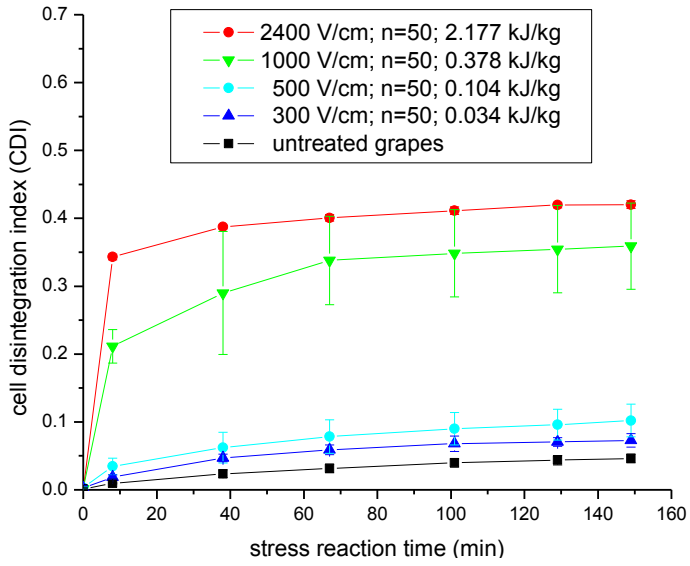


Figure 4-20: Impact of PEF treatment on the extent of membrane permeabilization in grape berries (*Vitis vinifera,* cultivar "Spät Burgunder"), measured as cell disintegration index (CDI), during 150 minutes stress reaction period.

The cell disintegration index of untreated grapes slightly increased from 0.01 (8 minutes after sample preparation) to 0.05 after 150 minutes (Figure 4-20). Observed increase over the measured time can be attributed to sharp cut of grape tissue, necessary step of sample insertion into impedance measurement equipment. Similar observations

were made during determination of CDI in other tested fruits (see subchapters 4.1.1 and 4.2.1). Identification of the degree of membrane permeabilization in soft fruit is more difficult, due to tissue damage that occurs during sample handling and consequently influences observations on cellular level. However, after application of 50 pulses at 300 V/cm CDI values were slightly larger than values of untreated control and increased from 0.02 after 8 minutes to 0.07 after 150 minutes (Figure 4-20). Increase in CDI over time most probably occurred due to release of electro conductive substances in extracellular surroundings caused by cut of grape tissue (sample preparation step) and possibly due to extension of pores induced by PEF treatment. Similar observation can be made after 50 pulses at 500 V/cm. Larger permeabilization of the cell membrane was observed after the application of 50 pulses at higher electric field strength, and CDI reached value of 0.21 and 0.34 immediately after treatment with 1000 V/cm and 2400 V/cm, respectively.

The cell disintegration level of grapes was shown to be dependant on treatment intensity. At low electric field strengths used (up to 500 V/cm) minimal level of permeabilization was achieved, whereas a CDI of 0.42 was obtained 150 minutes after PEF treatment at 2400 V/cm. Interestingly, large standard deviation was noticed after PEF treatment at 1000 V/cm, while similar effect with other tested treatment intensities was not observed (Figure 4-20). Since complex fruit tissue contains cells of different size, it seems that chosen treatment intensity irreversibly permeabilized larger cells. Due to variability of cell size in grape berry, from about 10 to 500 μm (Tyler, Matthews & Shackel, 2006; Wada, Matthews, Choat & Shackel, 2011), number of irreversibly permeabilized cells also varies between different tested samples, which can be attributed to large standard deviation after imposed treatment with 1000 V/cm.

4.3.2. TP concentration in grape juice and skin extracts

Total polyphenol concentration was determined in juice and in grape skin extracts obtained after pressing of PEF treated grapes in comparison to corresponding untreated samples. In juice obtained from PEF treated grapes (*Vitis vinifera*, red cultivar Spät Burgunder) at field strength of 500 V/cm and 50 pulses (total energy input of 0.104 kJ/kg), 13 % larger TP concentration was observed than in corresponding juice obtained from untreated samples (Figure 4-21). After application of 50 pulses at 1000 and 2400 V/cm (total energy input of 0.378 and 2.177 kJ/kg, respectively), 22 and 28 % larger TP concentration was reached. With increasing electric field strength (consequently increasing total energy input) larger concentration of total polyphenols in fresh pressed

juice occurred. Enhanced diffusion of intracellular substances from grapes by PEF can be suggested, which is in accordance with observations made by other authors (Eshtiaghi & Knorr, 2000; Lopez et al., 2008; Grimi, Lebovka, Vorobiev & Vaxelaire, 2009).

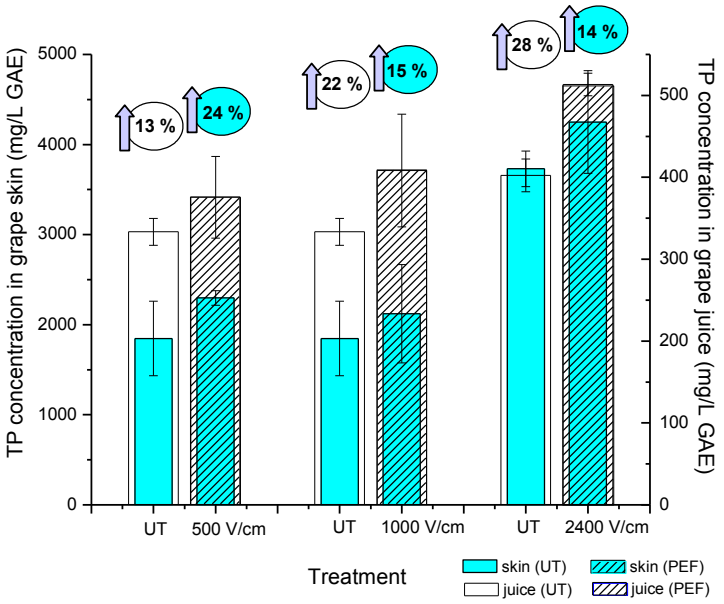


Figure 4-21: Total polyphenol (TP) concentration of grape juice and skin extracts obtained from untreated and PEF treated grape clusters (50 pulses at corresponding field strength). 13, 22 and 28 % denotes increase in TP concentration of juice and 24, 15 and 14 % of grape skin extracts, respectively, obtained from PEF treated grapes, in comparison to untreated reference sample (UT).

In grape skin extracts 24, 15 and 14 % larger TP concentration was reached after application of 50 pulses at 500, 1000 and 2400 V/cm, respectively, than in corresponding extracts obtained from untreated samples (Figure 4-21). With increasing treatment intensity, increased diffusion of intracellular substances during extraction of skin would be expected, due to larger poration of the cell membrane (Figure 4-20). However, the largest difference in TP concentration of skin residue extracts between PEF treated and untreated samples occurred at the lowest field strength used.

Overall TP concentration (from whole grapes) can not be evaluated by the addition of TP concentration in juice and skin, since polyphenolic content of grape skin represents about 80 % in 100 g berry sample. Thereafter, any difference of TP concentration measured in the juice could be overlooked if evaluated as a whole sample. Comparing TP concentration in juice and corresponding grape skin extracts, obtained after pressing of the grape sample treated with 2400 V/cm, it was noticed that expressed juice contained the highest difference in TP concentration, while the lowest difference was observed in grape skin extracts (Figure 4-21). Since PEF was imposed as a pretreatment before pressing, intracellular compounds might have migrated through the pores into extracellular space

and larger polyphenolic content was expressed from the skin into the juice. Following that explanation it can be assumed that less phenolic substances from grape skin leaked into the juice after 500 V/cm, and lower difference of TP concentration found in the juice (consequently higher difference in the grape skin extract) has been observed. Since grape berry comprises cells of different sizes, which varies from about 10 µm near the epidermis to 500 µm in mesocarp tissue (Wada et al., 2011), cell permeabilization with imposed treatment intensity might exhibited different impact. PEF treatment applied to multicellular tissue could have attributed to reversible permeabilization of smaller cells and irreversible permeabilization of larger cells. A stress induction of the smaller cells in grape skin may be therefore proposed as another reason for increased TP concentration in grape skin extracts after 500 V/cm. Since available method for CDI determination was not applicable to determinate PEF impact on different cells in grape berry tissue, further investigation was conducted to reveal possible stress response in grape berries after low treatment intensities. Subchapter 4.4 involves modified experimental design with stress reaction period after imposed PEF stress.

4.3.3. TP concentration of grape must

The application of PEF pre-treatment was shown to be efficient for the enhanced TP concentration in fresh pressed juice. However, to investigate polyphenol retention in young wine, TP concentration has been determined after fermentation. Fresh juice obtained after pressing of PEF treated grapes was kept for 8 weeks at 16 °C in plastic wine containers. Total polyphenol concentration of fermented must measured after 6 and 8 weeks of storage are shown in Figure 4-22.

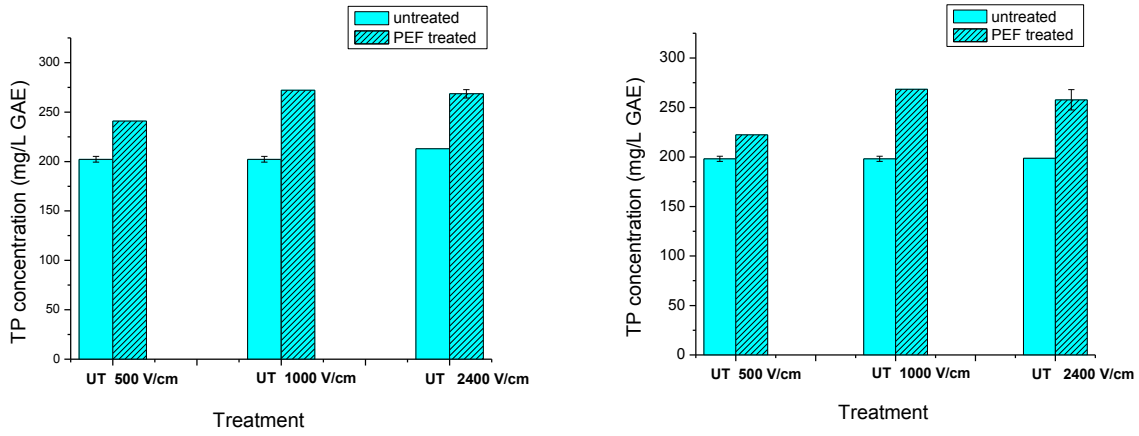
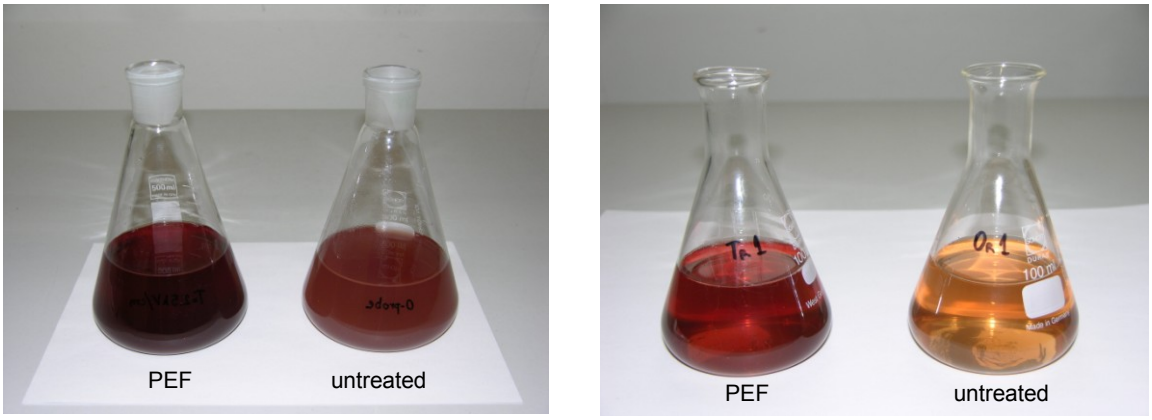


Figure 4-22: Total polyphenol (TP) concentration of grape must obtained from untreated (UT) and PEF treated (50 pulses at 500, 1000 and 2400 V/cm) grape clusters after: (A) 6 and (B) 8 weeks of storage.

Larger TP concentration observed in juice obtained from PEF treated grapes immediately after treatment (Figure 4-21) remained over the storage time of 8 weeks (Figure 4-22). The obtained results indicate that the application of PEF treatment contributes to larger concentration of valuable polyphenolic compounds in expressed juice, which was kept constantly high during 8 weeks storage. Similar results have been reported after the application of PEF treatment during vinification process and increase in anthocyanin content, total polyphenolic index and color intensity in grape juice was observed (Lopez et al., 2008; Puertolas et al., 2010).

Usual practice in traditional wine production includes maceration step, which allows leaching of polyphenolic compounds from grape skin into the must, through which dark red color of wine is obtained. Although maceration step was not included within experimental design of this study, PEF treated must obtained dark red color (Figure 4-23). No additional changes in pH value, total sugar and titrable acidity were noticed (results not shown), suggesting PEF as an effective pretreatment method for must enrichment with polyphenolic compounds, shortening vinification process by excluding time consuming maceration step. Furthermore, depending on the treatment intensity PEF offers potential application for increased extraction of phenolic substances from residue that could be recovered as functional food ingredients.



(A) (B)

Figure 4-23: Grape must after: (A) 4 weeks, and (B) 6 weeks of storage; obtained from PEF treated and untreated grape clusters.

4.4. PEF impact on stress response of grapes

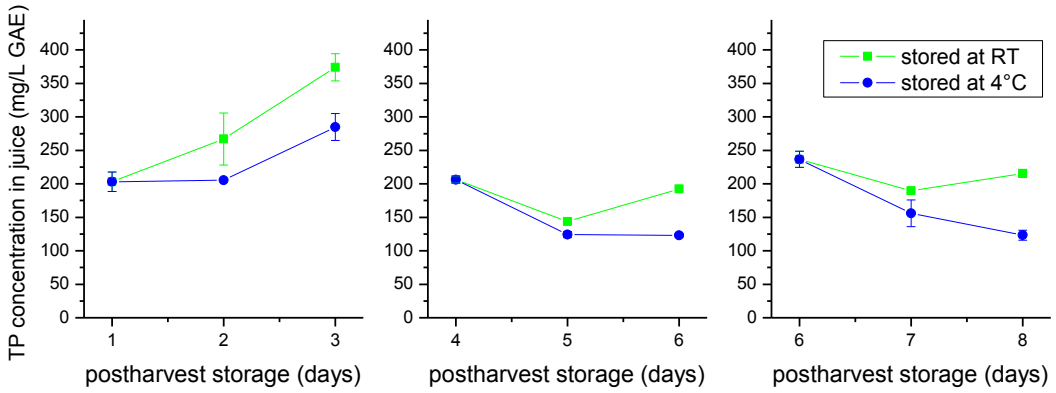
TP concentrations of juice and skin residue extracts, observed immediately after PEF treatment of red grape cultivar Spät Burgunder suggested possible induction of stress reactions (subchapter 4.3). In order to detect suggested changes additional parameters were investigated during 48 h after imposed stress. Polyphenol oxidase can convert phenolics into more reactive species and moreover has been linked to defense responses caused by diverse external stressors (Rivero et al., 2001; Thipyapong et al., 2007). Therefore, PPO activity was next to polyphenols assigned as additional stress indicator. Furthermore, temperature is very important factor affecting postharvest life since it has profound effect on biological reactions and has also been considered during this experimental setup. To detect plant reaction on PEF as external stressor, wine grapes (*Vitis vinifera*, red grape variety, cultivar Dornfelder) were manually harvested and taken directly to lab facilities in order to control postharvest storage conditions before being further processed.

4.4.1. Impact of postharvest storage temperature on TP concentration in grape juice and skin extracts

Cultivated table and wine grapes for either fresh consumption or wine production undergo specific changes of postharvest deterioration. Extensive evaluation of postharvest treatments in order to preserve table grapes and maintain quality aspects during storage, acceptable for consumers, have been studied (Artes-Hernandez, Artes & Tomas-Barberan, 2003; Gonzalez-Barrio, Salmenkallio-Marttila, Tomas-Barberan, Cantos & Carlos Espin, 2005). However, most of the studies concerning wine grapes referred to harvesting time for optimal polyphenolic content and concentration, as well as juice extraction and release of polyphenolic substances during pressing and maceration (Cantos et al., 2003; Amarowicz, Carle, Dongovski, Durazzo, Galensa, Kammerer et al., 2009). In order to evaluate impact of temperature during postharvest storage of wine grapes, total polyphenol concentration has been measured from the first day after the harvest over the stress reaction period. Grapes were kept in clusters during postharvest storage period of 8 days at 4 °C and on day 1, 4 and 6 were taken out from cold room and kept for 48 h at room temperature. Each 24 h during postharvest storage grape clusters were pressed and TP concentration was determined in juice and skin residue extracts.

Total polyphenol concentration of grape juice measured from grapes pressed one day after the harvest was 203.3 mg/L GAE (Figure 4-24A). After 3 days postharvest

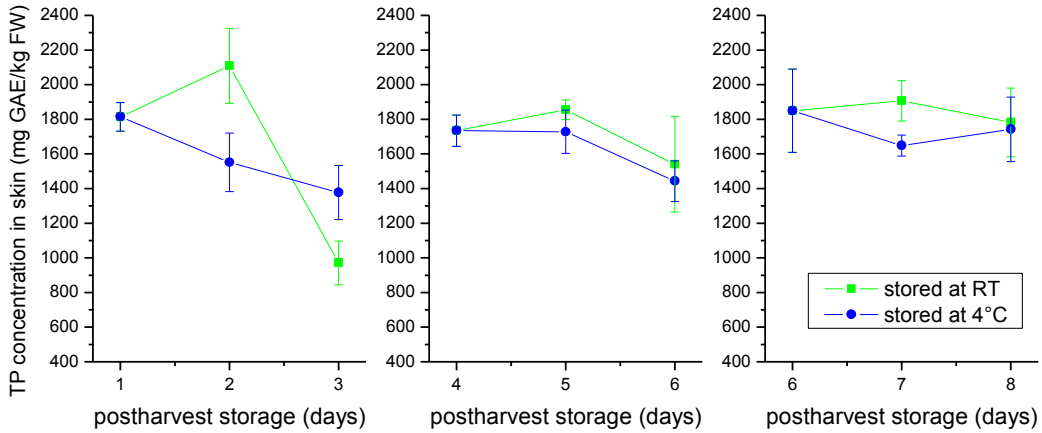
storage at room temperature, TP concentration in fresh pressed grape juice increased to 373.9 mg/L GAE. When grapes were stored at 4 °C, plant postharvest metabolism was slowed down, and therefore a slower gradual increase to 284.9 mg/L GAE in juice was observed (Figure 4-24A).



(A) (B) (C)

Figure 4-24: Total polyphenol (TP) concentration of grape juice obtained from grape clusters (after pressing) during 8 days postharvest storage at room temperature (RT) and at 4°C. Postharvest storage period: (A) 1 – 3, (B) 4 – 6 and (C) 6 – 8 days.

Gradual decrease of TP concentration measured in corresponding skin residue extracts obtained from grapes kept at 4 °C during first 3 days of postharvest storage was observed (Figure 4-25A). Increase in TP concentration of grape juice and decline in grape skin extracts from day 1 to day 3 of postharvest storage at 4 °C, might have occurred due to migration of water soluble pigments from skin into the pulp, which was then presumably expressed into the juice. However, when grapes were stored at RT, increase in TP concentration was observed 2nd day of postharvest storage in juice (Figure 4-24) and in corresponding skin residue extracts (Figure 4-25). Thereafter, cold storage must have induced metabolic changes which resulted in lower TP concentration.



(A) (B) (C)

Figure 4-25: Total polyphenol (TP) concentration of grape skin residue extracts obtained from grape clusters (after pressing) during 8 days postharvest storage at room temperature (RT) and at 4°C. Postharvest storage period: (A) 1 – 3, (B) 4 – 6 and (C) 6 – 8 days.

On the 4th and 6th day of postharvest storage at 4 °C, grape clusters were taken out of the cold room and kept in subsequent storage at room temperature before pressing. TP concentration of grape juice (Figure 4-24B and C) and skin residue extracts (Figure 4-25B and C) obtained from grapes kept in subsequent storage at room temperature was larger than in corresponding samples obtained from grapes kept at 4 °C. Since low temperatures regulate cellular metabolism directly by reducing the rate of biochemical reactions, it can be assumed that aforementioned change occurred due to metabolic activity of harvested organs favored by temperature in subsequent storage. Campos-Vargas et al. (2012) observed oxidative stress during cold storage of Red Globe table grapes, which might be alternative stimulus suggested for physiological disorder and different polyphenol accumulation in tested grapes.

Plant productivity generally declines under cooling conditions, whereas the time of exposure and the extent of plant ability to withstand strained conditions are determining alterations in plant metabolic activity. Some authors reported increase in phenolic substances after cold storage, e.g. increase in anthocyanin content in grape skin due to cold stress measured after 12 days storage at 0 °C (measurements between day 1 and 12 were not conducted), followed by a decline on day 22 (Romero, Sanchez-Ballesta, Maldonado, Escribano & Merodio, 2008), while others reported decrease in total polyphenol concentration measured after 17 days of storage at 0 °C (Meng, Qin & Tian, 2010). However, these studies can not be directly compared with results presented above since storage time and/or temperatures do not correspond.

4.4.2. Impact of stress by coldness and PEF on TP concentration

The evaluation of stress response was studied in order to modify polyphenol concentration of grape juice and skin residue extracts. Since stress can be caused by any external factor and stress tolerance is dependant on the plant life cycle (Romero et al., 2009), grapes were obtained from the same field and harvested at the same time of maturity. Collected grape clusters were stored for 1, 4 and 6 days at 4 °C before being stressed with PEF treatment. To observe possible changes, grape clusters were left after imposed stress for reaction times of 0, 24 and 48 h at room temperature (RT) and at 4 °C, before being pressed. To investigate impact of storage time after the harvest, PEF and temperature during stress reaction period, one batch of grapes was used to perform replications of PEF treatments and also for the corresponding untreated controls.

