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Effect of pulsed electric field on the germination of barley seeds

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ABSTRACT

This study explores metabolic responses of germinating barley seeds upon the application of pulsed electric fields (PEF). Malting barley seeds were steeped in aerated water for 24 h and PEF-treated at varying voltages (0 (control), 110, 160, 240, 320, 400 and 480 V). The seeds were then allowed to finish germination in saturated air. It is shown that exposure of germinating barley to PEF affects radicle emergence without significantly affecting the seeds' gross metabolic activity, as quantified by isothermal calorimetry. An exploration of protein 2-DE profiles of both the embryo and the starchy endosperm showed that, at the studied time scale, no significant changes were found in proteins present at concentrations higher than the detection limit. However, western blotting demonstrated that α-amylase concentration decreases in the PEF-treated seeds.

1. Introduction

Seed germination comprises those events that start with water uptake by the quiescent dry seed and terminate with the extension of the embryonic axis. This process may take 24–36 h depending on the temperature, and it is followed by radicle elongation and growth of the new plant (Bewley & Black, 1994, p. 147–197).

Upon imbibition, which corresponds to the steeping process of industrial malting, metabolic activity needed for seed growth is immediately resumed by embryo and aleurone cells. The embryo synthesizes the phytohormone giberellic acid (GA) which is secreted to the scutellum and aleurone layer, where it induces the production of hydrolytic enzymes, α-amylase being the most abundant (Zentella, Yamauchi, & Ho, 2002). These enzymes are synthesized at increasing rate during germination and early seedling growth (MacGregor, 1978; MacGregor, MacDougall, Mayer, & Daussant, 1984). An adequate level of α-amylase activity is a desired property of a good quality barley malt, as its activityduring mashing is a key issue in the brewing process (Montanari, Floridi, Marconi, Tironzelli, & Fantozzi, 2005). High activity of β-amylase is also desired for starch conversion during mashing. Overall, α- and β-amylase are capable of converting 60–80% of the available starch into fermentable sugars (Montanari et al., 2005). Once sufficient enzymes have been produced within the grain to allow starch modification, but before the hydrolysed storage products in the starchy endosperm are used as a food source by the developing roots and shoot that will form the barley plant, the maltster stops the metabolic activity of the seed by increasing the temperature.

Other important enzymes active during germination and radicle elongation are stress protective enzymes such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), which are part of a tight redox control system in the barley seed, preventing cellular damage caused by reactive oxygen species (ROS) (Bønsager, Shahpiri, Finnie, & Svensson, 2010). During germination and radicle elongation, the seed has the capacity to respond to a diversity of abiotic stresses such as oxidative, salt and osmotic stress (Bønsager, Finnie, Roepstorff, & Svensson, 2007). In this paper, we have investigated the response of germinating barley seeds to the application of reversible electropermeabilization.

The application of pulsed electric fields (PEF) may cause lethal damage to cells, or induce sublethal stress by transient permeabilization of cell membranes and electrophoretic movement of charged species between cellular compartments (Teissié, Golzio, & Rols, 2005). The technique is, in its lethal version, at very high

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field strength, used for the inactivation of microorganisms in foods (Góngora-Nieto, Pedrow, Swanson, & Barbosa-Canovas, 2003; Toepfl, Heinz, & Knorr, 2007) and can, at lower field strength, be used for the improvement of extraction yield (Chalermchat & Dejmek, 2005; Fincan, DeVito, & Dejmek, 2004) or increased rate of drying (Ade-Omowaye, Rastogi, Angersbach, & Knorr, 2003; Lebovka, Shynkaryk, & Vorobiev, 2007). In its non-lethal version, the nature and order of events leading to the physiological responses to PEF-induced stress are still largely unknown. It has been reported that PEF affects metabolism, including an oxidative burst with the consequent generation of ROS (Gabriel & Teissié, 1994; Sabri, Pulissier, & Teissié, 1996), and the stimulation of the production of secondary metabolites such as increased yields of a cytostatic compound in cell culture of *Taxus chinensis* (Ye, Huang, Chen, & Zhong, 2004) and of antioxidants and phytoesterols from oil seeds and fruits (Guderjan, Elez-Martínez, & Knorr, 2007; Guderjan, Töpfl, Angersbach, & Knorr, 2005). Several studies have shown that the application of an electric field with low intensity and low frequency can modify the activity of biosystems (organisms or tissue and cell cultures) with consequences on proliferation, growth and differentiation phenomena (Berg, 1993). The bioeffects induced by the electric fields are reported to depend on both electrical parameters and physiological state of the treated cells (Cogalniceanu, Radu, Fologea, & Brezeanu, 1998).

It is shown that exposure of germinating barley to pulsed electric fields affects radicle emergence without significantly affecting the seeds' gross metabolic activity, as quantified by isothermal calorimetry. Calorimetric measures of the rate of heat production are proportional to metabolic rates and provide a direct indication of integrated metabolic responses such as respiration and reaction to stress (Criddle, Breindenbach, & Hansen, 1991). An exploration of protein 2-DE profiles of both the embryo and the starchy endosperm showed that, at the studied time scale, no significant changes were found in proteins present at concentrations higher than the detection limit. However, western blotting demonstrated that α-amylase concentration decreases in the PEF-treated seeds.

2. Materials and methods

2.1. Raw material handling

Malting barley seeds (*Hordeum vulgare* cv. Prestige, 6.5 g/100 g humidity) were provided by the Swedish University of Agricultural Sciences (SLU), Alnarp, Sweden and stored at room temperature in a dry place before the experiments. Undamaged seeds were manually selected and four groups of 35 seeds were formed. Each group of seeds was placed in a wire mesh basket before the steeping process.

2.2. Steeping

Steeping was performed in a temperature-controlled room at 20 °C in a laboratory steeping device built at SLU, Alnarp, Sweden. The wire mesh baskets with the selected seeds were coupled to the steeping machine. The machine was programmed to soak the grains for 6 h in 20 L of aerated distilled water, lift the grains from the water to be exposed to atmospheric air for 12 h, and then soak them again for 6 h. During the soaking periods, air was bubbled through the wire mesh baskets in which the seeds were placed. The design of the steeping machine did not allow dehydration of the seeds during the time of exposure to air.

2.3. Electrical treatment

Immediately after steeping, the seeds from the different vessels were mixed and 4 seeds were selected based on visual homogeneity of germination. The seeds were placed in an electroporation cuvette (4 mm gap, EP-104, Cell Projects, Harrietsham Kent, UK) filled with enough distilled water (conductivity 2.5 μS/cm) to totally cover the electrodes and PEF treated. Electric pulses were delivered using a CEPT pulse generator (Arc Aroma Pure, Lund, Sweden). A digital oscilloscope (Fluke 123, Washington) was connected to the system to monitor the delivery of the pulse to the sample.

Seeds were treated at varying nominal electric field strengths of 0, 275, 400, 600, 800, 1000 and 1200 V/cm. Based on the results of preliminary experiments (not shown), fifty 1 ms, rectangular electric field pulses were used. The space between the pulses was 2 s. These conditions showed to affect the radical elongation of the germinating seeds. The specific energy delivered to the seeds in the cuvette is given by the applied energy per treated mass and was calculated by $Q = V^2t/Rm$ (Zhang, Barbosa-Canovas, & Swanson, 1995) where V is the voltage of the square pulses, R is the effective resistance, t is the treatment time and m the mass of the sample. Applied field strengths corresponded to Q values of 48, 100, 227, 405, 630 and 910 J/Kg.

The treatments were repeated in 3 groups of 4 seeds. In this way, 12 seeds were treated with the same PEF conditions.

2.4. Germination set-up

Immediately after PEF treatment, germination was completed in calorimetric ampoules using a set-up described by Panarese (2009). Twelve seeds were placed in a 20 ml glass ampoule. The internal wall of the ampoule was covered by a cellulose based tissue (Wettex, Vileda, Germany) soaked with 6 ml of distilled water to keep saturated atmosphere inside the ampoule. The ampoule containing the seeds was sealed with a silicon stopper through which two needles were running. The needles allowed the ampoule to be aerated during the measurements. The aeration was made by bubbling a 3 ml min^{-1} air stream through a saturation ampoule with 7 ml of water before it was led into the ampoule with the barley grains. A four channel peristaltic pump was used to pump the air.