4.4.2.1. Treatment with 300 V/cm one day after the harvest

Grape clusters were subjected to 10 pulses at 300 V/cm (resulting in total energy input of 0.007 kJ/kg) one day after the harvest. Total polyphenol concentration measured in juice obtained immediately after pressing of PEF treated grapes was 15 % lower than in corresponding untreated samples (Figure 4-26). Reduced initial TP concentration, after 24 h stress reaction time at RT (which in graph corresponds to day 2 after the harvest) did not only reach values of untreated samples, but 13 % larger TP concentration was observed in juice obtained from PEF treated grapes. TP concentration measured in fresh pressed juice from untreated and PEF treated grapes increased over 48 h stress reaction period at RT. However, reached value was not exceeded by TP concentration in juice obtained from PEF treated grapes. When grape clusters were kept at 4 °C for 24 h after imposed PEF treatment (2 days after the harvest), TP concentration in juice obtained from PEF stressed grapes was 14 % larger than in untreated samples stored at 4 °C, which is in agreement to increase observed within grapes stored at room temperature (Figure 4-26). 48 h after PEF treatment (stored at 4° C) TP concentration in juice was 30 % lower than in untreated samples stored at equivalent conditions. Although plant metabolism is reduced at cold temperatures, coldness can be attributed as additional stress factor, which together with PEF treatment leads to cumulative effect. Responses obtained from multiple stressors do not involve only simple reactions (Mittler, 2006). Joint activity of two or more strategies to overcome imposed stress is present, which makes it very difficult to clearly differentiate impact from one another.

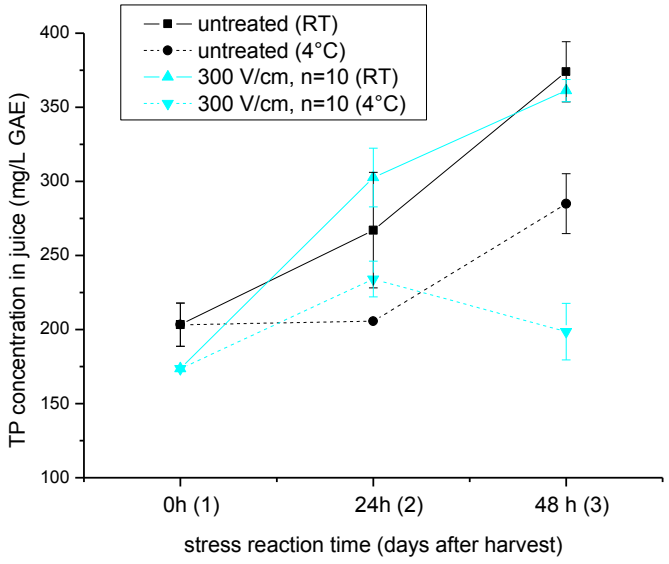


Figure 4-26: Total polyphenol (TP) concentration in fresh pressed grape juice obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (300 V/cm, 10 pulses, 0.007 kJ/kg) one day after the harvest.

TP concentration in juice obtained from untreated grapes 1st day after the harvest was 203.3 mg/L GAE and after 24 h stress reaction time (2nd day) at RT increased to 267.1 mg/L GAE (Figure 4-26). An increase of 31 % from day 1 to day 2 can be observed. Much larger difference in TP concentration, from 173.6 to 302.6 mg/L GAE (within same period of time at RT) and increase of 74 % was observed in juice obtained from PEF stressed grapes. Furthermore, TP concentration of untreated samples stored at 4° C within first 24 hours remained at initial value (from day 1 to 2 after the harvest). However, 1 day after the harvest TP concentration in juice obtained from PEF treated grapes was 173.6 mg/L GAE and increased to 234.1 mg/L GAE after 24 h stress reaction time at 4 °C (Figure 4-26). These facts indicate that PEF treatment contributed to marked differences and larger increase in TP concentration during first 24 h after imposed stress was observed.

Total polyphenol concentration in skin residue extracts obtained from PEF treated grapes immediately after treatment was 22 % lower than in corresponding reference sample (Figure 4-27). Since 15 % lower TP concentration in corresponding juice (Figure 4-26) has been obtained, diffusion and relocation of polyphenolic substances from skin to juice can be excluded. After 24 h stress reaction time (which corresponds to day 2 after the harvest), unlike increased TP concentration observed in juice, further decrease was observed in skin extracts obtain from PEF treated grapes to 1133.7 and 908.7 mg GAE/kg FW, kept at room temperature and 4 °C, respectively.

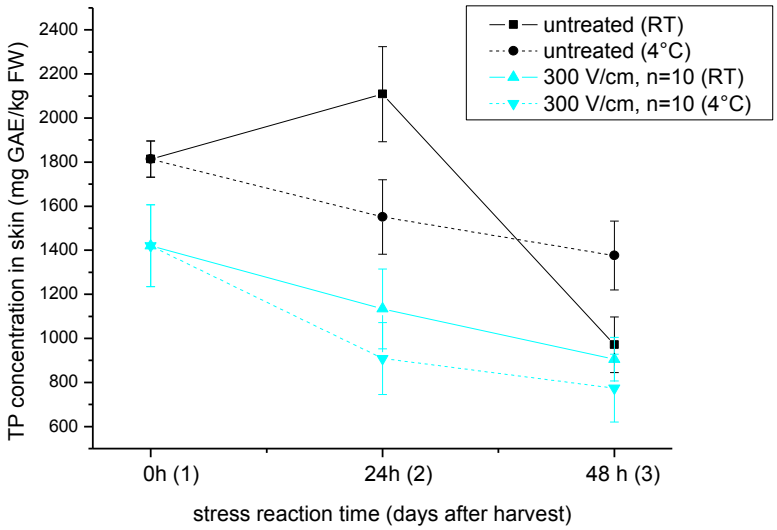


Figure 4-27: Total polyphenol (TP) concentration in grape skin residue extracts obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (300 V/cm, 10 pulses, 0.007 kJ/kg) one day after the harvest.

When untreated grapes were stored at 4 °C for stress reaction period, similar decrease was observed (Figure 4-27) demonstrating effect of temperature stress during

postharvest storage. 48 h after imposed stress (day 3 after the harvest) decrease of TP concentration in skin residue extracts obtained from untreated samples stored at RT was observed and reached values of PEF treated grapes.

4.4.2.2. Treatment with 300 V/cm four days after the harvest

After 4 days storage at 4 °C, grape cluster were subjected to pulsed electric fields with the same treatment intensity (300 V/cm, 10 pulses, 0.007 kJ/kg) as grapes which were treated one day after the harvest (see subchapter 4.4.2.1). Total polyphenol concentration of grape juice and skin residue extracts were examined immediately after pressing of PEF treated and untreated grapes, assigned as 0 h stress reaction time, which corresponds to day 4 after the harvest. Furthermore, whole grape clusters were kept at room temperature and 4 °C for 24 and 48 h stress reaction time after PEF treatment (which corresponds to day 5 and 6 after the harvest) before being pressed and analyzed.

Total polyphenol concentration measured in juice obtained from PEF treated grapes immediately after treatment did not differ from reference sample (Figure 4-28). After 24 h stress reaction time at room temperature (which corresponds to day 5 after the harvest), TP concentration of juice from PEF stressed grapes was 20 % higher than in corresponding reference stored at same conditions. After 48 h stress reaction time (day 6), TP concentration of untreated and PEF treated samples (measured in juice) increased with regard to day 5. Difference of 18 % larger TP concentration of PEF stressed samples in comparison to untreated grapes stored at same conditions remained.

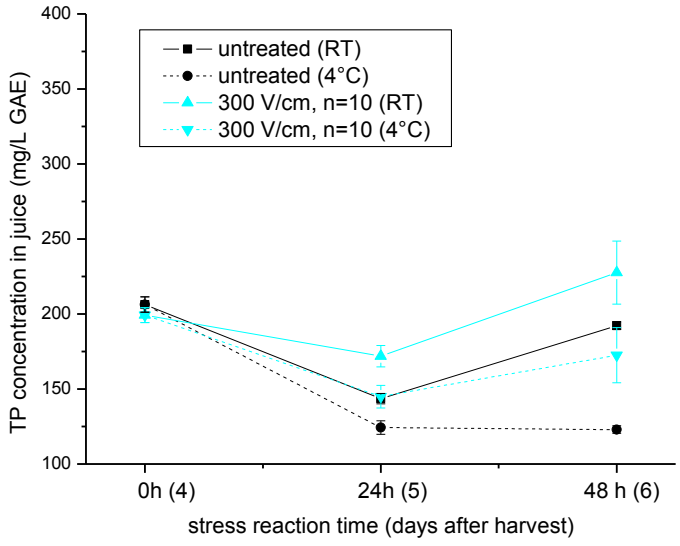


Figure 4-28: Total polyphenol (TP) concentration in fresh pressed grape juice obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (300 V/cm, 10 pulses, 0.007 kJ/kg) four days after the harvest.

Total polyphenol concentration in grape skin residue extracts obtained immediately after PEF treatment (0 h stress reaction time on 4th day after the harvest) was 17 % larger than from untreated grapes (Figure 4-29). Initial larger TP concentration remained constant during storage at RT and at 4 °C; however, these values were below TP concentration of untreated control stored for 24 h at RT and above untreated control stored at 4 °C. After 48 h stress reaction time (6th day after the harvest) TP concentration of all stressed samples reached values of untreated grapes.

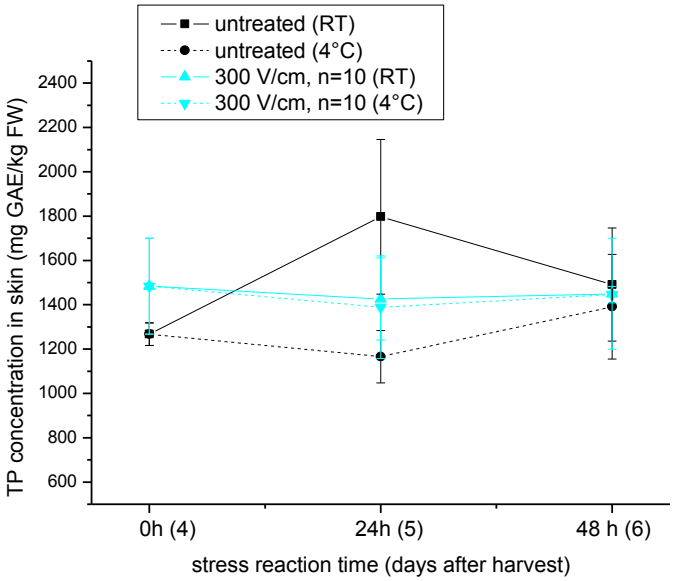


Figure 4-29: Total polyphenol (TP) concentration in grape skin residue extracts obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (300 V/cm, 10 pulses, 0.007 kJ/kg) four days after the harvest.

Grape clusters stored for 4 days at 4 °C after the harvest (before being subjected PEF) exhibited different response in comparison to grapes treated one day after the harvest. Fruits fitness decreased over storage time and physiological changes of cell membranes must have occurred due to low temperature storage. Reduced fluid structure of the cell membrane at cold temperatures (Chinnusamy et al., 2007) and changed initial physiological fruit fitness must have attributed to different TP concentrations. As discussed in previous subchapters, fruit maturity and physiological fitness have been often reported to influence stress response and consequently TP accumulation and/or relocation (Basra & Basra, 1997; Larcher, 2003; Prono-Widayat et al., 2003; Beck et al., 2005), which was observed during postharvest stress induction of wine grapes on day 1 and 4.

4.4.2.3. Treatment with 700 V/cm four days after the harvest

After 4 days storage at 4° C, grape cluster were subjected to pulsed electric field treatment at 700 V/cm and 10 pulses (resulting in total energy input of 0.037 kJ/kg), and

TP concentration of grape juice and skin residue extracts were examined as described in subchapter 4.4.2.2. Total polyphenol concentration measured in grape juice obtained from PEF treated grapes immediately after treatment (0 h stress reaction time, which corresponds to 4th day after the harvest) was 11 % higher than in reference samples stored at equivalent conditions (Figure 4-30). After stress reaction times of 24 and 48 h at room temperature (5th and 6th day after the harvest), difference increased to 19 and 53 %, respectively. When grape clusters were kept at 4 °C for stress reaction period after imposed PEF stress, such a large difference of polyphenol concentration in fresh pressed grape juice was not detected.

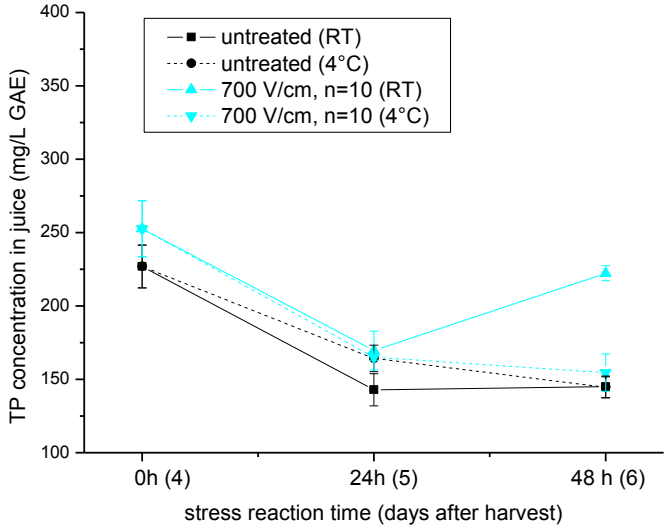


Figure 4-30: Total polyphenol (TP) concentration in fresh pressed grape juice obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (700 V/cm, 10 pulses, 0.037 kJ/kg) four days after the harvest.

37 % larger TP concentration was observed in grape skin residue extracts obtained immediately after PEF treatment, followed by decrease after 24 h stress reaction time (day 5), stored at room temperature and at 4 °C (Figure 4-31). After longer stress reaction time of 48 h, TP concentration in grape skin extracts obtained from grapes stored at RT remained below values of untreated corresponding reference. However, when grapes were stored at 4 °C for 48 h, 34 % higher TP concentration was observed than in corresponding referent sample stored at same conditions. Coldness must have induced additional impact of already stressed fruits and cumulative effect of PEF and coldness can be recognized, indicating that coldness can be assigned as additional stress factor and increase stress intensity when imposed together with PEF treatment. Furthermore, changes in TP concentration of grape skin residue extracts (Figure 4-31) and corresponding juice (Figure 4-30), obtained after pressing of grape clusters that underwent equivalent stress/storage conditions, can not be directly correlated.

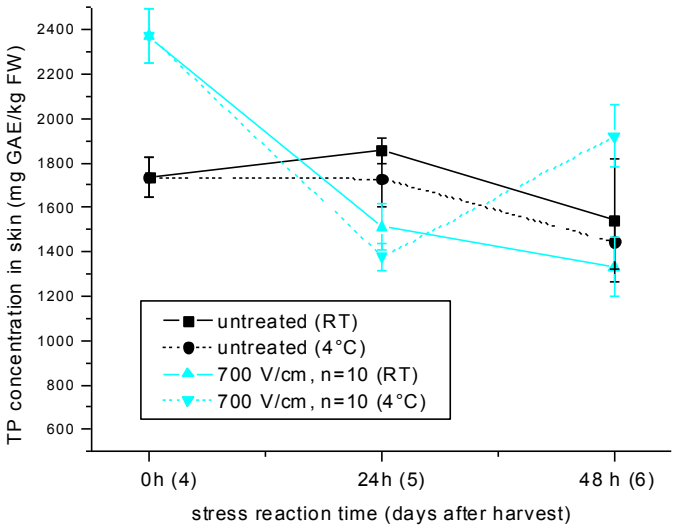


Figure 4-31: Total polyphenol (TP) concentration in grape skin residue extracts obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (700 V/cm, 10 pulses, 0.037 kJ/kg) four days after the harvest.

4.4.2.4. Treatment with 700 V/cm six days after the harvest

Due to experimental design, grape clusters were kept for 6 days at 4 °C after the harvest before being treated with 10 pulses at 700 V/cm (total energy input 0.037 kJ/kg). Total polyphenol concentration measured in grape juice obtained from PEF treated grapes immediately after treatment and during stress reaction period did not significantly differ from reference samples stored at the equivalent conditions (Figure 4-32). After 48 h stress reaction time (day 8 after the harvest), slightly lower TP concentration were observed during subsequent storage at room temperature, whereas slightly larger TP concentration occurred at 4 °C.

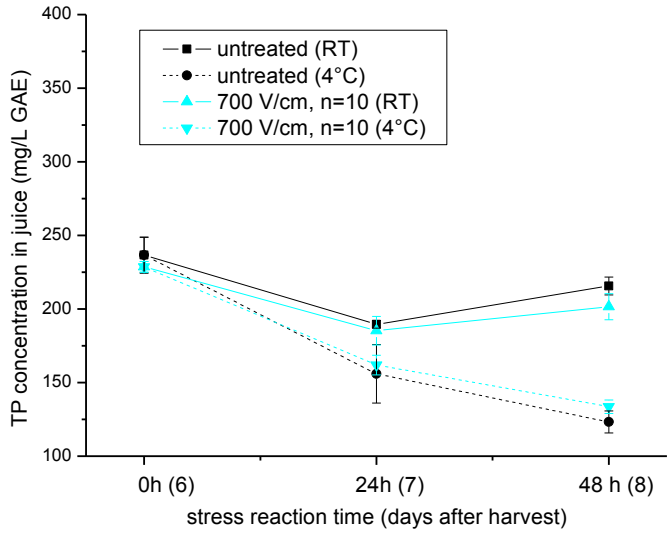


Figure 4-32: Total polyphenol (TP) concentration in fresh pressed grape juice obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (700 V/cm, 10 pulses, 0.037 kJ/kg) six days after the harvest.

TP concentration in grape skin extracts did not significantly differ from reference immediately after imposed PEF treatment (Figure 4-33). A decline followed after 24 h stress reaction time at room temperature (day 7), when 14 % lower TP concentration was observed in PEF treated samples. After 48 h stress reaction time at RT (day 8), TP concentration in PEF stressed samples increased and was 10 % larger than in corresponding untreated grapes stored at equivalent conditions. However, when PEF treated grapes were stored during stress reaction period at 4 °C, TP concentration observed in grape skin extracts was kept at higher level than in skin extracts of untreated samples stored at equivalent conditions. Again two abiotic stress factors: PEF treatment and coldness exhibited different stress response as each by itself.

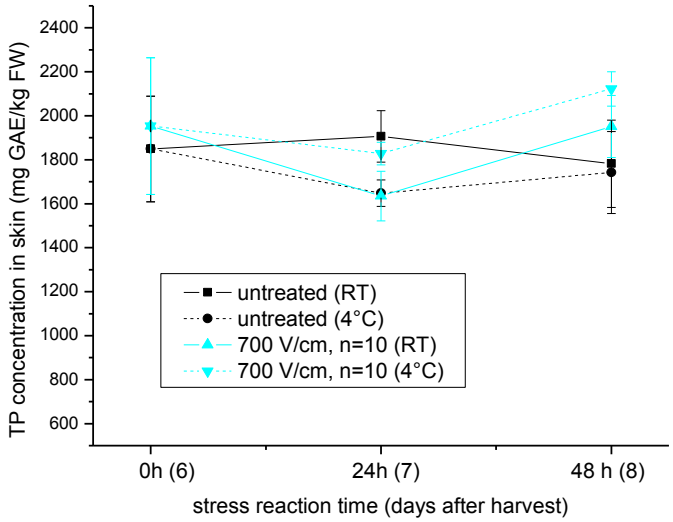


Figure 4-33: Total polyphenol (TP) concentration in grape skin residue extracts obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (700 V/cm, 10 pulses, 0.037 kJ/kg) six days after the harvest.

When comparing grape clusters obtained from the same vintage and stored for either 4 or 6 days at 4 °C before subjected to PEF treatment at 700 V/cm, impact of storage time before imposed PEF stress can be noticed. Different response in terms of TP accumulation was observed when the same treatment was applied. Postharvest quality deterioration which include loss of turgidity, weight loss, color changes and accelerated softening of grape berries are well known (Takeda, Saunders & Saunders, 1983; Moreno, Cerpa-Calderon, Cohen, Fang, Qian & Kennedy, 2008; Romero et al., 2008), which was shown to affect plant response to stress induction by PEF treatment. Similar observations were made after stress induction by 300 V/cm imposed to grape clusters stored for 1 and 3 days after the harvest (subchapters 4.4.2.1 and 4.4.2.2).

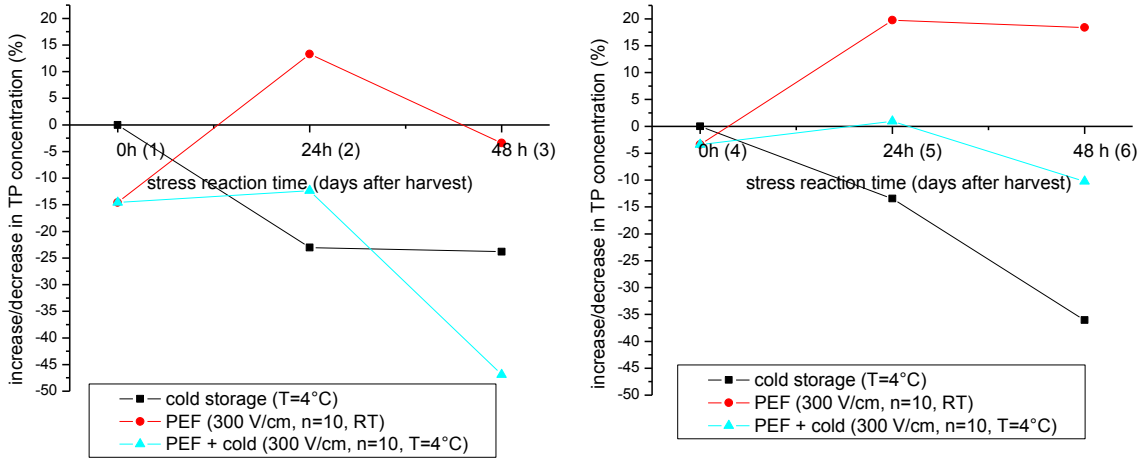
4.4.3. Changes in TP concentration due to PEF intensity and postharvest storage time

To observe the impact of postharvest storage, PEF treatment, coldness and interaction among these abiotic stressors, stress exposed fruits were compared to untreated fruits stored at room temperature. Induced changes in TP concentration are expressed as percentage of increase/decrease in respect to untreated samples and presented through biological stress concept. When the grape clusters are being pressed, first juice is released from the berry pulp followed by the juice from the sections closer to the skin. Therefore, total polyphenol concentration of fresh pressed juice can be considered as polyphenolic pool enclosed within pulp, and grape skin extracts as polyphenolic content of a grape skin. These terms will be used in further discussion for easier understanding of plant response in multicellular fruit tissue.