Ampoules containing untreated grains (controls) were measured in the same way as the treated grains.

2.5. Measurement of the rate of heat production and radicle elongation

The rate of heat production after the application of PEF was continuously measured in a TAM Air isothermal calorimeter (Thermometric AB, Järfälla, Sweden), as described by Rocculi et al. (2007). This calorimeter has a sensitivity (precision) of ±10 μW (Wadsö, 2005) and contains eight twin calorimeters. In each calorimeter, heat is allowed to flow between the reaction vessel containing the sample and a heat sink, the temperature of which is kept essentially constant. The heat transfer takes place through a heat flow sensor that is located between the vessel and the heat sink.

The described germination set-up was placed in the calorimeter at 20 °C. Each calorimeter had its own reference that consisted of a sealed 20 ml glass ampoule containing 4 ml of water. The eight calorimeters permitted simultaneous measurements of the rate of heat production of the ampoules containing the seeds. Four calorimeters contained the ampoules with the seeds and the other four contained the connected water saturation ampoules. In this way,

controls and treated samples were distributed in the calorimeters. Two measurements at each PEF condition were done.

The isothermal measurements were performed for a period of 22 h. Baselines (BL) were recorded before or after each measurement. The primary output from the heat flow sensors in the calorimeters (a voltage) was recorded by computer from the digitized output of the calorimeters. The corresponding thermal powers (heat production rates) were calculated by the following equation:

$$P = \varepsilon * \frac{V_S - V_{BL}}{M} \quad (1)$$

where P (mW g^{-1}) is the specific thermal power of a sample, ε the calibration coefficient of the calorimeter (mW mV^{-1}), V_S the voltage signal from the calorimeter (mV), V_{BL} the corresponding voltage (mV) recorded for the baseline and M is the mass (g) of the sample. The calibration coefficients were calculated from electrical calibrations made at 20 °C.

After calorimetric measurements, each group of seeds was taken out from the ampoules and the length of the longest root of each seed was measured manually using a digital calliper.

Measurements were done twice. In each replicate (12 seeds) the whole procedure, starting from the selection of the dry seeds, was repeated.

2.6. Evaluation of the effect of electrical treatments on seed proteins

2.6.1. Sample preparation

Seeds were steeped for 24 h as previously described. Immediately after steeping, 12 seeds were submerged in liquid nitrogen (Time 0). From the rest of the steeped seeds, 12 were PEF treated with fifty 1 ms, rectangular electric field pulses of 1200 V/cm and 12 were not PEF treated (control). Both groups of seeds were then germinated as previously described.

The germination set-up was placed in a thermostatically controlled room at 20 °C. Eight hours after the PEF treatment, at the time at which germination is finished and the radicle is not longer than 0.5 mm, the seeds were manually dissected separating the embryo from the rest of the seed (mainly containing starchy endosperm and aleurone layer) with a scalpel. Immediately after dissection, the parts of the seed were frozen in liquid nitrogen, freeze dried (Hetsicc, Heto, Birkerød, Denmark) for 48 h and stored at −18 °C until the time of analysis.

Freeze-dried samples of seeds were ground to a fine powder using a mortar and pestle. Each sample was then placed in a 1.5 ml Eppendorf tube at −18 °C until the time for protein extraction.

2.6.2. Protein extraction and quantification

Unless otherwise indicated, all steps below were performed at 4 °C. For the extraction of proteins, the sample powder in the Eppendorf tube was mixed with an ice-cold solution containing 5 mM/L Tris–HCl, pH 7.5 and 1 mM/L $CaCl_2$, as described by Finnie, Melchior, Roepstorff, and Svensson (2002). The ratio weight of sample: volume of solution was 1:5 (mg:µl). Samples were mixed by stirring for 30 min before centrifugation at 16,000 rpm for 15 min (JA rotor, Beckman Instruments, Fullerton, CA). The supernatant containing the soluble protein fraction was aliquoted and stored at −80 °C until use.

Protein concentration in the extracts was estimated using the Popov, Schmitt, Schulyeck, and Matthies (1975) method using bovine serum albumin as standard, so that the same amount of protein could be loaded in the polyacrylamide gel. This assay was reported to have high reproducibility for small quantities of protein (1–40 µg) without suffering interference from sugars, which made it suitable for extracts from barley seeds (Finnie & Svensson, 2003).

2.6.3. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as described by Bønsager et al. (2007). For each treatment, 200 µg of protein were precipitated from the extracts by the addition of 80 ml/100 ml acetone at −20 °C for >12 h. Precipitated proteins were redissolved in a buffer containing 7 M/L urea, 2 g/100 ml CHAPS, 2 M/L thiourea, 0.5 ml/100 ml IPG buffer 4–7 (GE Healthcare Bioscience AB), 20 mM/L DTT and a trace of bromophenol blue. The first dimensional IEF was run on 18 cm, Immobiline™ DryStrip (GE Healthcare) IPG strips with a linear gradient of pH 4–7 using an IPGphor (GE Healthcare) for a total of 63 000 V h^{-1} (Finnie et al., 2002).

The IPG strips were soaked for 20 min in equilibration buffer (50 mM/L Tris–HCl pH 8.8, 6 M/L urea, 30 ml/100 ml glycerol, 2 g/100 ml SDS and a trace of bromophenol blue) containing 10 mg/ml DTT, followed by 20 min incubation in equilibration buffer containing 25 mg/ml iodoacetamide. The second dimension SDS-PAGE (12–14 g/100 g, 18 × 24 cm, GE Healthcare) was run on a Multiphor (GE Healthcare) according to the manufacturer's recommendations and stained with colloidal Coommassie Blue (Rabilloud and Chermont, 2000).

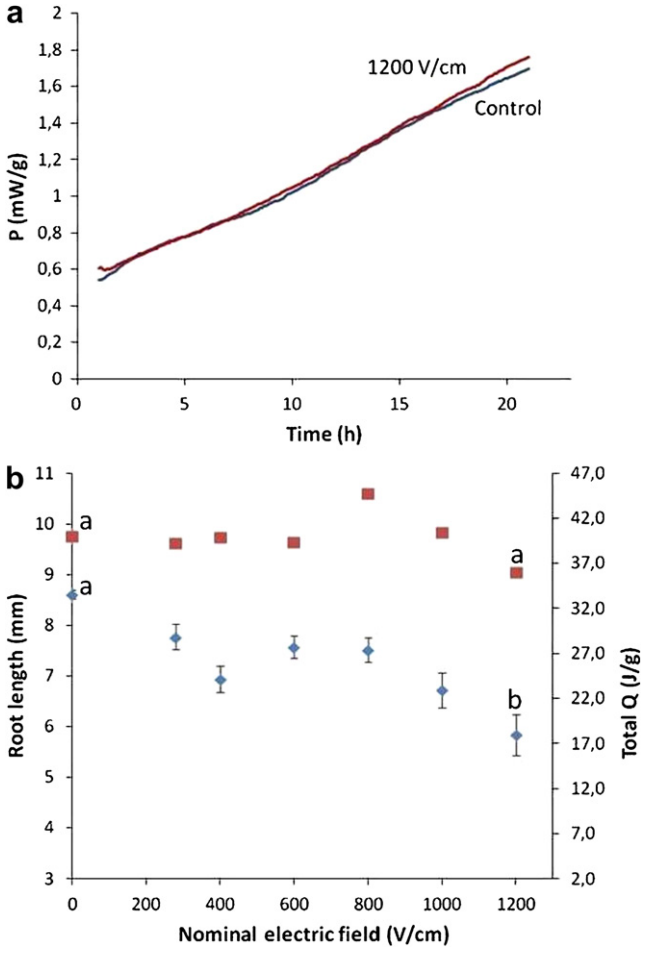

Fig. 1. Effect of PEF on the gross metabolic activity and radicle elongation of barley seeds. (a) Typical calorimeter results from germinating seeds subjected to pulsed electric treatment. Thermal power (P) was calculated according to Eq. (1). PEF conditions are indicated next to the curve. Average of duplicates is shown. (b) Comparison of calorimetrically measured metabolic heat production of PEF-treated seeds (squares) and the length of the longest root 22 h after steeping (circles). Each given value of root length is the average of 24 measurements, vertical bars represent the standard deviation. Each given value of total heat is the average of 2 measurements. Values with different letter in a given series of data are significantly different ($p < 0.05$).