Changes of TP concentration in grape pulp after 300 V/cm

Lower TP concentration in PEF stressed grape pulp obtained from grapes treated with 300 V/cm ***one day after harvest*** can be observed immediately after treatment (Figure 4-34A). It displays a plant effort to sustain stress through utilization of intracellular ingredients in alarm phase of biological stress concept. After 24 h stress reaction time at RT, adaptation and *de novo* synthesis of protective substances can be observed through increase of TP concentration in PEF stressed samples. Recovery phase has been recognized, which leads to increased resistance to stress. Plant adjustment after 48 h stress reaction time took place, when TP concentration retrieved to normal range. Gradual decrease of TP concentration in untreated grape pulp was observed during 24 h stress reaction time due to cold storage at 4 °C (Figure 4-34A). After 48 h cold storage, metabolic system remained in alarm phase, accompanied by destabilization of structural and functional properties of plant systems stimulated by low temperature. The effect of coldness can be observed through depletion of TP concentration that occurred after longer period of time, in comparison to lower TP concentration that occurred immediately after PEF treatment. When grapes were stored at 4 °C after PEF treatment, depletion of phenolic substances that occurred immediately after imposed stress was prolonged over 24 h (Figure 4-34A). After 48 h stress reaction time at 4 °C, grapes suffered additional stress caused by coldness, which resulted in repeated descend of polyphenol concentration. Cumulative effect of PEF and cold resulted in different response than each stress factor by itself. Since TP concentration was not measured longer than 48 h after imposed stress, it can not be concluded weather the functional state of fruit metabolic

activity was restored, or due to persistence of stressor (in this case coldness) reached exhaustion.



(A) (B)

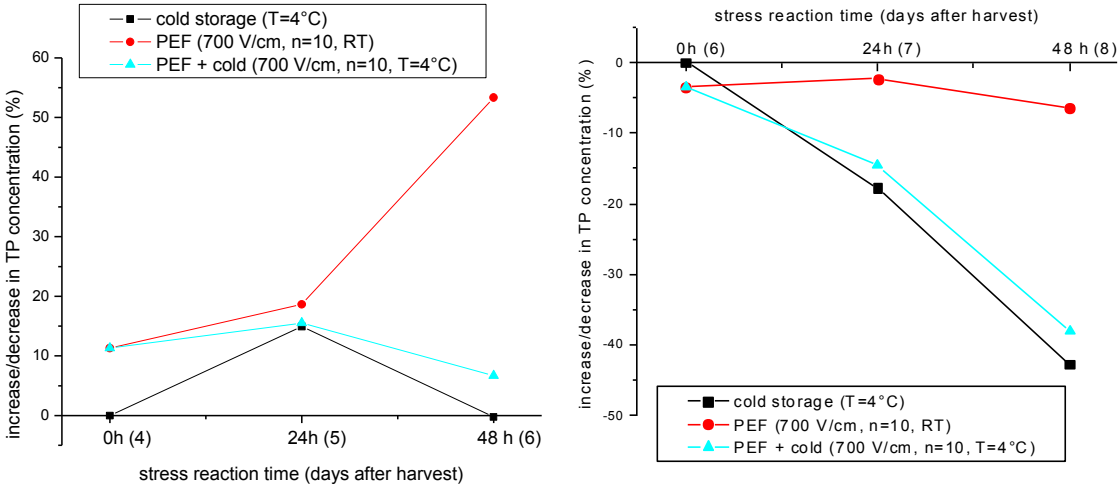
Figure 4-34: Changes of total polyphenol (TP) concentration during stress reaction period of 48 h, in juice (pulp) obtained from grapes treated (A) 1 and (B) 4 days after the harvest; due to cold storage at 4 °C, PEF treatment (300 V/cm, 10 pulses, 0.007 kJ/kg) and their cumulative effect (PEF+cold).

De novo synthesis of protective substances can be observed 24 h after stress induction by PEF treatment of 300 V/cm, imposed to grapes ***four days after the harvest*** (Figure 4-34B). Similar response was observed 24 h after application of PEF with same treatment intensity one day after the harvest (Figure 4-34A). However, after 48 h stress reaction time (day 6) plant metabolism remained in elicited state, where prolonged resistance phase can be observed in comparison to PEF stressed samples one day after the harvest (Figure 4-34A and B). Impact of subsequent cold storage was observed through gradual decrease of TP concentration during stress reaction period, which corresponds to day 5 and 6 after the harvest (Figure 4-34B). Multiple stress factors, which included former exposure to coldness (grape clusters were stored for 4 days at 4 °C before being subjected to PEF), PEF treatment of 300 V/cm and coldness during stress reaction period after PEF, most probably triggered joint activity of two or more strategies to overcome imposed stress. Simultaneous degradation and production of plant protective substances could have occurred exhibiting insignificant changes in TP concentration during 24 h stress reaction period (Figure 4-34B).

Changes of TP concentration in grape pulp after 700 V/cm

Figure 4-35A represents changes of TP concentration after stress reaction period obtained from grapes subjected to 700 V/cm four days after the harvest and Figure 4-35B six days after the harvest. Increase in TP concentration during first 24 h after all tested stressors (cold storage, PEF and PEF+cold) imposed to grapes ***four days after the***

harvest, has been observed (Figure 4-35A). Larger TP concentration in grape pulp was observed immediately after treatment with 700 V/cm (Figure 4-35A; 0 h, 4th day), indicating that due to larger treatment intensity (larger stress) plant reaction was faster and *de novo* synthesis of protective substances occurred earlier than after 300 V/cm (Figure 4-34B). TP concentration of PEF treated samples at 700 V/cm continued to rise up to 48 h after stress induction (at RT), prolonging recovery phase, while TP concentration of samples left during 48 h subsequent cold storage retrieved to normal range (Figure 4-35A).



(A) (B)

Figure 4-35: Changes of total polyphenol (TP) concentration during stress reaction period of 48 h, in juice (pulp) obtained from grapes treated (A) 4 and (B) 6 days after the harvest; due to cold storage at 4 °C, PEF treatment (700 V/cm, 10 pulses, 0.037 kJ/kg) and their cumulative effect (PEF+cold).

De novo synthesis of protective substances in samples obtained from grapes kept for ***six days after the harvest***, was not observed (Figure 4-35B). Depletion of TP was observed during subsequent storage at 4 °C, while no significant changes were obtained after PEF during stress reaction period at RT. Six days of postharvest storage brought on metabolic changes in grape berries, which resulted in different response to PEF treatment with 700 V/cm, as grapes stored for 4 days before stress induction (Figure 4-35A).

Changes of TP concentration in grape skin after 300 V/cm

Immediately after treatment with 300 V/cm applied to grapes ***one day after the harvest***, lower TP concentration in PEF stressed grape skin can be observed (Figure 4-36A). TP concentration in PEF stressed samples continued to decline during 24 h stress reaction time at RT and at 4 °C, displaying catabolism predomination and utilization of protective substances through alarm phase of the biological stress concept. After 48 h stress reaction time, adaptation and *de novo* synthesis of protective substances can be

observed through increase of TP concentration in PEF stressed samples. Repair and recovery phase has been recognized between 24 and 48 h stress reaction time after all tested stressors (cold, PEF and PEF+cold). However, increase in TP concentration after cold storage exceeded values of untreated control (stored at RT), while in PEF stressed samples it retrieved into normal range.

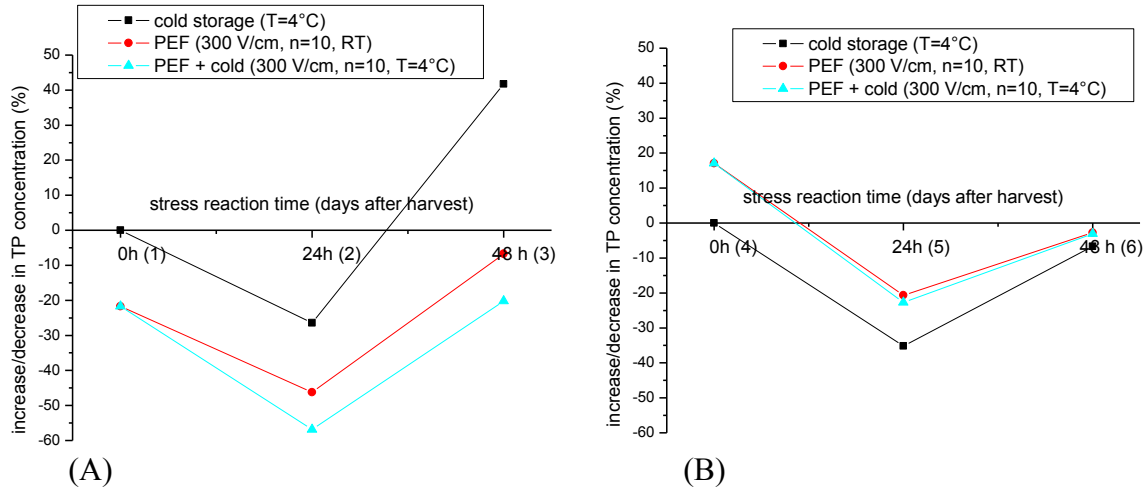


Figure 4-36: Changes of total polyphenol (TP) concentration during stress reaction period of 48 h, in grape skin obtained from grapes treated (A) 1 and (B) 4 days after the harvest; due to cold storage at 4 °C, PEF treatment (300 V/cm, 10 pulses, 0.007 kJ/kg) and their cumulative effect (PEF+cold).

When equivalent stress factors were imposed to grapes stored for ***four days after the harvest***, similar observations concerning biological stress concept can be made as within samples treated one day after the harvest. Decrease of TP concentration during first 24 h, interpreting early stage of stress through alarm phase, followed by increase between 24 and 48 h stress reaction time (day 5 and 6, respectively) in recovery phase, reaching normal range (Figure 4-36B). Although, same phases of plant response have been recognized, different amount of phenolic substances have been utilized and/or produced in fruits that underwent postharvest senescence. These results are consistent with previous observations where fruit physiological fitness was found to influence abiotic stress response (Larcher, 2003; Hodges & Toivonen, 2008; Romero et al., 2009).

Changes of TP concentration in grape skin after 700 V/cm

Immediately after PEF application of 700 V/cm to grapes stored for ***four days after the harvest*** larger TP concentration in grape skin was observed (Figure 4-37A). Larger treatment intensity must have provoked larger stress and *de novo* synthesis of protective substances occurred within very short period of time. Gómez Galindo *et al.* (2007) reported that initial reaction of responsiveness may occur within time scale from seconds to days. Higher productivity level was not kept over stress reaction period.

Depletion of PEF treated samples after 24 h was observed, indicating utilization of phenolic substances in order to overcome stress and adapt to strained conditions. However, when additional stressor was imposed (coldness during stress reaction time) repeated increase between 24 and 48 h can be observed, displaying cumulative effect of both stressors.

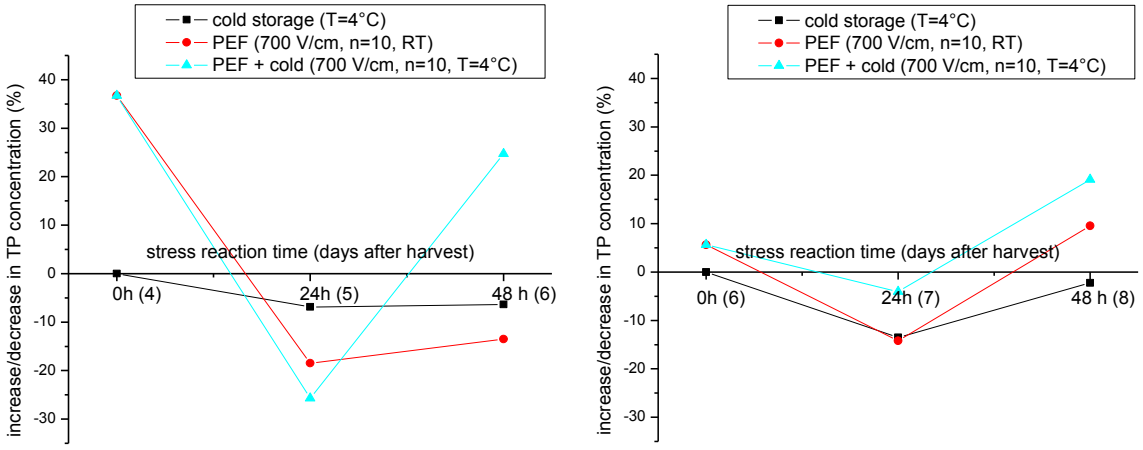


Figure 4-37: Changes of total polyphenol (TP) concentration during stress reaction period of 48 h, in grape skin obtained from grapes treated (A) 4 and (B) 6 days after the harvest; due to cold storage at 4 °C, PEF treatment (700 V/cm, 10 pulses, 0.037 kJ/kg) and their cumulative effect (PEF+cold).

Concerning biological stress concept, similar observations can be made after same PEF treatment intensity imposed to grapes stored for ***six days after the harvest*** (Figure 4-37B). However, different amount of phenolic substances have been produced and utilized in fruits that underwent 4 and 6 days of postharvest storage. As indicated previously, physiological fruit fitness affected plant response on imposed stressors.

A correlation between TP accumulation in grape pulp and skin obtained from corresponding grape clusters, that underwent equivalent stress conditions, can not be derived. Since impact of PEF is shown to be cell size dependant (Heinz et al., 2002) and cell size varies from about 6.5-10 µm of the grape skin to 500 µm in pulp tissue (Wada et al., 2011; Chervin, Aked & Csrisosto, 2012), it can be assumed that application of same treatment intensity induced different impact to grape pulp and skin. Furthermore, dense cuticle layers, thick-walled anatomy of grape skin cells and its natural protective function must have contributed to different response observed. Supporting these observations, Becatti et al. (2010) used a molecular approach and showed that short-term postharvest carbon dioxide treatment induced different accumulation of total polyphenolics in grape skin and pulp. The presence of altered metabolism was indicated by the number of differentially expressed genes in grape skin and pulp, which clearly highlighted the marked difference in reactivity to external stimuli between these two tissues. Schulze et

al. (2005) reported that optimal intensities of various abiotic stressors may differ for particular organs of the same organism and Considine (1981) reported thickness of epidermal cell walls contributed to a positive correlation with resistance to physical stress. Intensive research on mechanisms involved in plant protection in this field to contribute to a deeper understanding is under current study.

4.4.4. Postharvest PPO activity of grapes

Since oxidation of phenolic compounds is catalyzed by polyphenol oxidase (PPO), changes it TP levels might be associated with PPO activity and therefore it has been evaluated upon chosen stressors. Polyphenol oxidase activity was measured in the whole berries obtained from grape clusters stored for 1, 4 and 6 days at 4 °C before being imposed to different stress factors. Diversities of PPO activity have been detected in untreated grapes stored at different temperatures during postharvest storage of 8 days (Figure 4-38). Variations of response magnitude were observed between single fruits (which can be seen through large standard deviations) indicating instability of the enzyme.

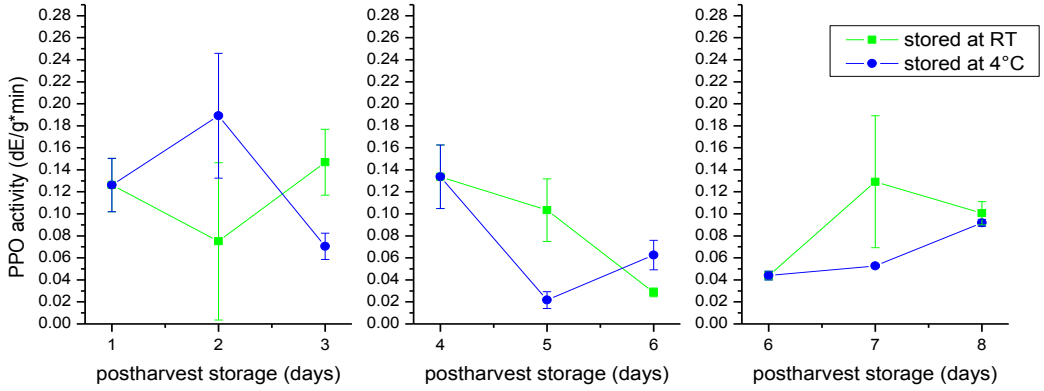


Figure 4-38: Polyphenol oxidase (PPO) activity of grapes during 8 days postharvest storage at room temperature (RT) and at 4°C. Postharvest storage period: (A) 1 – 3, (B) 4 – 6 and (C) 6 – 8 days.

Grape clusters taken directly after the harvest were stored at RT and at 4 °C for 3 days. An increased PPO activity was observed in samples kept at 4 °C for 2 days (Figure 4-38A). After 4 and 6 days storage time at 4 °C, grape cluster were taken out of the cold room and kept at RT and at 4 °C during subsequent storage. This time, an increase in PPO activity was observed in samples stored at RT (Figure 4-38B and C). It seems that adjustment of fruits to storage conditions occurred and changes in environmental temperature was recognized as stress. Thereafter, it can be suggested that PPO activity was stimulated by temperature change after 24 h stress reaction time (days: 2, 5 and 7 in

Figure 4-38). Impact of temperature change on TP concentration in grape juice and skin residue extracts obtained from grape clusters during same storage regime was observed as well (subchapter 4.4.1). Since decrease in TP concentration was observed after 24 h subsequent storage at 4 °C in all samples (days 2, 5 and 7 in Figures 4-24 and 4-25), direct correlation with changes in PPO activity can not be made. Changes in the enzyme activities and/or their *de novo* synthesis have been reported as a result of gene expression for resistance (Schnablová et al., 2006; Rizzini et al., 2009). Moreover, characterization of genes responsible for carbohydrate and secondary metabolism, transport, transcription factors and hormone metabolism in grape berries indicated their involvement in response to biotic and abiotic stressors (Grimplet, Deluc, Tillett, Wheatley, Schlauch, Cramer et al., 2007; Rizzini et al., 2009).

4.4.5. Impact of stress by coldness and PEF on PPO activity

4.4.5.1. Treatment with 300 V/cm one day after the harvest

Grape clusters were subjected to 10 pulses at 300 V/cm (total energy input 0.007 kJ/kg) one day after the harvest and PPO activity was measured from grape berries immediately after treatment and after stress reaction period. Lower PPO activity was observed immediately after PEF treatment suggesting temporally inhibition of PPO (Figure 4-39). After 24 h stress reaction time, two-fold increase in PPO activity of all stressed samples (cold storage, PEF and PEF+cold) was observed, indicating that phenolic oxidation system might be involved in the plant resistance.

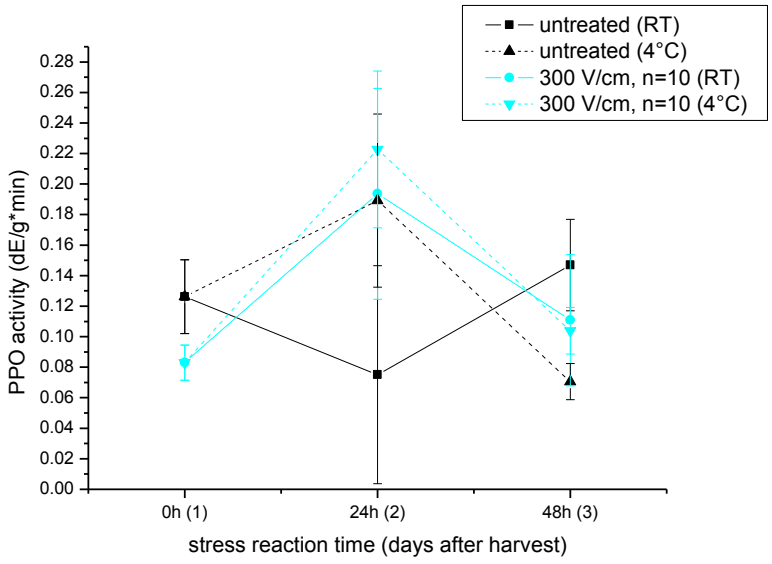


Figure 4-39: Polyphenol oxidase (PPO) activity of grape berries obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (300 V/cm, 10 pulses, 0.007 kJ/kg) one day after the harvest.

Examples of increased PPO activity have been frequently reported after different biotic and abiotic stressors (Thipyapong et al., 1995; Rivero et al., 2001; Wu & Lin, 2002; Mayer, 2006), where PPO´s defensive role was attributed to quinines (PPO´s primary products), highly reactive electrophiles undergoing complex secondary reaction pathways. Since larger TP concentration was observed after 24 h stress reaction time in corresponding grape juice (Figure 4-26) and lower in residue extracts (Figure 4-27), increased PPO activity can not be directly associated to changes of phenolic substances caused by PEF treatment. Thereafter it can be assumed that more than single biosynthetic pathway was stimulated by PEF application during postharvest storage.

4.4.5.2. Treatment with 300 V/cm four days after the harvest

Stored grape clusters for 4 days at 4 °C after the harvest were subjected to 10 pulses at 300 V/cm (0.007 kJ/kg) as grapes which were treated one day after the harvest. Slightly larger PPO activity was observed immediately after PEF treatment (0 h), and increased after 24 h stress reaction time at RT (day 5), which resulted in 2.5 fold larger PPO activity than in corresponding control (Figure 4-40). Since grape berry remained metabolically active during 4 days postharvest storage, metabolic changes that occurred in fruit resulted in different response observed immediately after PEF treatment (0 h), as in grape berries treated one day after the harvest (Figure 4-39). Thipyapong et al. (1995) reported increased PPO activity within 48 h after wounding of potato tissue and suggested that only developmentally competent tissues were capable on responding to stress signal by increased accumulation of PPO mRNA.

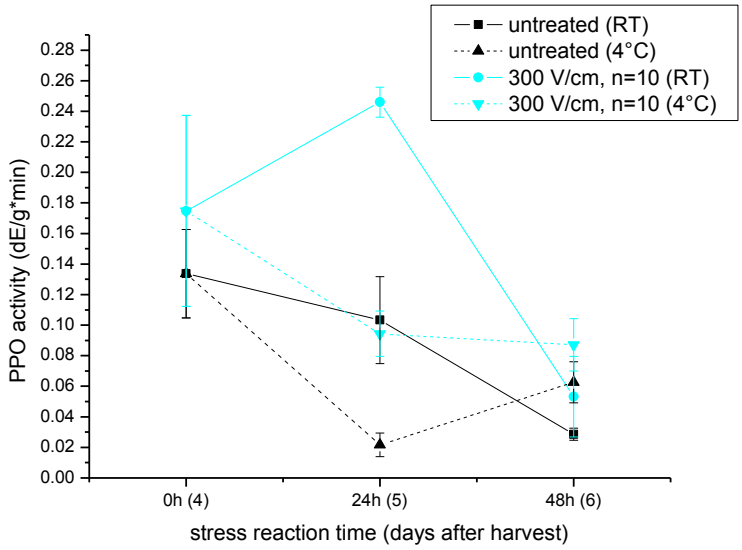


Figure 4-40: Polyphenol oxidase (PPO) activity of grape berries obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (300 V/cm, 10 pulses, 0.007 kJ/kg) four days after the harvest.

Although enzymatic activity of PEF treated grapes stored at RT declined between 24 and 48 h, larger PPO activity of all stressed samples in comparison to corresponding control was observed after 48 h stress reaction time (Figure 4-40).

4.4.5.3. Treatment with 700 V/cm four days after the harvest

Larger PPO activity can be observed immediately after PEF treatment at 700 V/cm and 10 pulses (0.037 kJ/kg), imposed on grapes stored for 4 days after the harvest (Figure 4-41). Similar observations were made immediately after 10 pulses at 300 V/cm imposed on grapes after equivalent postharvest storage conditions (Figure 4-40). After 24 h stress reaction time, unlike enhanced enzymatic activity observed after treatment with 300 V/cm, lower PPO activity was observed after 700 V/cm, and reached values of untreated control after 48 h (Figure 4-41). When grapes were stored at 4 °C for 48 hours after PEF treatment (day 6 after the harvest), 6.5 and 2.2 fold larger PPO activity was observed than in corresponding control stored at RT and at 4 °C, respectively.

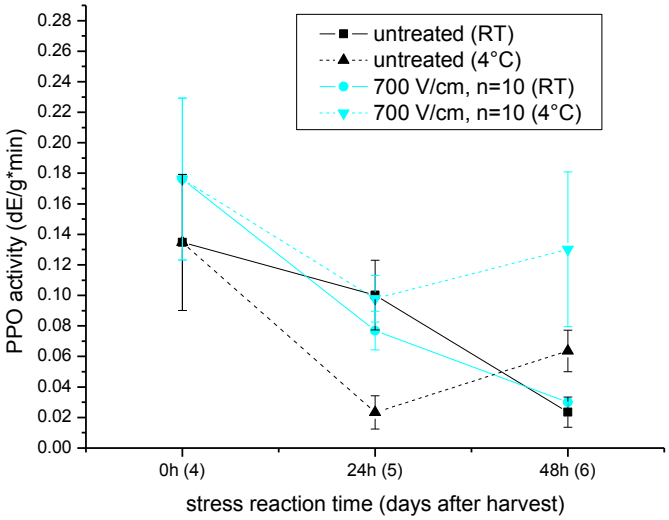


Figure 4-41: Polyphenol oxidase (PPO) activity of grape berries obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (700 V/cm, 10 pulses, 0.037 kJ/kg) four days after the harvest.

4.4.5.4. Treatment with 700 V/cm six days after the harvest

Grapes were stored for 6 days at 4 °C before being subjected to PEF treatment of 700 V/cm and 10 pulses (0.037 kJ/kg). Enhanced PPO activity in PEF treated grapes was observed immediately after treatment (Figure 4-42). After 24 h subsequent storage at RT (corresponds to day 7 after the harvest) PPO activity of PEF treated grapes was below values of corresponding control, which remained over 48 h stress reaction period. Similar observation can be made for samples kept at 4 °C during stress reaction period. Unlike

grape clusters kept for 4 days after the harvest, no additional rise in PPO activity after 48 h was observed, indicating once again importance of initial plant fitness. Due to postharvest storage, biochemical transformations in fruit tissue occurred and different response was observed after application of the same PEF treatment intensity.

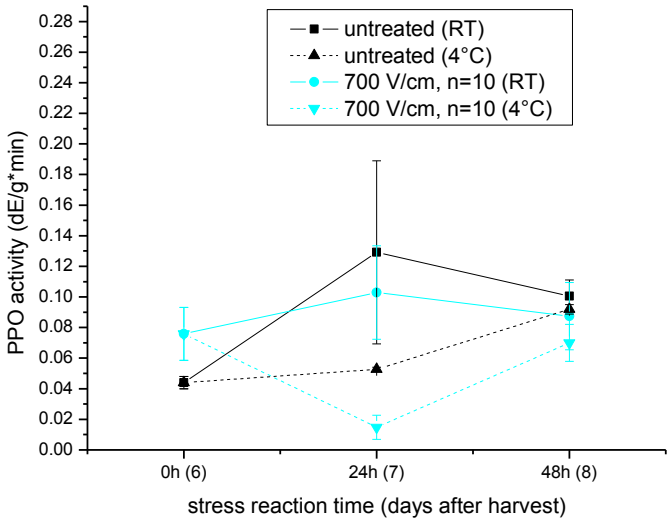


Figure 4-42: Polyphenol oxidase (PPO) activity of grape berries obtained after 0, 24 and 48 h stress reaction time from untreated and PEF treated grape clusters (700 V/cm, 10 pulses, 0.037 kJ/kg) six days after the harvest.

4.4.6. Changes in PPO activity due to PEF intensity and postharvest storage time

Seeking for a pattern of nonuniform plant response to stress imposed by PEF on grapes treated after 1, 4 or 6 days of postharvest storage, PPO activity was expressed as percent of increase/decrease, considering reference sample as plant normal behavior. Initial inhibition of PPO activity observed immediately after treatment with 300 V/cm, applied on grapes stored for ***one day after the harvest***, rapidly increased over 24 h stress reaction time (Figure 4-43A). An increase in PPO activity of untreated grapes stored at 4 °C for 24 h stress reaction time (day 2) was observed as well, indicating similarity in plant response to coldness and PEF treatment imposed on grapes one day after the harvest. When grape clusters were stored for ***four days after the harvest*** before being exposed to 300 V/cm, increased PPO activity was observed immediately after treatment (0 h, day 4 in Figure 4-43B), followed by additional rise during 24 h stress reaction time (day 5). Larger enzyme activity was maintained during 48 h (day 6), unlike drop observed 48 h after PEF treatment of same intensity applied to grape clusters one day after the harvest (day 3 in Figure 4-43A). The same stress intensity provoked increased PPO

activity over longer period of time when imposed to fruits that already underwent deteriorative metabolic changes. Thus it indicates that longer time was necessary for plant adjustment. Coldness as second stress factor imposed to grapes during subsequent storage (days 4 – 6) induced delayed response (Figure 4-43B) in comparison to grapes treated one day after the harvest (Figure 4-43A). PPO inhibition can be observed in untreated grapes after 24 h stress reaction time (day 5, Figure 4-43B) at 4 °C, followed by increase in PPO activity observed after 48 h (day 6). Similar response was noticed in PEF treated samples during same stress reaction period at 4 °C. Although similarity in these events can be recognized when comparing changes in PPO activity due to subsequent cold storage (black line in Figure 4-43) and multiple stress factors PEF + cold (blue line in Figure 4-43), additional elicitation of PPO activity by PEF can be observed.

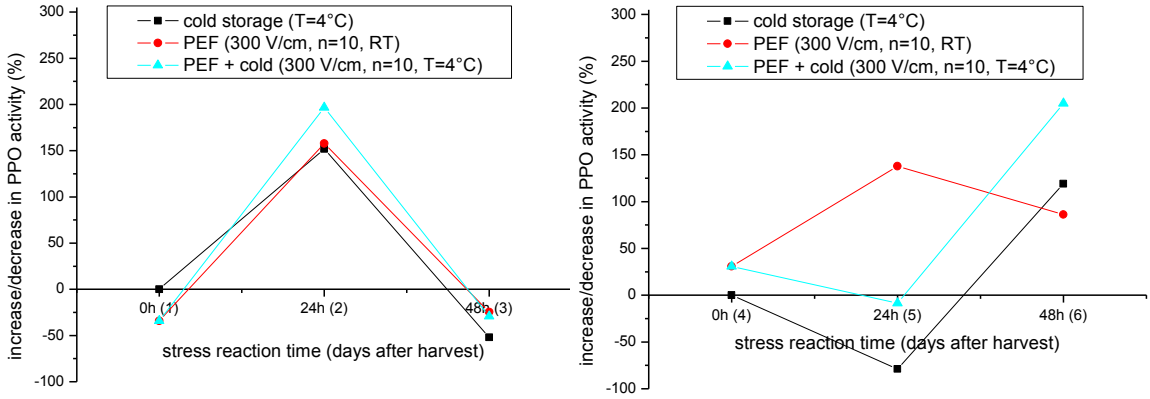


Figure 4-43: Changes of PPO activity during stress reaction period of 48 h, in grape berry obtained from grape clusters treated (A) 1 and (B) 4 days after the harvest; due to cold storage at 4 °C, PEF treatment (300 V/cm, 10 pulses, 0.007 kJ/kg) and their cumulative effect (PEF+cold).

When 700 V/cm were imposed to grapes stored for ***four days after the harvest***, increase in PPO activity was observed immediately after treatment (Figure 4-44A), similar to response observed immediately after 300 V/cm (Figure 4-43B). However, 24 h after PEF treatment at 700 V/cm (kept at RT), inhibition of PPO activity could be noticed (Figure 4-44A). Enzyme inhibition might be associated to predomination of catabolic processes through prolongation of plant adjustment to strained conditions. Subsequent cold storage after PEF treatment at 700 V/cm induced increased PPO activity after 48 h (Figure 4-44A) to a larger extent than after 300 V/cm (Figure 4-43B), indicating dose dependent response.

Increase in PPO activity was observed immediately after treatment with 700 V/cm applied to grape clusters stored for ***six days after the harvest*** (Figure 4-44B), similar to response observed after 700 V/cm imposed to grapes stored for four days (Figure 4-44A).

However, plant adjustment observed through inhibition in PPO activity during 24 h, remained over 48 h stress reaction period (Figure 4-44B). The lowest PPO activity was observed after multiple stress factors from PEF and subsequent cold storage, indicating cumulative effect of both stressors. Physiological fruit fitness that grape clusters attained after 6 days of postharvest storage must have affected plant response on the same stress intensity and no additional rise in PPO activity was observed.

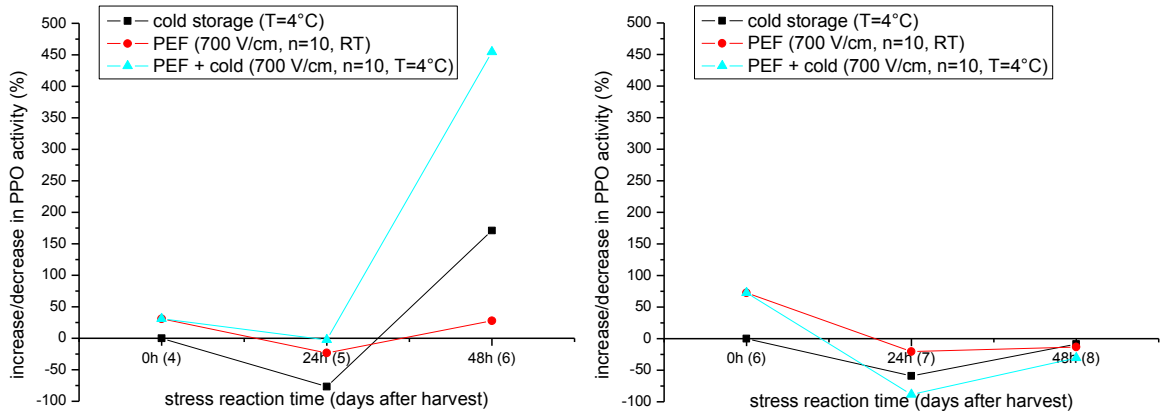


Figure 4-44: Changes of PPO activity during stress reaction period of 48 h, in grape berry obtained from grape clusters treated (A) 4 and (B) 6 days after the harvest; due to cold storage at 4 °C, PEF treatment (700 V/cm, 10 pulses, 0.037 kJ/kg) and their cumulative effect (PEF+cold).

As noticed previously, PEF treatment was not the only elicitor that induced stress reactions in plant system. Temperature change was shown to affect PPO activity and TP concentration of grapes as well. Both elicitors combined together could exhibit cumulative effect of imposed stressors, which resulted in different response. The measurements of PPO activity indicated an influence of PEF on postharvest metabolic changes in grape berry tissue. Comparing increase and decrease in PPO activity with changes of total polyphenol concentration after PEF treatment imposed to grape clusters stored for 1, 4 and 6 days (subchapter 4.4.3), no relation could have been found which would bring this two in direct dependence. Since enhanced mass transfer of intracellular components is achieved after exposure to PEF (Knorr & Angersbach, 1998; Ade-Omowaye et al., 2001b), it could be assumed that release of polyphenol substances from vacuoles came in contact with PPO located in cytosol. However, changes in enzymatic activity did not influence the amount of total phenols in the same manner. Thereafter, changes in TP concentration were not influenced only by phenolic oxidation system catalyzed with PPO, but must have been involved in the expression of plant resistance to strained conditions. Thereafter it could be proposed that at least two metabolic pathways concerning synthesis and oxidation of secondary metabolites must be included in fruit

response on PEF treatment. Positive correlation between PPO activity and resistance to stress has been made in a number of studies (Rivero et al., 2001; Mayer, 2006; Thipyapong et al., 2007; Pinto, Siqueira, Oliveira & Fernandes, 2008). However, the exact function of PPO has still not been clearly demonstrated, which makes it very difficult to subjoin its role after application of PEF treatment as external stress elicitor. Due to complexity of biological system and involvement of many other enzymes (e.g. PAL, POD), amino acids and cofactors in phenylpropanoid metabolic pathway responsible for generation of secondary metabolites, further investigation is necessary to clarify possible mechanism of action caused by PEF treatment. Some of these will be discussed in subchapters 4.5 and 4.6.

4.5. Apple (*Malus domestica*) cell suspension culture

During the course of this study, cell culture suspensions of corresponding higher plants were used as model systems to eliminate uncontrollable environmental interactions. Since plant cells grown in culture suspension posses the complete genetic information, but not the morphological complexity of a higher plant (Rao & Ravishankar, 2002), they are suitable for basic studies. Furthermore, higher rate of metabolism than intact differentiated plants and fast proliferation of cell mass within short cultivation time (2 to 4 weeks) offers an advantage of model system for this study. Due to similarity of metabolic processes comparable to parent plant, cell cultures were used to investigate polyphenolic accumulation and activity of related enzymes.

4.5.1. Growth cycle of apple (*Malus domestica*) cell culture

Malus domestica cell culture was used as model system for apple fruit tissue to acquire more information about impact of PEF stress on the biosynthetic pathway. Obtained cell culture was subcultured in regular cycles of 7 days to maintain stable cell line as batch culture, grown in culture flasks. To characterize properties of apple cell culture, the growth period was analysed in order to select most suitable time during cultivation with adequate enzyme activity to ensure biosynthetic capacity of the cells for an exposure to the electric field treatment. Several growth criteria were chosen (fresh and dry weight increase, polyphenol oxidase activity, total polyphenol concentration, pH, conductivity and cell viability) and monitored over 10 days of cultivation (Figure 4-45 and 4-46).

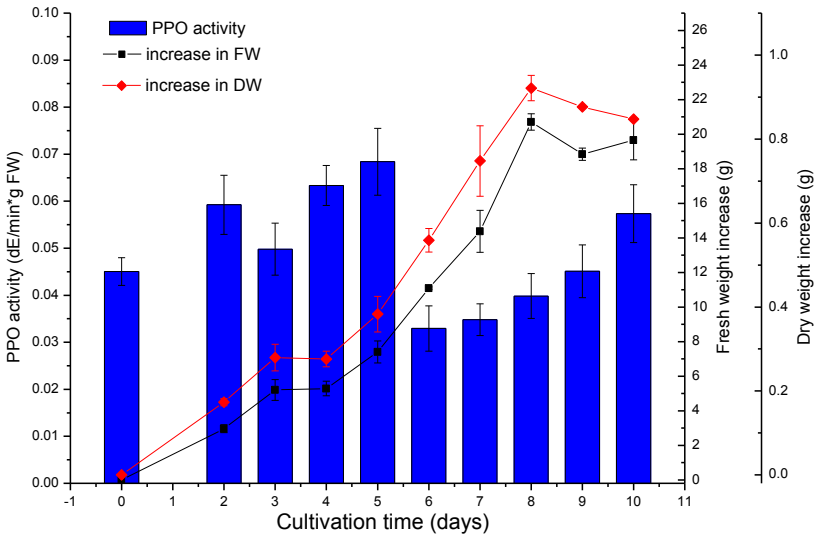


Figure 4-45: Growth curves containing polyphenol oxidase activity, fresh and dry weight of *Malus domestica* cell suspension culture during 10 days cultivation.

A short incubation time is necessary for cell adaptation to new environment after cell inoculation into new fresh medium (Stepan-Sarkissian & Grey, 1990). This was observed through increased PPO activity 2 days after inoculation (Figure 4-45) and decreased cell culture viability correlated to reductase activity (measured by TTC reduction) at the same period after subcultivation (Figure 4-46). After short adaptation period initial lag phase was observed between 3rd and 4th day of cultivation (Figure 4-45). The cell biomass at that time remained constant, during which cells regained ability to divide. Entering exponential phase of growth, cells underwent rapid cell division, and from day 4 to 8 biomass triplication was observed (Figure 4-45). From day 8 progressive deceleration and stationary phase were detected through decrease of fresh and dry weight of the cells. During stationary phase cell growth is retarded and eventually ceases, wherein intra- and extracellular ingredients are available for other metabolic processes, such as secondary metabolite production. This is supported with larger total polyphenol concentration measured in cells cultured for 9 and 10 days, simultaneously with decreased conductivity of cell suspension (Figure 4-46). Since nutrient growth media are supplemented with micro- and macronutrients such as ions and salts, nutrient intake can be observed through decreased conductivity during whole cultivation period.

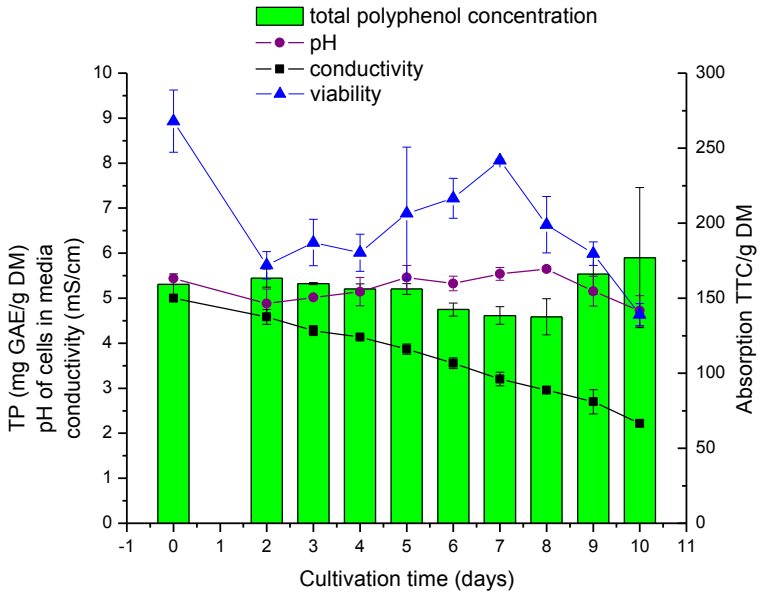


Figure 4-46: Total polyphenol concentration, pH, conductivity and viability of *Malus domestica* cell suspension culture during 10 days cultivation.

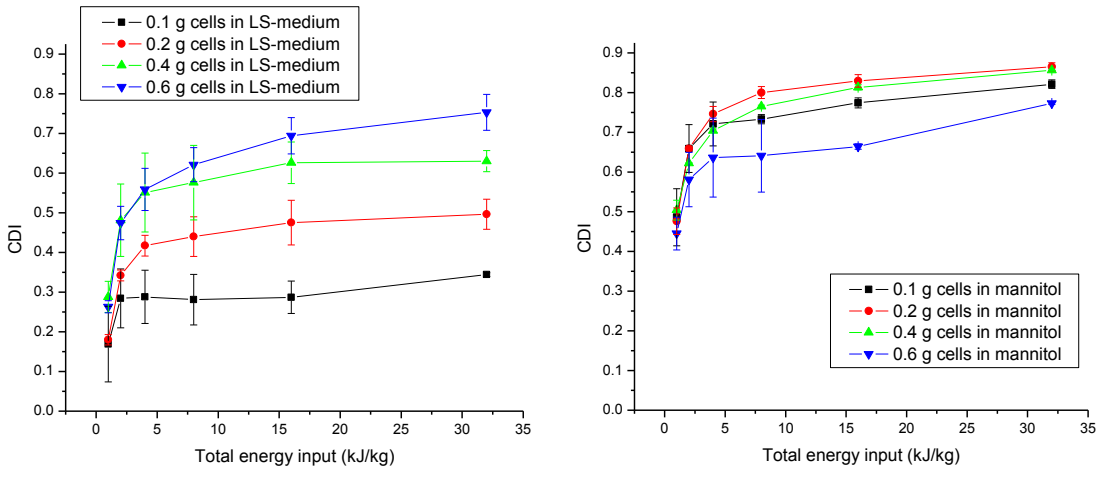
Growth kinetic of apple cell suspension cultures showed stable properties with stagnation during lag phase and highest cell viability at day seven after inoculation (Figure 4-46). Loss of cell viability observed in stationary phase of growth is typical occurrence in the cell cultures due to changed environmental conditions, i.e. due to

nutrient exhaustion from media and cellular waste accumulation (Hossein & Mulligan, 2002). In the early cell growth period larger PPO activity was observed than in later developmental stage and reached highest activity 5 days after inoculation (Figure 4-45). Although many studies concerning specific functions of PPOs have been published, complete mechanism of action and its involvement in modulation of developmental process is still not fully understood (Mayer, 2006; Richter, Lieberei, Strnad, Nóvak, Gruz, Rensing et al., 2012). PPO activity was reported to be considerably high in plants containing significant amount of phenolic compounds, and suggested to play main role during growth protection against infection and injury (Mayer, 2006).

Due to obtained constant growth properties, *Malus domestica* cell culture was shown to be suitable for investigation of stress induction. Two different growth periods have been chosen during which cells were exposed to PEF treatment: during exponential phase of growth, when the highest enzyme activity was observed (5 days after inoculation), and in the early lag phase (3 days after inoculation), just after the adaptation period to new environment.

4.5.2. Induction of membrane permeabilization by PEF application

The impact of different pulsed electric field treatment parameters on cell membrane permeabilization of *Malus domestica* cells has been investigated. Since cell density and treatment medium conductivity was assumed to influence membrane permeabilization effect and CDI measurements, PEF treatment of cultured cells was performed in growth medium and in mannitol. Extent of membrane permeabilization after different PEF treatment intensities and cell density is shown in Figure 4-47.



(A) (B)

Figure 4-47: Extent of membrane permeabilization in *Malus domestica* cultured cells, measured as cell disintegration index (CDI) after application of PEF treatment (field strength 7.1 kV/cm) in: (A) LS-medium; and (B) mannitol.