2.6.4. Analysis of 2-D gels

2-D gels from controls and PEF-treated samples were compared with the PROGENESIS SAMESPOTS software (Nonlinear Dynamics, Newcastle, UK). The reproducibility was ensured by 4 replicates (In each replicate the whole procedure, starting from the selection of the dry seeds, was repeated). Statistical comparison of the spot intensity of the different treatments, based on one-way ANOVA ($p < 0.05$) and maximum fold changes ($fc > 1.5$) was verified using the false discovery rate (FDR) analysis ($q < 0.05$). FDR is a bioinformatic tool used to improve the process for estimating error rates of peptide identification. It is used for testing thousands of null hypothesis simultaneously, correcting for any possible statistical errors of the previously applied comparison methods (Tab, 2007).

2.6.5. Western blotting for α-amylase detection

Fifteen μg of protein extracted from the seed endosperm were loaded in polyacrylamide gels and separated by SDS-PAGE followed by electroblotting to a nitrocellulose transfer membrane (Hybond™ ECL™, Bioscience, Amsterdam) before being probed with rabbit polyclonal antibodies raised against barley α-amylase (Søgaard & Svensson, 1990). Secondary goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase and "BCIP/NBT" Western blotting reagent (Sigma Fast™) were used according to the manufacturer's protocol to detect binding. The analysis was done four times. In each replicate the whole procedure, starting from the selection of the dry seeds, was repeated.

Integrated lane profiles of protein bands were obtained and their area measured by ImageJ (Wayne Rasband, Maryland, USA) gel analysis tool.

3. Results

3.1. Gross metabolic activity and radicle elongation

An example of the results from the calorimetric measurements of germinating seeds is shown in Fig. 1a, where the curves corresponding to the control and to the sample treated at 1200 V/cm are reported. In the figure, the thermal powers corresponding to the initial disturbance of the calorimeter have been subtracted. The integrals of the thermal power curves between 1 and 21 h were used to compare the different treatments. Fig. 1b shows the results of the total heat obtained at different PEF treatments compared with the variations of the root length, as a measure of radicle elongation. The results show that while there are no statistically significant differences in the gross metabolic heat of germination, there is a significant reduction of radicle elongation when 1200 V/cm was applied.

3.2. Effect of PEF treatment on seed proteins

The electrical conditions at which radicle elongation was affected were chosen to study possible effects on the overall pattern of proteins in the seeds.

Representative 2-D gels of embryo proteins from the control and PEF-treated seeds are shown in Fig. 2a and b. Statistical comparison of the spot intensity using one-way ANOVA showed no significant differences ($p < 0.05$) between the samples. ANOVA analysis of the spot intensity in the starchy endosperm from the control and PEF-treated seeds (Fig. 2c and d) showed statistically significant differences in some of the spot intensities (data not shown). However, this result was not verified by the false discovery rate (FDR) analysis ($q < 0.05$) and, therefore, it was concluded that there are no differences in the protein pattern caused by PEF treatment under the studied time scale.