The disintegration index measured after treatment of cells in cultured LS-medium was shown to be cell density dependant, while detection of CDI within mannitol solution (after PEF treatment with the same processing conditions) showed less sensitivity to cell density (Figure 4-47). By increasing cell density in LS-medium, increased permeabilization of cellular tissue was observed, most probably due to lower conductivity of treated cell solution. Increased density of cells in mannitol increased conductivity of PEF treated solution and lower permeabilization of cells was observed.

Growth medium normally contains large variety of charged macromolecules and ions that interfere with the electrical pulses, therefore iso-osmolar solutions such as mannitol solution, with low conductivity, are often used for investigation of cell permeabilization properties (Mally, McKnight & Glassy, 1992). Since conductivity of LS-medium is high and CDI determination is based on the frequency dependence of electrical conductivity of biological cells, changes in measured conductivity due to PEF induced cell disintegration are less detectable. Therefore, mannitol was chosen as media for measurements of the impact of electric field strength, pulse number and total energy input on the cell membrane permeabilization of cell cultures.

Malus domestica cells have been exposed to PEF treatment in growth LS-medium and suspended in mannitol for further detection of cell membrane permeabilization. To obtain similar total energy input with three chosen electric field strengths (540, 1240 and 2540 V/cm) various number of pulses have been applied. Extent of membrane permeabilization expressed as cell disintegration index is shown in Figure 4-48.

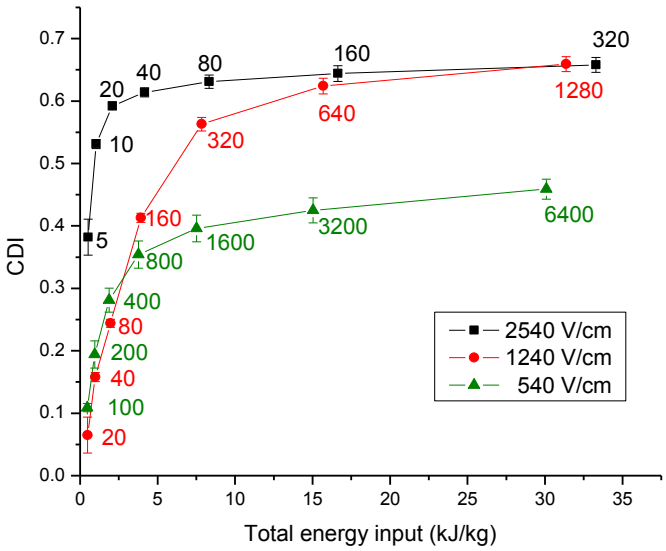


Figure 4-48: Extent of membrane permeabilization in *Malus domestica* cultured cells, measured as cell disintegration index (CDI), after application of PEF treatment, depending on energy input with different field strength and number of pulses.

The cell disintegration index of approximately 0.4 can be obtained after 5 pulses at 2540 V/cm (total energy input of 0.5 kJ/kg); after 160 pulses at 1240 V/cm (total energy input of 4 kJ/kg); and after 3200 pulses at 540 V/cm (reaching total energy input of 15 kJ/kg). Therefore, it can be noticed that low electric field strength with sufficient number of pulses can reach permeabilization degree of larger field strength applied. However, much larger energy input is required to reach same degree of permeabilization.

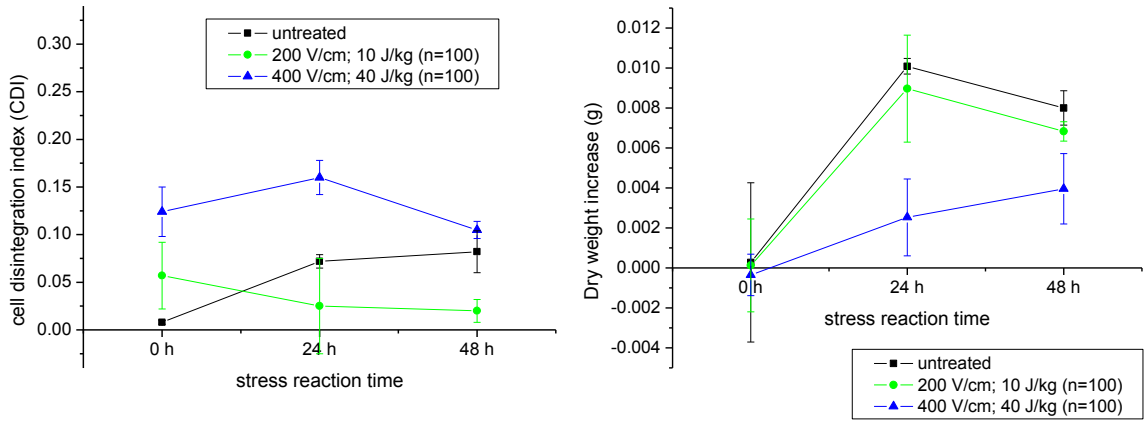
Increasing treatment intensity by increasing number of pulses at corresponding field strength, the cell disintegration index increased to a certain level, after which no drastic disintegration was observed (Figure 4-48). Although similar total energy input was applied, significant difference in permeabilization can be observed between electric field strength of 540 and 2540 V/cm. Higher PEF efficiency for the cell membrane permeabilization can be observed with increase in the electric field intensity. Therefore, the cell disintegration level obtained was shown not to be dependant only on the total energy input.

4.5.3. Impact of stress by PEF in the early lag growth phase

Apple plant cell cultures (*Malus domestica*) have been subjected to 100 pulses at 200 and 400 V/cm (total energy input of 10 and 40 J/kg, respectively) on the third growth day. Due to experimental design and need to keep cells viable with least stress (other than PEF treatment) as possible, the cell cultures have been grown and treated in LS-medium, harvested after stress reaction time and analysis of chosen parameters was performed.

Disintegration of cellular material measured in apple cells resuspended in mannitol solution immediately after 100 pulses at 200 and 400 V/cm (0 h stress reaction time) was larger than in untreated cells (Figure 4-49A). After 24 and 48 h stress reaction time, CDI value of apple cells treated with 200 V/cm (total energy input of 10 J/kg) was lower than in untreated cells maintained at the same conditions, indicating reversible membrane permeabilization. Untreated suspension cultures were transferred into PEF treatment chamber to simulate possible mechanical or hydrodynamic stress caused by movement of cells, which might have caused damage to certain population of cells in suspension. Similar observation was made after 20 pulses at 300 V/cm (total energy input of 136 J/kg) applied on apple fruit tissue which was attributed to sample preparation step (see subchapter 4.1.1.). Larger impact to cultivated apple cells was noticed after 100 pulses at 400 V/cm, where CDI value of 0.124 was measured immediately after treatment (Figure 4-49A), representing membrane permeabilization of 12.4 % of the cells. 24 hours after

PEF induction CDI value reached 0.16 indicating extension of pores or increased number of cells that suffered damage after stress reaction period.



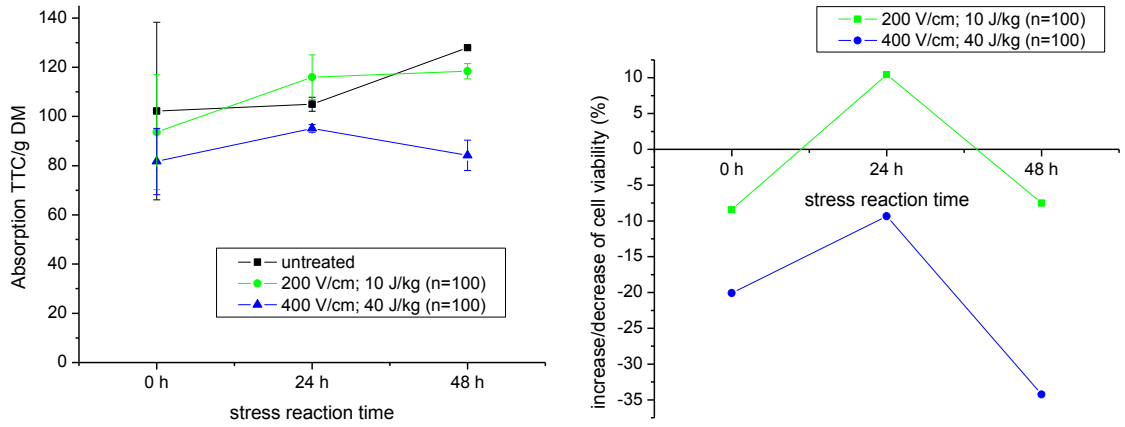
(A) (B)

Figure 4-49: Impact of PEF treatment on: (A) Extent of membrane permeabilization, measured as cell disintegration index (CDI); and (B) Dry weight of cells; in *Malus domestica* cell culture after 0, 24 and 48 h stress reaction times.

Changes in dry weight of PEF treated and untreated cells 24 and 48 h after the treatment (which corresponds to 4th and 5th day of cultivation) were investigated (Figure 4-49B). Much faster increase in dry weight of untreated and PEF treated cells with lower treatment intensity (200 V/cm) was noticed, than within cell cultures treated with 400 V/cm, indicating retarded growth caused by PEF treatment. This is supported with lower cell viability measured in cells after 400 V/cm (Figure 4-50). Cell viability of untreated cultures differed between batches which can be observed from large standard deviation at 0 h stress reaction time (Figure 4-50A), indicating that transfer to treatment chamber and back induced short destabilisation of cells in suspension culture. However, cell activity remained constant after 24 h (4th day of cultivation) and increased after 48 h indicating normal cell growth as observed during cultivation period (Figure 4-46).

To determine differences to normal cell metabolic activity during tested period, culture viability of untreated cells was taken as standard and difference to cells normal activity was calculated as percent increase or decrease of cell viability (Figure 4-50B). Since the use of TTC viability test relies on reductase activity within a live cell, and dead cell should not have mitochondrial activity to reduce TTC to red water insoluble triphenylformazin (which is spectrophotometrically measured), percentage of decrease can be regarded as percent of dead cell population. Thereafter, 80 % of cells remained their viability immediately after PEF treatment with 400 V/cm and only 66 % 48 h after the treatment (Figure 4-50B). Healthy cell population exhibits rapid increase in cell mass

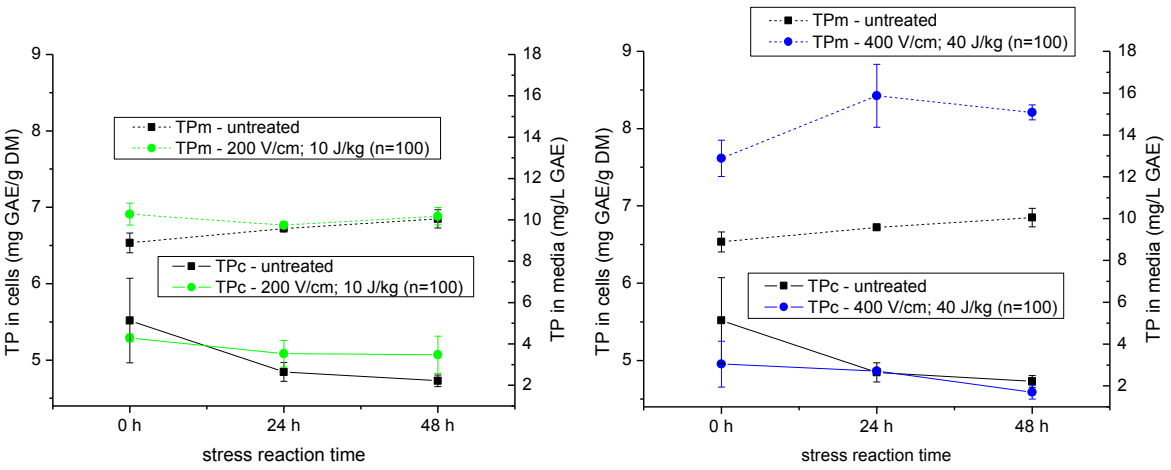
(Figure 4-45) and viability (Figure 4-46) on the 4th and 5th day of cultivation during exponential phase of growth, which was not observed in stressed culture with 400 V/cm (Figure 4-49B and 4-50). To gain information if and when cells regained ability to divide and grow, culture should be grown for a longer time after imposed stress. Modified experimental design was thereafter used for further investigation (see subchapter 4.6).



(A) (B)

Figure 4-50: Impact of PEF treatment on the *Malus domestica* cell culture viability, measured after 0, 24 and 48 h stress reaction times presented as: (A) reductase activity and (B) changes in cell viability expressed as percentage of increase/decrease in respect to untreated samples.

Although polyphenolic substances do not directly affect basic metabolism, they support physiological activation of cells and are engaged in defence mechanisms against various factors (Kim, Chun & Kim, 2000). Therefore, total polyphenol concentration was measured in the plant cells and within cultured media after 100 pulses at 200 V/cm (Figure 4-51A) and at 400 V/cm (Figure 4-51B).



(A) (B)

Figure 4-51: Impact of PEF treatment: (A) 100 pulses at 200 V/cm, total energy input of 10 J/kg; and (B) 100 pulses at 400 V/cm, total energy input of 40 J/kg; on total polyphenol concentration in cells (TPc) and in growth medium (TPm) of *Malus domestica* cell culture, measured after 0, 24, and 48 h stress reaction times.

Immediately after PEF treatment (0 h stress reaction time) lower polyphenol concentration was measured within the cells, and higher in cell growth media for both treatment intensities used (Figure 4-51), most probably due to altered transport through the cell membrane. However, after 400 V/cm much larger depletion in cells and increase in cultured media were observed. During stress reaction times of 24 and 48 h after the treatment with 200 V/cm, increase of TP concentration within cells was observed which exceeded concentration values of untreated cells, while no alterations were detected within treated media (Figure 4-51A). Due to cell culture recovery observed after lower PEF intensity (Figure 4-49B and 4-50), initiation of polyphenol production during stress reaction time can be assumed.

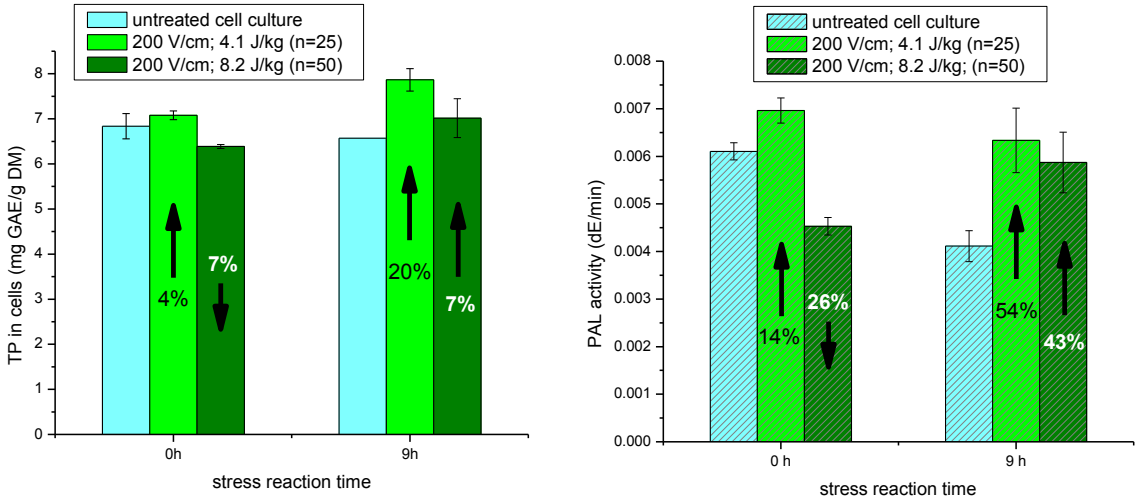
When larger PEF intensity was applied (400 V/cm), TP concentration in cells reached value of untreated samples after 24 h (Figure 4-51B). However, certain population of cells did not regain its viability (Figure 4-50B) and leak through damaged membrane occurred (Figure 4-49A), which resulted in much larger concentration of polyphenolic substances in growth medium (Figure 4-51B). These observations are indicating that stress response of *Malus domestica* cell culture to PEF treatment is dose dependant.

4.5.4. Impact of stress by PEF in the exponential phase of growth

Apple cell culture were further grown and treated with 200 V/cm and lower number of pulses in order to observe changes of the action of phenylalanine ammonia-lyase (PAL) and TP concentration. PAL is the key enzyme of phenylpropanoid metabolism, through which all phenylpropanoids are formed (Dixon & Paiva, 1995) and therefore represents a good marker for polyphenol *de novo* synthesis. Exponential phase of culture growth was chosen to be suitable for this set of treatments, since cells passed adaptation to new environment (due to subculturing) and regained ability to divide. By exposing cells to external stimuli in exponential phase, multiple stress effect (adaptation and PEF) which may cause different response can be avoided. Furthermore, shorter period of time after imposed stress was chosen according to literature data, since accumulation of PAL mRNA was observed with peak value 9 hours after the elicitation (Gómez-Vásquez et al., 2004).

The total polyphenol concentration in apple cells significantly increased 9 h after imposed PEF treatment of 25 pulses at 200 V/cm, resulting in total energy input of 4.1 J/kg (Figure 4-52A). 14 % larger PAL activity was determined immediately after treatment, indicating cell response to stress signal (Figure 4-52B). 9 h after the treatment

PAL activity was even larger and exceeded value of untreated cells for 54 %. Increased PAL activity immediately after treatment preceded increase in TP concentration observed 9 h after imposed stress, which indicates *de novo* synthesis of polyphenolic substances. Similar observations were made in cassava (*Manihot esculenta*) cell culture, after elicitor induced PAL mRNA accumulation, with peak value 9 hours after the elicitation, which preceded induction of PAL activity that reached maximum 15 hours after elicitation (Gómez-Vásquez et al., 2004). Furthermore, stress response has been reported in minimally processed lettuce leaves through increased phenolic content after wound induction of PAL (Saltveit, 2000; Gegl`Innocenti et al., 2005).



(A) (B)

Figure 4-52: Impact of PEF treatment on: (A) total polyphenol (TP) concentration in cells; and (B) phenylalanine ammonia-lyase (PAL) activity; in *Malus domestica* cell culture after 0 and 9 h stress reaction times.

*Black arrows denote percent increase or decrease of values compared to untreated sample.

The impact of 50 pulses at 200 V/cm (total energy input of 8.2 J/kg) resulted in lower TP concentration measured immediately after treatment (Figure 4-52A). At the same time lower PAL activity was determined (Figure 4-52B). After 9 h, PAL activity increased and was 43 % higher than in untreated cells, which resulted in 7 % larger TP concentration at the same stress reaction time. Since biosynthesis of polyphenolics starts with the conversion of phenylalanine by the PAL, larger increase in TP concentration could be expected after longer stress reaction period than measured within this set of trials.

Enzyme activity can be affected by various parameters such as chemical environment i.e. due to change of pH. However, no alterations in pH and cell suspension conductivity were observed immediately after treatment or after 9 h (data not shown),

indicating that PEF treatment was through different triggering mechanism responsible for observed changes. Triggering signals for different abiotic factors are till date not entirely identified, due to complexity of biosynthetic pathways and large number of involved precursors. However, changes in TP concentration after PEF induction can be correlated to PAL activity and were found to be dose dependant. Thereafter it can be suggested that phenylpropanoid metabolism was triggered by low intensity PEF application. Similar observations were made after stress induction in apple skin by UV irradiation, where *de novo* synthesis of anthocyanins and increased PAL activity were observed (Dong et al., 1995). The authors reported increase in PAL transcript, suggesting that the increase in PAL enzymatic activity was due to *de novo* synthesis of the enzyme.

4.6. Grape (*Vitis vinifera*) cell suspension culture

The induction of stress by PEF treatment on *Vitis vinifera* cell culture abundant with polyphenolic substances was investigated. This cell culture suspension was used as a model system of corresponding higher plants to observe possible changes during longer stress response period. To detect plant reaction on PEF as external stressor different growth parameters were analyzed together with cell viability, impact on the cell membrane, polyphenolic accumulation and activity of related enzymes.

4.6.1. Growth cycle of *Vitis vinifera* cell culture

Cultured cells were grown *in vitro* with the growth cycles of 14 days before further subcultivation in order to maintain cell line stability. To determine growth and culture viability, measurements of the cell mass, enzyme activity, pH, conductivity and anthocyanin concentration was performed in order to determine optimal time during growth cycle for stress induction. Figure 4-53 represents growth curves of the cell suspension expressed through increase in fresh and dry weight of harvested cells. Linear growth of cultured cells in fresh nutrient medium up to 9th day after inoculation can be observed through steady increase in fresh weight. More than double fresh weight biomass increase of the cell population from day 2 to 9 was observed, representing exponential phase of growth. Decay was noticed on the 10th day of inoculation, indicating progressive deceleration phase and start of the stationary phase.

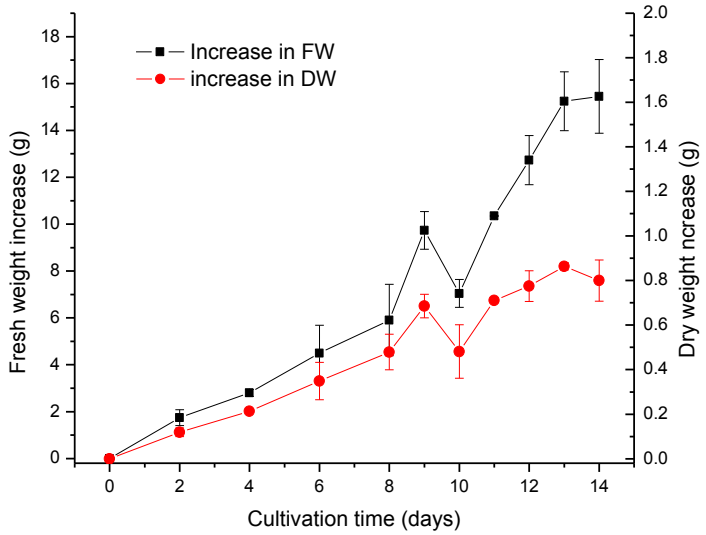


Figure 4-53: Growth curves containing fresh and dry weight of *Vitis vinifera* cell suspension culture during 14 days cultivation.

Corresponding increase in dry weight was observed up to 9th day after inoculation, with sharp declination on 10th day, followed by stagnation and slower gradual increase. Increase in fresh weight and stagnation in dry weight during stationary phase represents reduction of cell division and single cell enlargement, since cells seize tendency to trap culture medium, which leads to increase in fresh weight (Stepan-Sarkissian & Grey, 1990). At that time the cells are utilizing intracellular ingredients and energy for other metabolic processes, such as secondary metabolite production. This impression is supported by the increase of anthocyanins concentration in the cells at the same cultivation period (Figure 4-54).