In the studied time scales (32 h after imbibition), the intensity of spots containing α-amylases were below the threshold for spot detection during image analysis and therefore the effect of PEF on these particular enzymes could not be determined. As α-amylase is of high industrial interest, a western blot for specific detection of this enzyme was performed. Western blots of the proteins extracted from the starchy endosperm showed that, as expected, the

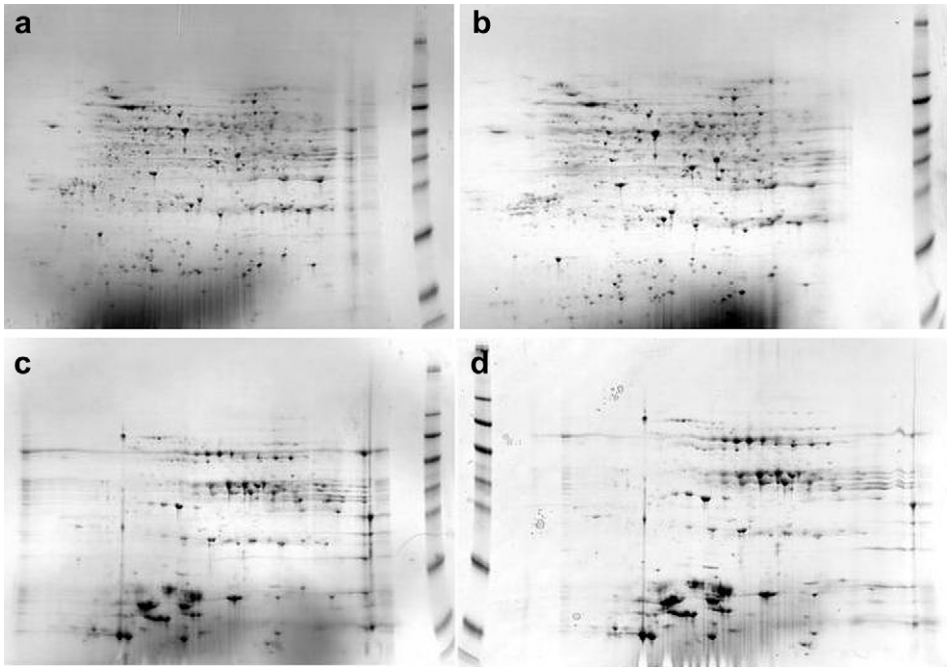

Fig. 2. Typical 2-DE gel patterns in the 4–7 pH range of PEF-treated germinating barley seeds. (a) untreated isolated embryo, (b) PEF-treated isolated embryo. The seeds were treated with fifty 1 ms pulses of 1200 V/cm before embryo isolation. Eight hours after the treatment, the proteins were extracted and separated as described in the Materials and Methods Section, (c) untreated starchy endosperm, (d) PEF-treated isolated starchy endosperm. The seeds were treated with a single 1 ms pulse of 1200 V/cm before endosperm isolation. Eight hours after the treatment, the proteins were extracted and separated as described in the Materials and Methods Section.

concentration of α-amylase increased during the 8 h of germination (Fig. 3), showing good reproducibility in the replicates. Interestingly, the PEF-treated seeds accumulated less α-amylase than the controls in the same time scale.

4. Discussion

This study has explored metabolic responses of germinating barley seeds upon the application of PEF. At pulse width of 1 ms and field strength of 1200 V/cm, our results show evidence that radicle elongation is affected without significant changes in the gross metabolic activity of the seeds. With the application of PEF, opening of pores in the plasma membrane will result in the efflux and influx of polar molecules. After the pulse application, the resealing process is accompanied by oxidative stress with the consequent production of reactive oxygen species (ROS) (Teissié et al., 2005). In a study by Gómez Galindo, Wadsö, Vicente, and Dejmek (2008), oxidative stress caused by PEF affected the gross metabolic activity of potatoes, as measured by isothermal calorimetry. In the case of germinating barley, if it is assumed that the metabolic processes of the seeds did not change, we can conclude that the overall metabolic activity of the germinating barley, or its capacity to perform metabolic processes, was not impaired after the PEF treatments. This is probably due to the tight redox control system of the germinating barley, conferring metabolic resistance to PEF-induced stress. This result is supported by the 2-DE gels (Fig. 2) where no indications of a significant effect on protein patterns could be detected. However, decreased radicle elongation suggests an effect of PEF on root development and α-amylase level. Impaired root elongation may be a consequence of reduced availability of sugars released from starch degradation by α-amylase. It will be of interest to determine whether other, less abundant enzymes that are synthesized in the aleurone layer and released to the starchy endosperm in response to gibberellic acid (Finnie, Andersen, Shahpiri, & Svensson, 2011), are affected in the same way.

The impairment of root growth in barley seedlings has been attributed to oxidative stress (Ktitorova, Skobeleva, Kanash, Bilova, & Sharova, 2006). Impairment of root growth could be the consequence of increased cell wall rigidity related to the formation of oxidative cross-links in the apoplast. Decreased transport rate of assimilates from the starchy endosperm could be another cause of the detected decrease of root growth. Interestingly, our results provide evidence of a lower accumulation of α-amylase when compared with that of the control (Fig. 3).

Pulsed electric fields of high intensity applied in nanoseconds (nsPEF) have shown growth stimulating effects on seven days old *Arabidopsis thaliana* seedlings (Eing, Bonnet, Pacher, Puchta, & Frey, 2009). Therefore, several conditions including nsPEF were also tested with the germinating barley seeds (results not shown). Germinating seeds were treated with 35 ns at varying nominal electric field strength from 5000 to 15 000 V/cm at field strengths from 10 to 100 pulses and frequencies of 1 and 2.5 Hz. These treatments resulted in either no effect or an impairment of radicle elongation when the voltage exceeded 10 000 V/cm and 30 pulses at both frequencies.

Further investigations are needed to provide a deeper understanding of the effect of different PEF protocols on different developmental stages of barley seeds.

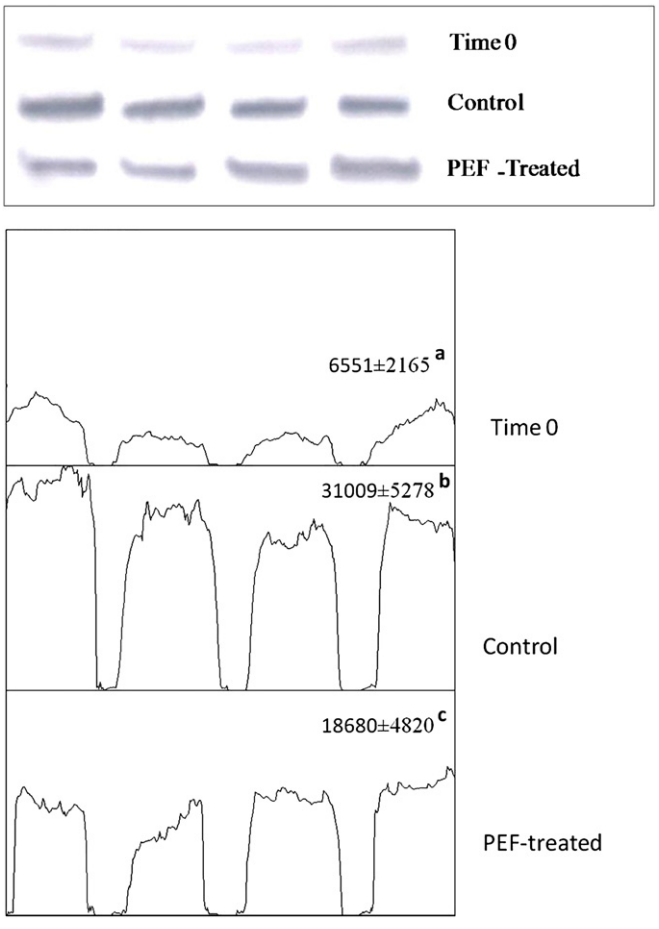

Fig. 3. Accumulation of α-amylase on the starchy endosperm of germinating barley seeds. Samples were treated and the enzyme analyzed as indicated in the Materials and Methods Section. Upper panel: Protein bands in each treatment represent replications. Lower panel: The integrated lane profiles from the protein bands shown in the upper panel. The average and standard deviation of the area of the curves, in pixels, are reported. The letters a, b and c represent statistically significant differences ($p < 0.05$).

5. Conclusions

The results of this investigation present evidence that exposure of germinating barley to pulsed electric fields, under the studied conditions, affects radicle emergence without significantly affecting the seeds' gross metabolic activity. Western blotting demonstrated that α-amylase concentration decreases in the PEF-treated seeds.

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