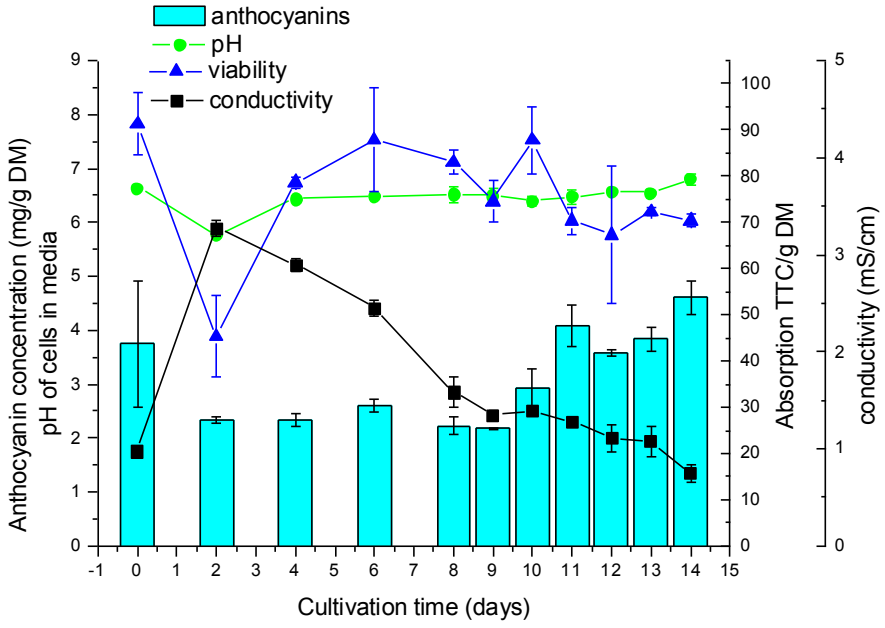


Figure 4-54: Anthocyanin concentration, pH, viability and conductivity of *Vitis vinifera* cell suspension culture during 14 days cultivation.

Difference in cell viability, pH and conductivity of the cell suspension between day 0 and 2 are attributed to cell transfer into fresh nutrient medium (Figure 4-54), representing short incubation time necessary for adaption to new environment. Metabolic activity of the cell suspension was retained during whole cultivation period, with highest viability level 6th day after inoculation, followed by typical decrease during stationary phase of growth.

Cells adsorb nutrients (ions/salts) from the growth medium required for normal cell development. Accordingly, conductivity of the growth medium decreases, which can be observed during the cultivation period of 14 days (Figure 4-54). Larger intake of nutrients necessary for cell division in the exponential phase of growth can be observed up to 9th day after inoculation.

Polyphenoloxidase (PPO) and peroxidase (POD) activity have been observed during 14 days of cultivation (Figure 4-55). Decrease in POD activity during lag phase of adaptation was observed (between day 0 and 2). However, increased PPO activity was observed during first 4 days after inoculation, indicating longer adaptation time to stress induced by transfer into new environment. Positive correlation between PPO activity and resistance to stress has been often made, however, the exact function of PPO has still not been clearly demonstrated (Mayer, 2006). Highest enzyme activity was reached during exponential phase of growth, after which a decrease during progressive deceleration and stationary phase was observed (Figure 4-55).

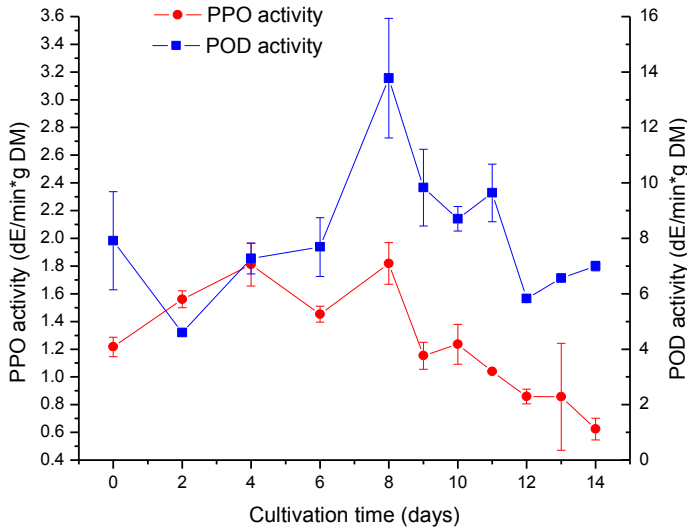


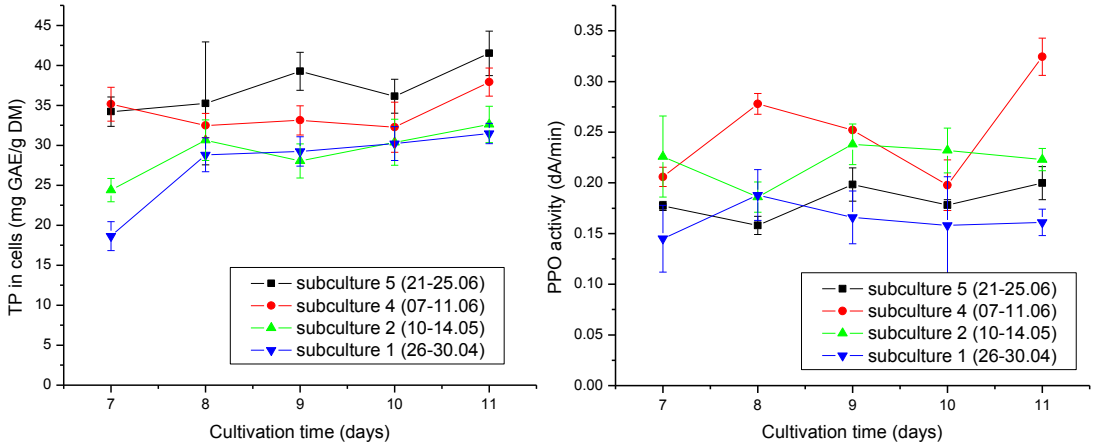
Figure 4-55: Polyphenol oxidase and peroxidase activity during 14 days cultivation of *Vitis vinifera* cell suspension culture.

Stable *Vitis vinifera* cell suspension cultures were obtained. Growth kinetics showed that high enzymatic activity and cell viability occurred before cells entered stationary phase, and therefore optimal time for stress induction by pulsed electric field treatment was chosen on 7th day of cultivation.

4.6.2. Differences between *Vitis vinifera* subcultures

Relative homogenous cell suspension cultures consist of cells and cell aggregates dispersed and grown in nutrient media. Over longer period of subcultivation changes in metabolite accumulation and enzymatic activity might occur (Mustafa et al., 2011), which was observed during subcultivation period of three months (Figure 4-56). Overall TP concentration in *Vitis vinifera* cells (measured between 7th and 11th day of cultivation) was larger after every further inoculation (Subculture 1 < 2 < 4 < 5). During subcultivation period, only representative cells with deep red colour were chosen for further inoculation,

which was the reason for larger TP concentration in cell cultures with increasing subculturing number (Figure 4-56A). Variations in PPO activity between subcultures was observed as well (Figure 4-56B). However, no correlation between cultivation time and enzyme activity can be drawn.



(A) (B)

Figure 4-56: (A) Total polyphenol concentration and (B) polyphenol oxidase activity of *Vitis vinifera* cell cultures obtained during subcultivation period.

Cell cultures exhibited stable growth with some divergences between subcultures, which was stimulated by the subculturing technique. Thereafter, to differentiate impact of additional external factor other than natural variability between subcultures, the PEF induced changes of every trial only refer to the untreated cells that were taken from the same subculture, processed and analyzed at the same time.

4.6.3. Degree of membrane permeabilization by PEF application

The impact of PEF treatment at different electric field strengths and 20 pulses on *Vitis vinifera* cells in nutrient media has been investigated. The cell disintegration index of PEF treated and corresponding untreated cultures was measured in mannitol, to avoid interference of high conductive growth medium (see subchapter 4.5.2). The impact of different treatment intensities is shown in Figure 4-57 after chosen stress reaction period (denoted as stress reaction time of 0, 24, 48, 72 and 96 h after imposed PEF treatment).

Disintegration of cellular material measured in *Vitis vinifera* cells (subculture A) after 200 and 500 V/cm (total energy input of 4.9 and 21.4 J/kg, respectively) did not significantly differ from corresponding untreated cultures (Figure 4-57). Disintegration of cellular material observed in untreated cell suspension can be attributed to a number of damaged cells that might have occurred due to cell transfer. These results indicate that PEF treatment at 200 and 500 V/cm did not cause disintegration of cellular tissue larger

than damage that occurred due to cell culture manipulation during sample preparation procedure. Similar observations were made after PEF treatment of cultured *Malus domestica* cell suspensions (see subchapter 4.5.3).

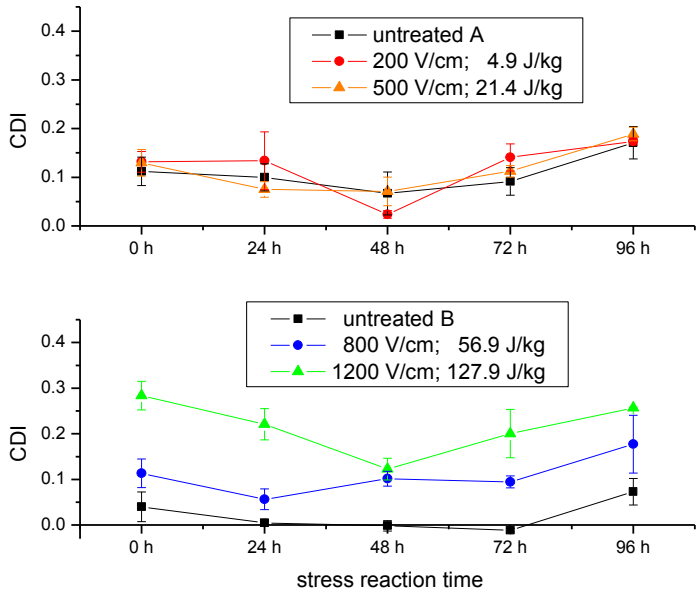


Figure 4-57: Impact of PEF treatment on the extent of membrane permeabilization in *Vitis vinifer* cell culture measured as cell disintegration index (CDI) after 0, 24, 48, 72 and 96 h stress reaction times.

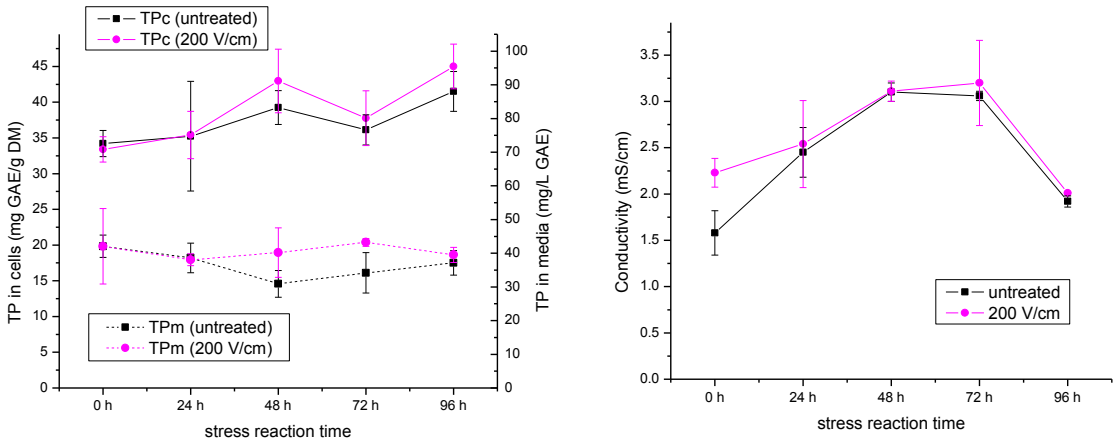
Larger impact on cultivated cells was noticed immediately after PEF treatment with 800 and 1200 V/cm, respectively, where CDI value of 0.114 and 0.284 were reached (Figure 4-57). The CDI observed immediately after treatment was kept over stress reaction period. Pores remained open for the flow of the polar molecules, representing membrane irreversible permeabilization for a certain percent of cell population. Since relative homogenous cell suspension cultures consist of larger and smaller cells dispersed in nutrient media, it can be assumed that portion of permeabilized cells were larger cells, which are more susceptible to PEF treatment.

4.6.4. Impact of stress by PEF on *Vitis vinifera* cell culture

Exponential phase of growth was chosen for further set of trials with *Vitis vinifera* cell culture, which corresponds to PEF induction on 7th day of cell cultivation. Since *Malus domestica* cell cultures was shown to be responsive to exposure of PEF treatment with lower number of pulses, 20 pulses at different electric field strengths were applied to *Vitis vinifera* suspension culture. Furthermore, to observe possible changes caused by imposed stress, total polyphenol concentration (in cells and media), polyphenol oxidase activity, medium pH and conductivity were investigated after longer stress response period (0, 24, 48, 72 and 96 h stress reaction times).

4.6.4.1. Treatment with 200 V/cm

Total polyphenol concentration of *Vitis vinifera* cell culture obtained immediately after 20 pulses at 200 V/cm (total energy input of 4.9 J/kg) did not differ from untreated samples (Figure 4-58A). However, after 48 h stress reaction time, although insignificant, trend of larger TP concentration in cells and in growth media was observed, and kept over 96 h after imposed stress. Polyphenolic content in growth media mostly depends on cell excretion and may occur due to leakage of intracellular components into surrounding media due to cell membrane permeabilization. Since no disintegration of cellular material was observed (Figure 4-57), it can be suggested that other mechanism was involved in increased concentration of polyphenolic substances in PEF treated culture.



(A) (B)

Figure 4-58: Impact of PEF treatment (20 pulses at 200 V/cm; total energy input of 4.9 J/kg) on: (A) Total polyphenol concentration in cells (TPc) and in growth medium (TPm); and (B) Suspension culture conductivity; of *Vitis vinifera* cell culture measured after 0, 24, 48, 72 and 96 h stress reaction times.

Conductivity of growth media depends among other factors on the amount of polyphenolic substances. After initial increase in culture suspension conductivity, observed immediately after treatment with 200 V/cm, no significant changes were noticed during stress reaction period (Figure 4-58B). Thereafter, larger concentration of TP in media after 48-96 h stress reaction time was not sufficient to affect conductivity of cell suspension. Since no change in TP concentration were observed during first 24 h after imposed treatment (Figure 4-58A), initial increase in conductivity (0 h) was not induced by TP leakage through the membrane. However, the external electric field might have caused enhanced transport of molecules and ionic species through the membrane associated with diffusion potentials and membrane pumps.

Since synthesized phenolic compounds are well known substrates for polyphenol oxidase (Mayer & Harel, 1979), PPO activity has been measured during stress reaction

period in parallel to TP concentration. Immediately after treatment with 200 V/cm lower PPO activity was observed (Figure 4-59). After 24 h stress reaction time, normalization took place and PPO activity approached values of untreated samples. PPO is intracellular enzyme probably located in plastids, while polyphenolics are stored in vacuoles or bound to polysaccharides in the cell wall (Basra & Basra, 1997; Mayer, 2006). After application of PEF treatment, occurrence of irreversible membrane permeabilization was not observed (Figure 4-57), however, transmembrane traffic of polar compounds has been altered, which might have caused substrate relocation. Phenolic substances or other compounds that affect the active enzyme site can inhibit PPO due to their structural similarities (Mayer, 2006), which was most probably responsible for lower activity of selected enzyme. Furthermore, no significant change of pH in extracellular surrounding was observed (Figure 4-59), which might display different kinetic behavior of *in vivo* enzyme. 48 h after the treatment lower PPO activity was observed and kept over 96 h after imposed stress. Certain perturbations of cell culture metabolism must have remained during stress reaction period, which was observed through larger TP concentration in cells and in media (Figure 4-58A), while PPO activity remained suppressed (Figure 4-59).

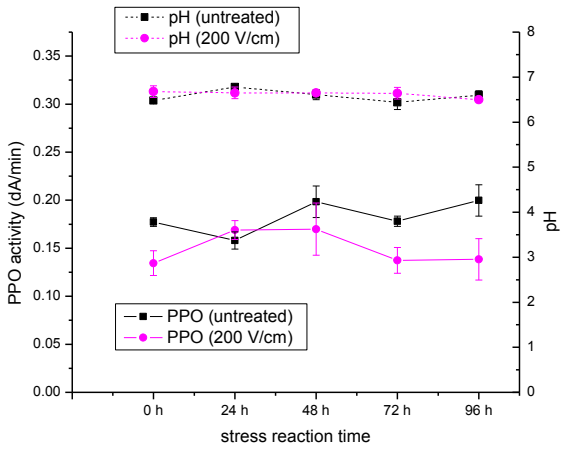


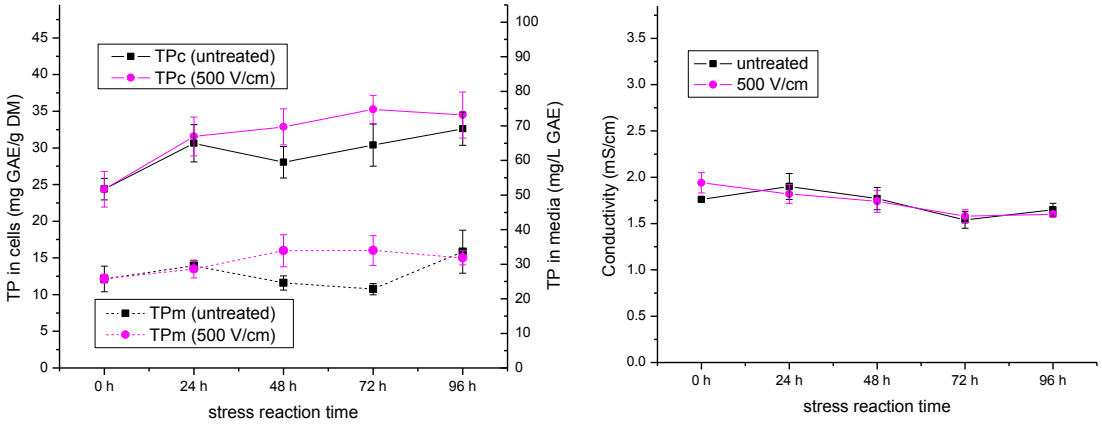
Figure 4-59: Polyphenol oxidase (PPO) activity and pH of untreated and PEF treated (20 pulses at 200 V/cm; total energy input of 4.9 J/kg) *Vitis vinifera* cell culture measured after 0, 24, 48, 72 and 96 h stress reaction times.

4.6.4.2. Treatment with 500 V/cm

Total polyphenol concentration of PEF treated cell culture suspensions during first 24 h after 20 pulses at 500 V/cm (resulting in total energy input of 21.4 J/kg) did not differ from untreated cultures (Figure 4-60A). Larger TP concentration in cells and in growth media was observed 48-96 h after imposed PEF stress. Similar observations were

made after PEF treatment at 200 V/cm (Figure 4-58A), however, larger difference to untreated cultures were observed after larger treatment intensity (Figure 4-60A).

Since disintegration of cellular material after 500 V/cm did not differ from corresponding untreated cultures (Figure 4-57), it can be assumed that phenolic substances synthesized in the cells were to certain extent released into the medium. Moreover, increased TP concentration in media did not occur immediately after PEF treatment, as would be expected due to altered membrane transport, but after 48 h, which was corresponding to the increase of TP concentration in the cells (Figure 4-58A).



(A) (B)

Figure 4-60: Impact of PEF treatment (20 pulses at 500 V/cm; total energy input of 21.4 J/kg) on: (A) Total polyphenol concentration in cells (TPc) and in growth medium (TPm); and (B) Suspension culture conductivity; of *Vitis vinifera* cell culture measured after 0, 24, 48, 72 and 96 h stress reaction times.

Slightly larger conductivity of *Vitis vinifera* suspension culture was observed immediately after PEF treatment, followed by no difference to untreated cultures over stress reaction period of 96 h (Figure 4-60B). As mentioned in previous subchapter, increased TP concentration in media did not affect conductivity of suspension culture.

Reduction of PPO activity was observed immediately after PEF treatment with 500 V/cm (Figure 4-61) in comparison to untreated cell culture. Decreased intracellular enzyme activity in PEF treated cultures was observed during whole stress reaction period. Similar observation was reported after thermal stress induction at 35 °C, when PPO inhibition simultaneously with polyphenol accumulation was observed (Rivero et al., 2001). No change in pH between PEF treated and untreated control cultures was observed (Figure 4-61), suggesting other factor responsible for suppression of PPO activity in PEF treated cultures.

Differences in PPO activity and total polyphenol concentration (in cells and media) of *Vitis vinifera* cell culture after 20 pulses at 500 V/cm were more pronounced

than after treatment at 200 V/cm (subchapter 4.6.4.1), in comparison to corresponding untreated culture. Imposed stress by different PEF treatment intensities affected plant metabolism and dose dependant response was observed.

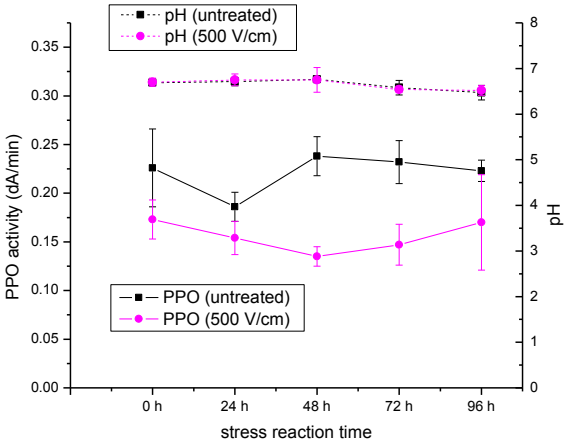


Figure 4-61: Polyphenol oxidase (PPO) activity and pH of untreated and PEF treated (20 pulses at 500 V/cm; total energy input of 21.4 J/kg) *Vitis vinifera* cell culture measured after 0, 24, 48, 72 and 96 h stress reaction times.

4.6.4.3. Treatment with 800 V/cm

Immediately after 20 pulses at 800 V/cm (total energy input of 56.9 J/kg) significantly larger TP concentration in cells was observed, in comparison to untreated control samples (Figure 4-62A). Disintegration of the cellular material was observed in 11.4 % of the cell population immediately after PEF treatment (0 h), while 4 % was observed in untreated control only due to cell culture manipulation (Figure 4-57). No significant change in cell suspension conductivity (Figure 4-62B), nor in TP concentration from corresponding growth medium (Figure 4-62A) was observed at 0 h stress reaction time, indicating no severe leakage of polyphenolics in treated media.

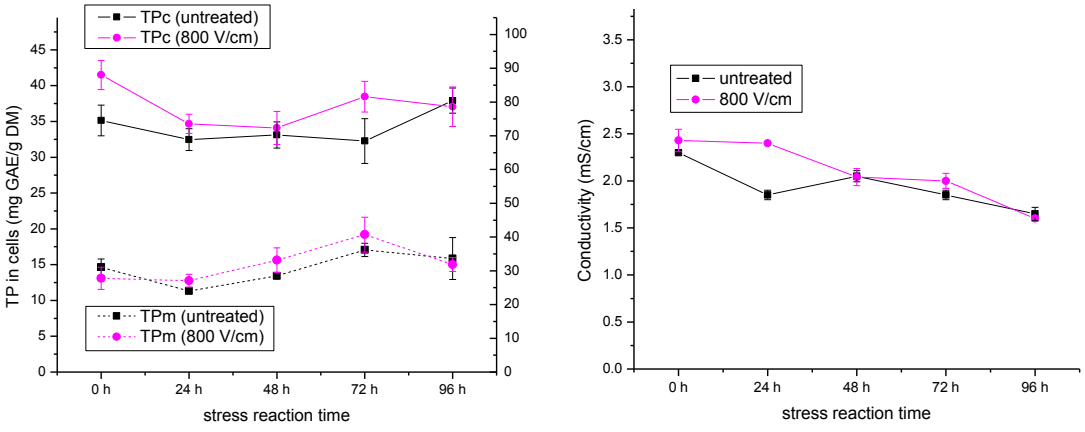


Figure 4-62: Impact of PEF treatment (20 pulses at 800 V/cm; total energy input of 56.9 J/kg) on: (A) Total polyphenol concentration in cells (TPc) and in growth medium (TPm); and (B) Suspension

culture conductivity; of *Vitis vinifera* cell culture measured after 0, 24, 48, 72 and 96 h stress reaction times.

24 h after the treatment slight decrease of TP concentration in PEF treated cells was observed, approaching values of control samples (Figure 4-62A). At the same time larger conductivity of PEF treated suspension culture was observed (Figure 4-62B), with increase of TP concentration in media (Figure 4-62A). Since difference in CDI of PEF treated and untreated cells during observed stress reaction period remained, it can be assumed that larger excretion of intracellular compounds into surrounding media occurred 24 h after the treatment from remained portion of damaged cells. Simultaneously other portion of non severely damaged cells continued production of polyphenolic compounds, which can be observed through larger TP concentration in the cells 72 h after the treatment (Figure 4-62A). No significant difference in conductivity of corresponding media was observed after aforementioned stress reaction period (Figure 4-62B). As observed previously, increased TP concentration in cell media did not affect cell suspension conductivity after PEF treatment with 200 and 500 V/cm (Figure 4-58 and 4-60). Thereafter, it can be assumed that leakage of other substances through damaged membrane caused larger conductivity 24 h after treatment with 800 V/cm.

Reduced intracellular PPO activity was observed immediately after PEF treatment with 800 V/cm (Figure 4-63). However, after 72 h stress reaction time PPO activity of PEF treated cultures approached values of untreated control, while no significant change in pH was detected.

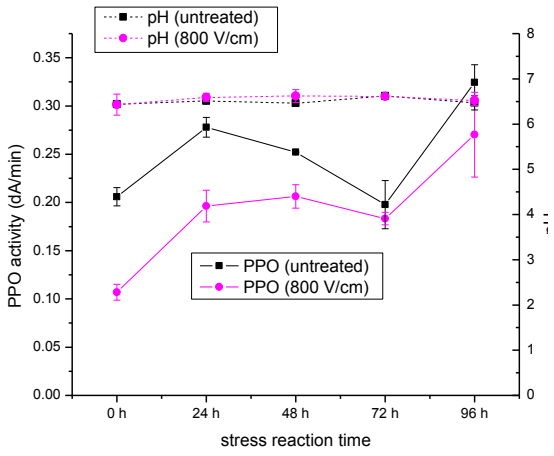


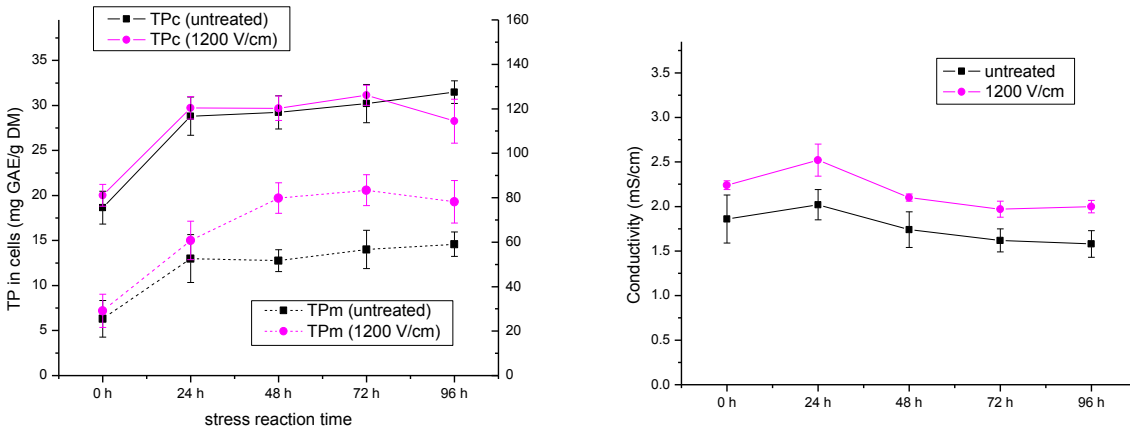
Figure 4-63: Polyphenol oxidase (PPO) activity and pH of untreated and PEF treated (20 pulses at 800 V/cm; total energy input of 56.9 J/kg) *Vitis vinifera* cell culture measured after 0, 24, 48, 72 and 96 h stress reaction times.

Increased PPO activity was found after PEF application due to tissue wounding, which was reported in number of studies after different physiological injuries caused by external stressors (Thipyapong et al., 1995). The application of PEF treatment at

800 V/cm induced irreversible permeabilization to a certain population of the cells (Figure 4-57), while other did not suffer severe damage (depending on the cell size and fitness). Thereafter, it is likely that reversibly damaged cells tolerated imposed stress through adaptation and *de novo* synthesis of polyphenolic substances, simultaneously with degenerative processes of irreversibly damaged cell population.

4.6.4.4. Treatment with 1200 V/cm

Total polyphenol concentration measured in cells after 20 pulses at 1200 V/cm (total energy input of 127.9 J/kg) did not significantly differ from untreated cell cultures (up to 72 h), while larger TP concentration in media was observed (Figure 4-64A). Over stress reaction period, TP concentration in media of PEF treated cultures increased; whereas after 96 h, depletion of TP concentration in corresponding cells was observed.



(A) (B)

Figure 4-64: Impact of PEF treatment (20 pulses at 1200 V/cm; total energy input of 127.9 J/kg) on: (A) Total polyphenol concentration in cells (TPc) and in growth medium (TPm); and (B) Suspension culture conductivity; of *Vitis vinifera* cell culture measured after 0, 24, 48, 72 and 96 h stress reaction times.

Large CDI values detected after PEF treatment with 1200 V/cm (Figure 4-57) and higher conductivity observed in culture suspensions subjected to the same treatment intensity (Figure 4-64B) indicate leakage of intracellular substances in extracellular environment. It seems that plat metabolic system suffered damage larger than cell culture was able to tolerate and consequently no increase in TP concentration within cells occurred. Due to methodological approach it can not be distinguished if some population of the cells managed to tolerate and synthesize *de novo* compounds, since it was not possible to separate cells with different degree of damage. Since PEF treatment was reported to be dependant on the cell size, as well as cell orientation in the field (Heinz et al., 2002; Toepfl, 2006; Chalermchat, Malangone & Dejmek, 2010), it can be further

hypothesized that larger cells suffered greater damage and consequently leakage of intracellular components occurred, which was observed through larger conductivity of cell suspension. Smaller cells would on the other hand manage to undergo imposed stress and keep regular metabolic activities with no additional decrease of TP concentration in PEF treated cells. Similar observation was made after PEF treatment with 800 V/cm (subchapter 4.6.4.3), where it seems that larger population of the cells managed to tolerate stress and increased TP concentration was observed within cells. However, after treatment with 1200 V/cm, larger population of cells underwent severe damage and no significant increase of TP concentration in cells was observed.

Lower PPO activity was observed immediately after PEF treatment with 1200 V/cm in comparison to untreated control, and was kept lower during whole stress reaction period (Figure 4-65). Change in pH of treated suspension culture in comparison to corresponding control was observed, with only statistical significance after 48 h (α=0.05). Although suboptimal pH levels can cause abnormalities in the cell metabolism, undamaged cells can maintain relatively constant cytoplasmic pH (Andersone & Ievinsh, 2008). However, much larger difference in pH value of medium is necessary to alter metabolic functions of the cell culture.

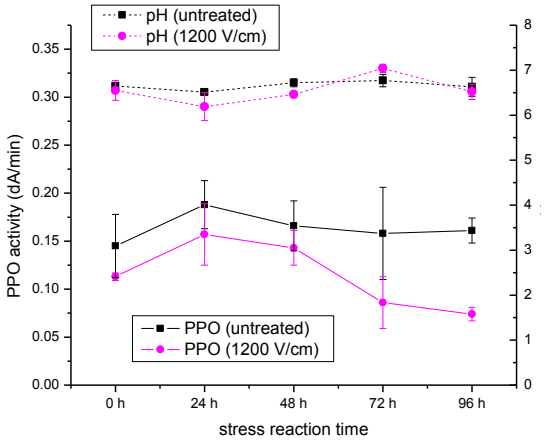
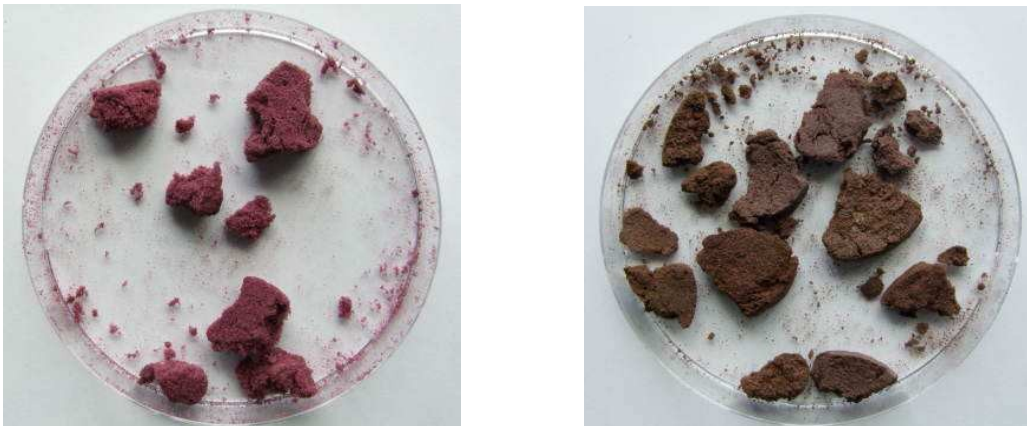


Figure 4-65: Polyphenol oxidase (PPO) activity and pH of untreated and PEF treated (20 pulses at 1200 V/cm; total energy input of 127.9 J/kg) *Vitis vinifera* cell culture measured after 0, 24, 48, 72 and 96 h stress reaction times.

4.6.4.5. Treatment with 2400 V/cm

Impact of PEF treatment at 2400 V/cm (20 pulses, total energy input 66.5 kJ/kg) on *Vitis vinifera* cell cultures in comparison to corresponding untreated samples was investigated up to 48 h after the treatment. Investigation after longer stress reaction period and measurements of CDI was not possible due to excessive decay of cellular tissue.

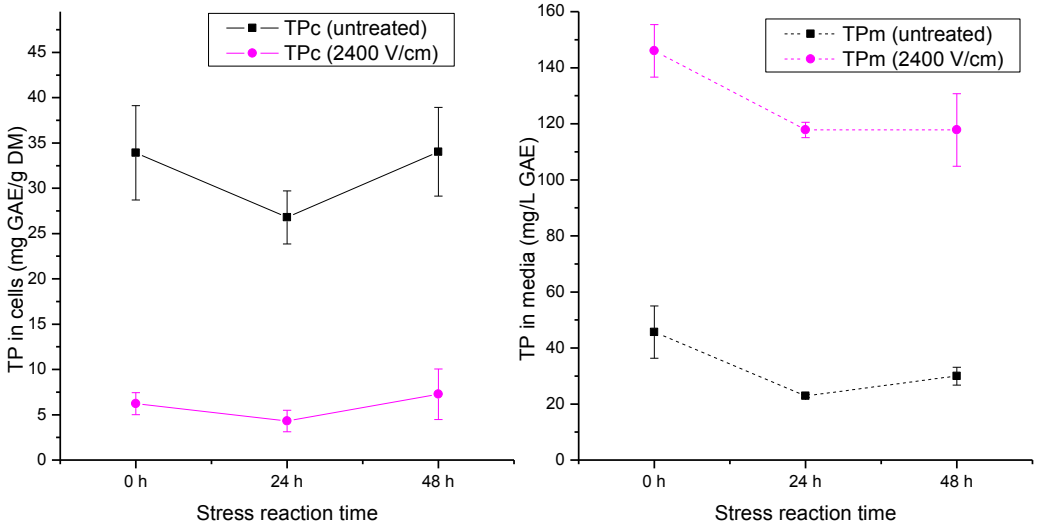
Detrimental effect of PEF treatment with chosen intensity was observed, which caused complete deterioration of the cells (Figure 4-66). PEF treated suspension cultures turned dark brown and loss of cell shape was observed.



(A) (B)

Figure 4-66: *Vitis vinifera* cells after lyophilization (A) untreated control (B) after PEF treatment with 2400 V/cm (20 pulses and 66.5 kJ/kg).

Significantly lower total polyphenol concentration in PEF treated cells and larger TP concentration in corresponding media, measured immediately after treatment and after stress reaction period was observed (Figure 4-67). Due to PEF induced complete disintegration of cellular tissue polyphenolic substances leaked through the damaged membrane into surrounding media.



(A) (B)

Figure 4-67: Impact of PEF treatment (20 pulses at 2400 V/cm; total energy input of 66.5 kJ/kg) on: (A) Total polyphenol concentration in cells (TPc); and (B) in growth medium (TPm); of *Vitis vinifera* cell culture measured after 0, 24 and 48 h stress reaction times.

Loss of intracellular components and liquids was confirmed, through increased conductivity of cell suspension after PEF treatment. Significantly larger conductivity of

Vitis vinifera suspension culture was observed immediately after PEF treatment and after stress reaction period of 48 h (Figure 4-68).

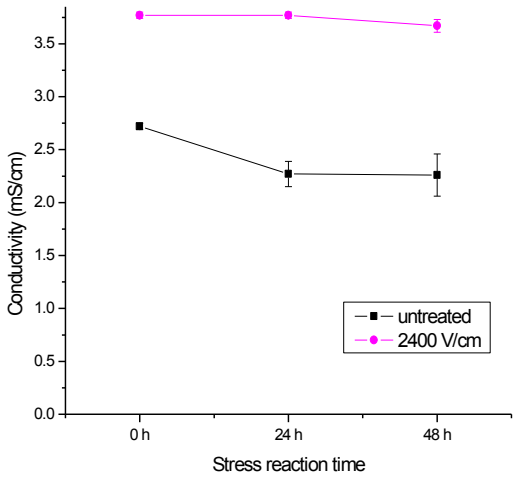
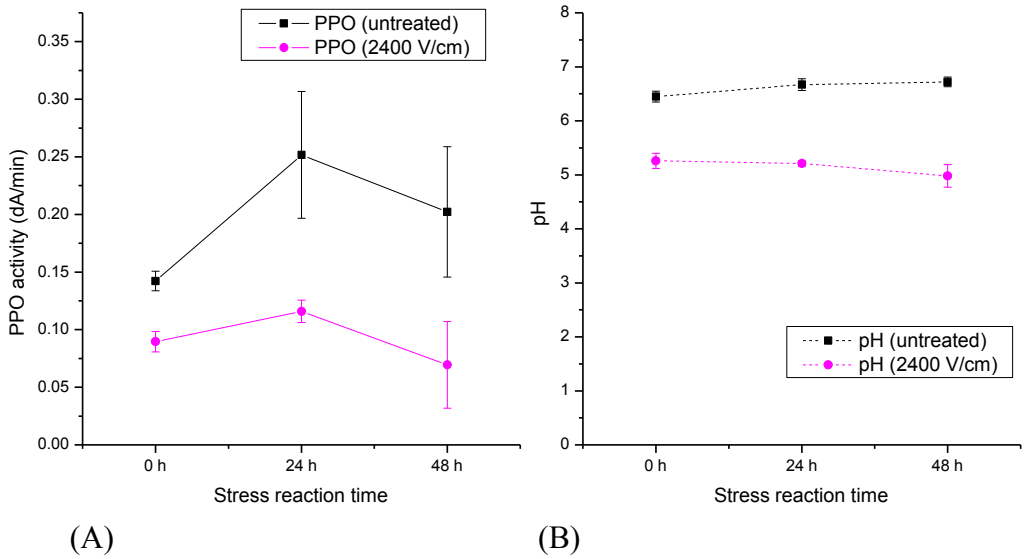


Figure 4-68: Impact of PEF treatment (20 pulses at 2400 V/cm; total energy input of 66.5 kJ/kg) on *Vitis vinifera* suspension culture conductivity measured after 0, 24 and 48 h stress reaction times.

Reduced PPO activity in PEF treated cultures was observed during 48 h stress reaction period (Figure 4-69A). Due to leakage of intracellular components (e.g. acids and salts) lower pH was observed in treated cultures, which was most probably the reason for lower PPO activity together with presence of other substances with inhibitory effect to selected enzyme.

Plant metabolic system suffered damage, larger than cell culture was able to tolerate. Cell membranes were directly affected by PEF treatment and the cell integrity collapsed before defence mechanism was established.



(A) (B)

Figure 4-69: Impact of PEF treatment (20 pulses at 2400 V/cm; total energy input of 66.5 kJ/kg) on: (A) Polyphenol oxidase (PPO) activity; and (B) pH; of *Vitis vinifera* cell culture measured after 0, 24 and 48 h stress reaction times.

4.6.5. PEF induced changes – dose dependence

It was previously shown that metabolite accumulation and enzymatic activity in tested *Vitis vinifera* suspension culture can differ over period of subcultivation (see subchapter 4.6.2). To reveal divergences caused only by PEF treatment as external stressor, chosen parameters (TP concentration in cells and in media) are expressed as percentage of increase/decrease in respect to corresponding untreated cultures.

The impact of 20 pulses at 200 and 500 V/cm was shown to induce positive effects (eustress) on treated cultures. The early stage of stress event (alarm phase) at which plant systems are destabilized can be observed during first 24 hours after imposed treatment with 200 and 500 V/cm (Figure 4-70 and 4-71). No significant changes of TP concentration in PEF treated cells and in growth media were observed during that time (0 – 24 h) in comparison to untreated control. However, tendency of slow increase of TP concentration in cells can be observed (Figure 4-70).

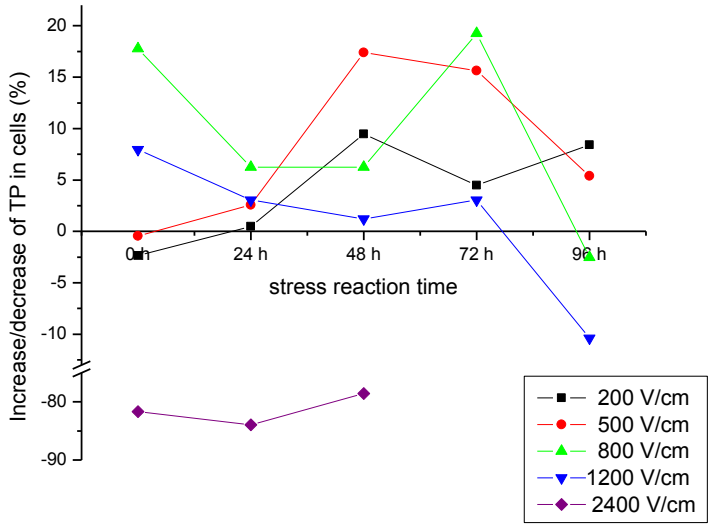


Figure 4-70: Changes of Total Polyphenol (TP) concentration in *Vitis vinifera* cells, after 20 pulses at 200, 500, 800, 1200 and 2400 V/cm, during stress reaction period.

Significant increase of TP concentration in PEF treated cells (200 and 500 V/cm) and corresponding media were noticed between 24 and 48 h, representing recovery phase of biological stress concept (Figure 4-70 and 4-71). It can be suggested that *de novo* synthesis of plant protective substances was induced after PEF treatment, observed through increase in TP concentration. Resistance phase in which higher productivity level is maintained can be observed between 48 and 96 h after PEF treatment with 200 V/cm, and between 48 and 72 h after PEF treatment with 500 V/cm. Larger stress impact was

obtained after application of 500 V/cm and consequently larger TP concentration in cells and in media was observed. Most likely greater amount of polyphenolic substances have been synthesized during that period in order to repulse imposed stress and repair damage to subcellular structures that stressor might have caused. Normalization phase of biological stress concept followed between 72 and 96 h stress reaction time, where TP concentration approached values of untreated control.

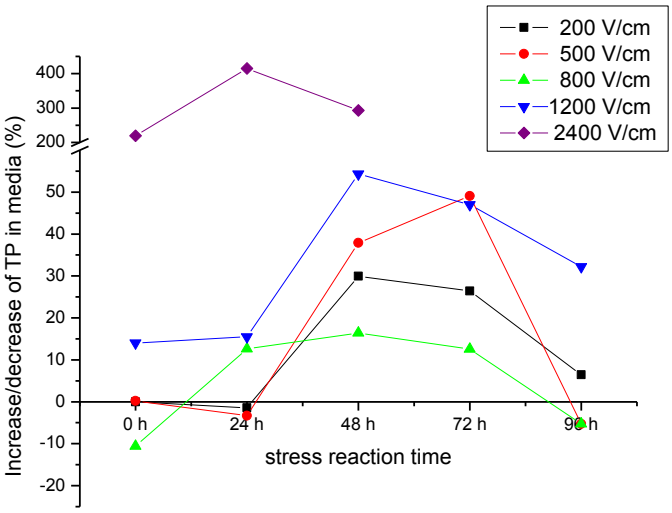


Figure 4-71: Changes of Total Polyphenol (TP) concentration in *Vitis vinifera* growth/treatment media, after 20 pulses at 200, 500, 800, 1200 and 2400 V/cm, during stress reaction period.

Much faster stress response was observed immediately after treatment with 800 V/cm, through significantly larger TP concentration in cells (Figure 4-70) and no significant change of TP concentration in media (Figure 4-71). It seems that PEF treated cell culture rapidly entered resistance phase due to larger stress imposed. Increased conductivity of suspension culture 24 h after the treatment (Figure 4-62B) indicated leakage of intracellular substances into the media presumably from certain population of cells with irreparable damage. However, larger population of PEF stressed cells maintained higher productivity level, and second peak can be observed after 72 h stress reaction time (Figure 4-70). After 96 h normalization took place and TP concentration in cells and media approached values of control cultures. Since larger TP concentration remained during stress reaction period, with certain damage observed through leakage of intracellular substances, it can be suggested that larger population of cells exhibited eustress, while smaller population of cells did not manage to recover from imposed PEF stress at 800 V/cm.

Very similar observations can be made after PEF treatment at 1200 V/cm. However, due to larger treatment intensity, larger population of cells was damaged

(Figure 4-64B) and after 96 h physiological resistance was surpassed and exhaustion took place (Figure 4-70 and 4-71). Thereafter, it can be observed that PEF treatment with 1200 V/cm was non-beneficial to plant productivity, causing distress and overcoming normal physiological capacity of cell cultures.

Complete depletion of TP concentration in cells (Figure 4-70) and marked increase in media (Figure 4-71) after PEF treatment at 2400 V/cm have been observed. This treatment intensity induced irreparable damage of cells in treated cultures, which did not allow the development of plant response and consequently acute damage and cell death occurred.

The results obtained after usage of PEF as abiotic factor to impose stress to *Vitis vinifera* suspension culture are in accordance to biological stress concept developed by Larcher (1987). However, suppressed PPO activity was observed after all treatment intensities used. PPO suppression was reported to increase stress tolerance to drought in tomato plants (Thipyapong, Melkonian, Wolfe & Steffens, 2004). Furthermore, PPO inhibition simultaneously with polyphenol accumulation was reported after thermal stress induction at 35 °C (Rivero et al., 2001). On the other hand, increased PPO activity was observed after stress induction due to tissue wounding caused by cutting or by low energy ultrasound treatment (Thipyapong et al., 1995; Wu & Lin, 2002). The experimental data indicated that this enzyme is a part of plants defense system against different abiotic and biotic stressors, contributing to defense response by its activation and/or suppression. However, no clear evidence is available today, nor are physiological specific functions of plant PPO explained in detail (Aniszewski, Lieberei & Gulewicz, 2008). The possible mechanism of PPO involvement in stress reactions remains unknown and hypothetical.

5. Conclusions and outlook

Pulsed electric field treatment applicability for stress induction in plant tissue has been investigated. Plant exposure to different abiotic stressors results in changed metabolic activity and consequently *de novo* synthesis of secondary metabolites, which contribute to plant defense response. Physiological events of abiotic stresses which were proposed to trigger production of protective substances are often connected to biomembrane destabilization, successive changes of the charges at the surface of membranes and consequently transmembrane potential. Due to similarity of specific impact on biological cell membrane, PEF treatment was applied on diverse biological tissues and changes in accumulation of protective substances and related enzymes were investigated.

The stress response to PEF treatment has been observed through sequences of events that are shown to be triggered as indirect evidence affecting metabolic activities and physiological fitness of the cells. For different plant tissue, it was shown that application of low treatment intensities, where no disintegration of cellular material occurred, induced largest accumulation of protective substances. Changes in TP concentration through biological stress concept were shown to be dependant on PEF treatment intensity. The impact of 300 V/cm (total energy input of 14 J/kg) on apple tissue induced 65 % larger TP concentration. This was attributed to the plant effort to sustain externally imposed stress through *de novo* synthesis of protective substances. By increasing treatment intensity to 700 V/cm (total energy input of 74 J/kg), larger stress impact was induced, which resulted in accumulation of phenolic substances and increase of 32 % in comparison to corresponding reference. Fruit adjustment to imposed stress at 700 V/cm, through simultaneous degradation and production of protective substances was suggested. The enforced stress was tolerated and fruit metabolic activity has been adapted to the change without experiencing permanent damage. When 1200 V/cm was applied (total energy input of 217 J/kg), the enforced stress reached its maximum of what metabolic activity of plant organs in the post-harvest phase are able to withstand. Exhaustion of a large number of cells was observed through loss of phenolic substances. PEF treatment at 4000 V/cm (total energy input of 10.29 kJ/kg) caused chronic damage with complete depletion of phenolic substances. In order to regulate metabolic processes and overcome external disturbances, similar diversity of responses were observed in tested fruit tissue and in plant cell cultures. Plant adaptation and tolerance on different

PEF treatment intensities has been described through biological stress concept, and universal summary model is shown in Figure 5-1.

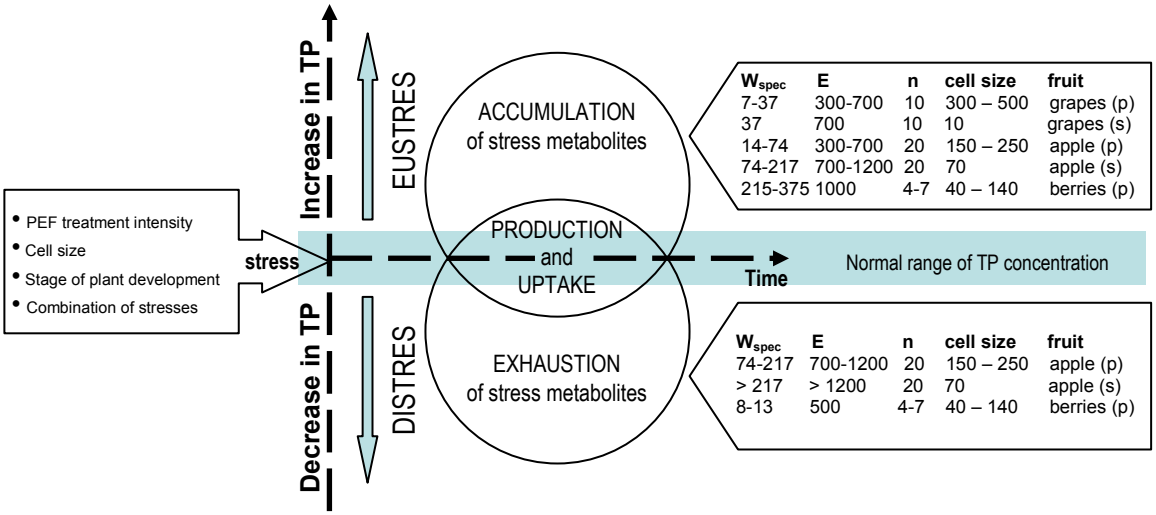


Figure 5-1: A universal summary model of different plant tissue responses (pupl (p) and skin (s)) to PEF stress through biological stress concept in correspondence with stress intensity and cell size. Specific energy input (W_{spec}) is expressed in J/kg, electric field strength (E) in V/cm, n in number of applied pulses, and cell size in µm.

Exposure of multicellular fruit tissue revealed distinguishable response in terms of polyphenol accumulation, supporting cell size and orientation dependant impact of PEF treatment. Lower electric field strength was necessary to induce TP accumulation in the inner regions of fruit, while smaller cells at the outer regions required larger electric field strengths to achieve same degree of TP accumulation (based on the results of TP concentrations in juice and skin of PEF treated fruits). Due to large variability in cell shape and size and natural protective function in fruit pulp and skin, local stress induction of multicellular tissue was suggested. Thereafter induction of stress response by PEF treatment onto multicellular systems needs to be optimized since it might represent an obstacle to achieve repeatable and controlled stress response in fruits.

Levels of secondary metabolites were affected by postharvest storage temperature and coldness was assigned as additional stress factor contributing to cumulative effect when applied together with PEF treatment. Addition of stressors (PEF and coldness) led to different response compared to the application of each single stress factor. Furthermore, due to different PEF intensity applied, fruits exhibited initial reaction of responsiveness within different time scale. Variation in response to the same stress intensity was furthermore attributed to the initial plant physiological fitness and several outcomes through different TP concentration in tested batches were observed.

The cell culture suspensions were used as model systems of corresponding higher plants to eliminate uncontrollable environmental interactions. Cultured cells were grown *in vitro* and stable cell lines were maintained over tested period. The impact of several PEF treatment intensities on stress induction in controlled growth and environmental conditions was observed through accumulation of polyphenolic compounds in PEF stressed cell suspension cultures. Dose dependant stress response has been observed during stress reaction period. PEF treatment at 200 and 500 V/cm (total energy inputs of 5 and 21 J/kg, respectively) were sufficient to induce production of protective substances in *Vitis vinifera* cell cultures. Increasing treatment intensity resulted in increased concentration of polyphenolics in the cell cultures. However, when the critical limit was achieved by PEF treatment at 1200 V/cm (total energy input of 128 J/kg), physiological resistance to this stress intensity was surpassed and exhaustion took place. These results indicated that cell metabolism suffered changes that allowed recovery and adaptation to enforced stress by low intensity PEF treatments through *de novo* synthesis of protective substances. After larger treatment intensity, when cell culture limit to withstand stress was surpassed, acute damage and cell death occurred.

The presence of altered metabolism was furthermore indicated by changes in enzyme activity. Phenylalanine ammonia-lyase (PAL) being the key enzyme of phenylpropanoid metabolism, through which all phenylpropanoids are formed, has been correlated to changes in total polyphenol concentration in *Malus domestica* cell culture. Increased PAL activity, after application of PEF treatment at 200 V/cm (total energy inputs of 4 and 8 J/kg), preceded increase in TP concentration, indicating *de novo* synthesis of polyphenolic protective substances through phenylpropanoid biosynthesis. Furthermore, since oxidation of phenolic compounds is catalyzed by polyphenol oxidase (PPO), changes in TP levels have been associated with PPO activity in grapes and in *Vitis vinifera* cell cultures. An influence of PEF on postharvest metabolic changes in fruit tissue through altered PPO activity was noticed, indicating that phenolic oxidation system might be involved in the plant resistance. However, PPO activity was not directly correlated to changes of phenolic substances, suggesting that more than single biosynthetic pathway was stimulated by PEF application during postharvest storage. Even though occurrence of irreversible membrane permeabilization was not observed, transmembrane traffic of polar compounds was presumably altered by PEF application, which might have caused substrate relocation. Phenolic substances or other compounds that affect the active enzyme site can inhibit PPO due to their structural similarities, which was most probably

responsible for lower activity of selected enzyme. However, no clear evidence about PPO biological function is available today and therefore the mechanism of its involvement in stress response remains unknown and hypothetical. In order to elucidate PEF impact on PPO, metabolite profiling and sequenced genome techniques offer possibility to further investigate PPOs physiological function in plant growth and development, followed by its involvement in stress response.

Application of external electric fields exerts forces on charges in processed material, which are redistributed (depending on the conductivity of the material) in a limited space. This additional polarization of membranes or any other material may result in cell stress undependable of cell permeabilization. Polyphenolic substances, recognized as strong antioxidants are involved in phytochemical defense through neutralization of reactive oxygen species. Plant exposure to different abiotic stressors results in increased production of ROS, which formation has been promoted after PEF exposure as well. Recent studies have indicated greater involvement of ROS as signal mediators upon stress, whose role could be also addressed to PEF stressor. Stress enforced to biological systems is not a single physical force commonly affecting the organism, but a load from many individual environmental factors and physical condition of the plant. Accumulation of secondary metabolites in plant tissue of different origin after low intensity PEF treatment has been identified in the course of this work. However, not every stress will provoke secondary metabolite reduction or increase in production of these valuable substances. Due to complexity of biological system simultaneous production and consumption might occur, to repair possible damage of the cells and prepare new protective substances. Competitiveness between production and utilization of protective substances results in increase or decrease of TP concentration in plant tissues at the chosen stress reaction period. Due to lack of understanding from trigger pathways of secondary metabolite synthesis, mechanism of stress induction by PEF treatment has been hypothesized and explained by the empirical nature of accumulated compounds.

A feasibility of PEF application to increase TP concentration in processed fruits was shown within the scope of this thesis. However, to reduce complexity and wide responsiveness of multicellular fruit tissue, further investigation with plant cell cultures as model systems are recommended. To evaluate and systematically quantify plant tolerance and detect metabolic response, application of genetic and molecular approach will accelerate efforts to understand biosynthetic pathways triggered by external stressor.

6. Industrial relevance

The greatest part of postharvest technology is devoted to retain metabolic reactions associated to quality deteriorations. Low temperatures in combination with SO_2 fumigation has been shown as an effective method for grape storage (Gabler, Mercier, Jimenez & Smilanick, 2010). The chemical growth regulators used in postharvest storage might be harmful to human health (Taylor & Bell, 1993) and therefore its application should be minimized and other ways of fresh fruit preservation through manipulation of external environment should be considered.

Results obtained within this study are suggesting that PEF treatment can be assigned to the list of abiotic stressors. Even though exact trigger mechanism is not elucidated, increased accumulation of polyphenolic substances has been observed in different fruits and plant cell cultures after application of various PEF treatment intensities. This effect could be used to improve or modify product properties during postharvest storage. Furthermore, low intensity PEF treatments which require low energy intake could improve polyphenol concentration in expressed juices, consequently altering product polyphenol profile. Physical stress could be thereafter utilized to prevent quality reduction or to promote changes that increase certain quality aspects. Pulsed Electric Field treatment offers a new tool to achieve optimal food quality by changing polyphenolic content and related enzyme activity.

Curriculum Vitae and List of Publications

PERSONAL INFORMATION

Name	**Ana**
Surname	**Balaša**
E-mail	**ana.balasa@gmail.com**

FORMAL EDUCATION

September 2006 – March 2011 **PhD**	**Technische Universität Berlin** Koenigin-Luise-Str. 22, 14195 Berlin, Germany Mentor: Prof. Dr. Dipl. Ing. Dietrich Knorr
September 1996 – May 2004 **University**	**University of Zagreb** **Faculty of Food Technology and Biotechnology** Pierottijeva 6, Zagreb, Croatia
August 2003 – February 2004 **Professional Internship**	**Technische Universität Berlin** Koenigin-Luise-Str. 22, 14195 Berlin, Germany
November 2001 - January 2002 **Professional Internship**	**Laboratory for quality control, Pharmaceutical company PLIVA** Ulica Baruna Filipovica, Zagreb, Croatia
September 1992 – June 1996 **Gymnasium**	**Nature science and mathematics gymnasium** Klaiceva 1, Zagreb, Croatia

ADDITIONAL SKILLS AND EXPERIENCE

AREAS OF EXPERTISE	Novel processing technologies Research methodology Data validation and analysis Process and product development Research collaboration Project management

SCIENTIFIC PROJECTS

June 2006 – March 2011 **NovelQ**	**Integrated Project: 'Novel processing methods for the production and distribution of high-quality and safe foods'** (http://www.novelq.org/Default.aspx) EC Framework Programme 6 Priority 5 "Food Quality and Safety"
September 2004 – June 2006 **DFG-project**	**'Entwicklung von Produktionskonzepten zur schonenden Gewinnung pflanzlicher, ernaehrungsphysiologisch wertvoller Oele unter Einsatz elektrischer Hochspannungsimpulse'**
August 2003 - February 2004 **Student research project**	Master thesis: **'Impact of solid-liquid phase transitions under high pressure on the permeabilization of Listeria innocua BGA 3532'**

EXTRACURRICULAR ACTIVITIES

2010 - 2011	**Chair of a Young Scientists SIG in EFFoST** (The European Federation of Food Science and Technology), a multi-disciplinary research network of scientists in Europe
2010	**Conference coordinator** First European PhD Conference in Food Science and Technology
2006 - 2011	**Reviewer activities** Innovative Food Science and Emerging Technologies; Journal of Food Process Engineering; Journal of Food Engineering; Trends in Food Science and Technology; LWT Food Science and Technology; Biotechnology Progress
2006 - 2011	**Member of a Training and Career Development (TCD)** a multi-disciplinary European research network of young scientists in NovelQ
2001 - 2003	**Member of a Committee for International Cooperation** at Faculty of Food Technology and Biotechnology, University of Zagreb

HONOURS / AWARDS

July 2009	First Prize winner of the scientific poster presentation *"Pulsed electric field treatment of plant tissue: an overview"* EuroFoodChem XV 2009 Food For the Future, Copenhagen/Denmark
September 2008	Third best presentation *"Impact of Pulsed Electric Field Treatment on stability and production of health related components in plant tissue"* Training and Career Development (TCD) Novel Q, Berlin/Germany
September 2007	Third Prize winner of the scientific poster presentation *"Production of Health Related Components Induced with Pulsed Electric Field Processing"* Training and Career Development (TCD) Novel Q, Zaragossa/Spain
September 2006	Research grant: Short Term scientific Mission (STSM), COST 928 Control and exploitation of enzymes for added-value products, Ljubljana/Slovenia

LANGUAGES

Croatian	Mother tongue
English	Fluently
German	Advanced knowledge

LIST OF PUBLICATIONS

Listed in appendix 1

APPENDIX 1: **LIST OF PUBLICATIONS**

Book chapters

Balasa, A., Janositz, A. and Knorr, D. (2011) Electric Field Stress on Plant Systems. In: Encyclopedia of Biotechnology in Agriculture and Food (EBAF), Taylor and Francis Group LLC.

Knorr, D., **Balasa**, A., Boll, D., Jäger, H., Mathys, A., Oba, E., Richter, J., Volkert. M. (2009) Alternative Processing Methods for Functional Foods. In: An integrated Approach to New Food Product Development. Moskowitz, Howard; Saguy, Sam; Straus, Tim (Eds.), 544 p., ISBN 9781420065534

Jaeger, H., **Balasa**, A. and Knorr, D. (2008) Food Industry Applications for Pulsed Electric Fields, In: Electrotechnologies for Extraction from Food Plants and Biomaterials, Series: Food Engineering Series, Vorobiev, Eugene; Lebovka, Nikolai (Eds.), VIII, 240 p. 157 illus., Hardcover, ISBN: 978-0-387-79373-3

Peer-reviewed publications

Moritz, J., **Balasa**, A., Jaeger, H., Meneses, N. and Knorr, D. (2012). Investigating the potential of polyphenol oxidase as a temperature-time-indicator for pulsed electric field treatment. Food Control, 26(1), 1-5.

Johnson, P. E., Van der Plancken, I., **Balasa,** A., et al. (2010). High pressure, thermal and pulsed electric-field-induced structural changes in selected food allergens. Molecular Nutrition & Food Research, 54(12), 1701-1710

Soliva-Fortuny, R., **Balasa,** A., Knorr, D. and Martín-Belloso, O. (2009). Effects of pulsed electric fields on bioactive compounds in foods: A review. Trends in Food Science and Technology, 20(11-12), 544-556.

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Luscher, C., **Balasa**, A., Fröhling, A., Ananta, E. and Knorr, D. (2004) Effect of high pressure induced ice I – ice III phase transitions on the inactivation of *Listeria innocua* in frozen suspension. Applied and Environmental Microbiology 70 (7), 4021–4029.

Presentations

Balasa, A., Janositz, A., and Knorr, D. (2009) Pulsed electric field treatment of plant tissue: an overview , EuroFoodChemXV 2009, Copenhagen/Denmark

Balasa, A. and Knorr, D. (2008) Impact of Pulsed Electric Field Treatment on stability and production of health related components in plant tissue, Training and Career Development (TCD) Novel Q, Berlin/Germany

Mackie, A., Husband, F., Johnson, P., Van der Plancken, I. and **Balasa**, A. (2008) Food allergens and non-thermal technologies, Innovative Applications of Nonthermal Technologies in Foods: Technology, Safety, Health and Consumer Acceptability, Madrid/Spain

Balasa, A., Heckelmann, A. K. and Knorr, D. (2008) Pulsed Electric Fields - Novel non thermal Technology and its Applications in Food Processing, 4th Central European Congress on Food (CEFood), Cavtat/Croatia, *Invited speaker*

Balasa, A., Heckelmann, A. and Knorr, D. (2007) Production of Health Related Components Induced with Pulsed Electric Field Processing, Training and Career Development (TCD) Novel Q, Zaragossa/Spain

Balasa, A., and Knorr, D. (2007) Novel methods in food and biotechnology: pulsed electric field processing and its application in winemaking process, From Grape to Wine - 1st International Junior Researchers Meeting, Ljubljana/Slovenia

Balasa, A., Toepfl, S., and Knorr, D. (2006) Impact of Pulsed Electric Field Treatment on Polyphenolic Content of Grapes, CAPPT 2006 (Control application in Post-Harvest and Processing Technology), Potsdam/Deutschland, ATB, Heft 55, 169-174, ISSN: 0947-7314

Poster presentations

Balasa, A, Heckelmann, A.K., Frandsen, H.B., Andersen, K. E., Sørensen, J. C., Sørensen, H. and Knorr, D. (2009) Pulsed electric fields processing and its potential to induce stress in plant systems, EuroFoodChemXV 2009, Copenhagen/Denmark

Balasa, A., Janositz, A., and Knorr, D. (2009) Pulsed electric field treatment of plant tissue: an overview , EuroFoodChemXV 2009, Copenhagen/Denmark

Balasa, A., and Knorr, D. (2007) Production and Extraction of Health Related Components Induced with Pulsed Electric Field Processing, 4th International Conference on polyphenol Application, Malta

Balasa, A., Toepfl, S. and Knorr, D. (2006) Extraction of Total Phenolics from Grapes in correlation with the Degree of Membrane Poration. COST meeting 928-300606, Cost, Reykjavik/Island

Balasa, A. and Knorr, D. (2006) Nutritional Improvement of Grape Products by Pulsed Electric Field Processing, BioPerspectives im Fokus: Nahrung für die Zukunft, Potsdam/Germany

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Luscher, C., **Balasa**, A., Ananta, E. and Knorr D. (2004) High pressure processing for microbial inactivation in frozen food. EFFoST Conference Food Innovations for an Expanding Europe FOODINNO, Warsaw/Poland

Luscher, C., Fröhling, A., **Balasa**, A. and Knorr, D. (2004) Inactivation of *Listeria innocua* and *E. coli* at high pressure and subzero temperature with consideration of the phase transitions of water. Proceedings on CD-Rom paper # 776. ICEF 9 - International Congress on Engineering and Food, Montpellier/France

Luscher, C., **Balasa**, A. and Knorr, D. (2003) Inactivation of *Listeria innocua* at high pressures and low temperatures: Observations in the subzero temperature range (centigrade) considering the state of aggregations of water. Joint IFT - EFFoST Workshop on Non - Thermal Food Preservation, Wageningen/The Netherlands

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass die vorliegende Dissertation in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind.

Weiter erkläre ich, daß ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Veröffentlichungen von irgendwelchen Teilen der vorliegenden Dissertation sind von mir wie folgt vorgenommen worden.

Berlin, 31.07.2013

Ana Balaša

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