



## Analysis of apoplastic and symplastic antioxidant system in shallot leaves: Impacts of weak static electric and magnetic field

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### ARTICLE INFO

#### Article history:

Received 3 November 2011

Received in revised form 10 March 2012

Accepted 13 March 2012

#### Keywords:

*Allium ascalonicum*

Antioxidant system

Apoplast

Electric field

Magnetic field

ROS

### ABSTRACT

Impacts of electric and magnetic fields (EFs and MFs) on a biological organism vary depending on their application style, time, and intensities. High intensity MF and EF have destructive effects on plants. However, at low intensities, these phenomena are of special interest because of the complexity of plant responses. This study reports the effects of continuous, low-intensity static MF (7 mT) and EF (20 kV/m) on growth and antioxidant status of shallot (*Allium ascalonicum* L.) leaves, and evaluates whether shifts in antioxidant status of apoplastic and symplastic area help plants to adapt a new environment. Growth was induced by MF but EF applied emerged as a stress factor. Despite a lack of visible symptoms of injury, lipid peroxidation and H<sub>2</sub>O<sub>2</sub> levels increased in EF applied leaves. Certain symplastic antioxidant enzyme activities and non-enzymatic antioxidant levels increased in response to MF and EF applications. Antioxidant enzymes in the leaf apoplast, by contrast, were found to show different regulation responses to EF and MF. Our results suggest that apoplastic constituents may work as potentially important redox regulators sensing and signaling environmental changes. Static continuous MF and EF at low intensities have distinct impacts on growth and the antioxidant system in plant leaves, and weak MF is involved in antioxidant-mediated reactions in the apoplast, resulting in overcoming a possible redox imbalance.

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### Introduction

All terrestrial organisms are exposed to the earth's electric and magnetic fields (EFs and MFs, respectively), which are natural components of their environment. However, interest in studying the effects of these natural phenomena on plants is strengthened by the increasing human activities that generate EF and MF. An EF is a field of force surrounding a charged particle, while a MF is a field of force surrounding a moving charged particle. A charged particle always has both a MF and an EF, and that is why EF and MF are associated with each other (Griffiths, 1999). They are two different fields with similar physical characteristics, and their effects on biological organisms show difference (McCann et al., 1993; Moon and Chung, 2000). Today, different intensities of MF and EF are used in

a wide range of areas including electronic appliances, food sterilization, medical diagnostics, medical therapeutics, and levitation. A large volume of literature is available on the effects of MF and EF on biological organisms. High intensity MF and EF have been utilized for direct biological applications due to their destructive effects on biological samples (McCann et al., 1993). On the other hand, weak MF and EF have been reported to have beneficial effects on living organisms (Nechitailo and Gordeev, 2001; Phirke et al., 1996). Knowledge of the mechanisms of the action of MF and EF on various biological systems may be effectively used as a means of regulating the biological activity of these systems. Stimulatory effects of weak intensity EF have been reported on early growth (Costanzo, 2008) and flowering (Nechitailo and Gordeev, 2001), even if small decreases in the germination ratio and slight disruption of meristem architecture with distracted cell division ratio were reported (Wawrecki and Zagorska-Marek, 2007). Positive effects of weak intensity MF on plant characteristics, such as seed germination and early growth (Cakmak et al., 2010a; Vashisth and Nagarajan, 2010), shoot development and flowering (Aladjadjyan, 2002) were reported. Moreover, effects of weak MF application on protein biosynthesis, cell division, nucleic acid content, and membrane ion movement were studied (Phirke et al., 1996; Stange et al., 2002). However, the underlying mechanism of these phenomena is

Abbreviations: MF, magnetic field; EF, electric field; CAT, catalase; GPOD, unspecific peroxidase; APX, ascorbate peroxidase; SOD, superoxide dismutase; GR, glutathione reductase; Asc, ascorbate; Glu, glutathione; G6PDH, glucose-6-phosphate dehydrogenase; MDA, malonyldialdehyde.

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still poorly understood because of the complexity of the biological responses.

Plants are fixed organisms exposed to environmental stresses. Efficient adaptive cellular mechanisms allow resistance to such stresses. When plants are exposed to different stress factors, a variety of free radicals and reactive oxygen species (ROS) are over-produced. Overproduction of ROS causes oxidative damage to DNA, lipids, and proteins, often leading to the cessation of the cell cycle, and apoptotic or necrotic cell death (Ahmad et al., 2008). On the other hand, at low levels, ROS are important signaling molecules and are effectively managed by several antioxidant molecules. To keep ROS levels in a balance, plants have evolved antioxidant defense mechanisms. These include enzymatic components such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), and glutathione reductase (GR, EC 1.6.4.2), as well as non-enzymatic components, such as ascorbate (ASC) and glutathione (GSH) pool (Mittler, 2002). Enzymatic reaction of SOD with superoxide radicals results in the formation of H<sub>2</sub>O<sub>2</sub>. Produced H<sub>2</sub>O<sub>2</sub> is then scavenged by CAT, nonspecific PODs and the ascorbate–glutathione cycle, where APX reduces it to H<sub>2</sub>O (Mittler, 2002). GR also plays a key role in antioxidant defense processes by reducing oxidized glutathione to GSH. Past research has focused mainly on the potential importance of symplastic antioxidant systems in the detoxification of the ROS. By contrast, relatively little attention has been paid to the potential for the detoxification of ROS in the apoplast. However, many well documented antioxidants such as APX, POD, SOD, and CAT are also located in the leaf apoplast (Cakmak and Atici, 2009; Polle et al., 1994). Therefore, adverse environmental factors are also capable of inducing the synthesis of ROS in apoplastic space as in the intracellular area. Thus, antioxidants located in the aqueous matrix of leaf cell walls constitute an important first line of defense against the environmental stress (Atici and Nalbantoglu, 2003).

Although some reports have investigated MF- or EF-induced oxidative stress and antioxidant response (Hajnorouzi et al., 2011; Sahebjamei et al., 2007; Wang et al., 2009), to our knowledge, there is no information available about MF- and EF-induced apoplastic antioxidant response, although initial events most likely occur in the apoplastic area of plant cells subjected to biotic and abiotic environmental factors. The objective of the present study was to assess the possible effects of weak static MF and EF on the antioxidant status of shallot leaves and to evaluate whether shifts in antioxidant status between apoplastic and symplastic area help plants adapt to a new environment. Shallot plants were chosen for effective evaluation of apoplastic and symplastic antioxidant status in response to weak MF and EF applications because the apoplastic space between cells in an onion leaf is larger than most other plant leaves, and more uniform exposure to leaf cells can be achieved because of the channeled cone-shaped structure of the leaves.

## Materials and methods

### Plant growth and sampling

Fresh shallot (*Allium ascalonicum* L.) bulbs were obtained from Fidanistanbul Inc. (Istanbul, Turkey). Six healthy bulbs for each group (control, MF applied, EF applied) were placed root down to the top of 50 ml flasks filled with nutrient solution after slightly cleaning and rinsing the root region. The nutrient medium was as previously described (Somerville and Ogren, 1982), but at half strength [2.5 mM KNO<sub>3</sub>, 1, 25 mM KH<sub>2</sub>PO<sub>4</sub> (pH 5.6), 1 mM MgSO<sub>4</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 25 μM Fe-EDTA], supplemented with the reported micronutrient mix at 1× concentration. Flasks were placed in coils and between plates where MF and EF were generated, respectively.

Control groups were placed in coils where no MF was generated. Shallots were sprouted under weak static MF and EF with the magnitudes 7 mT MF and 20 kV/m EF for 17 days. The nutrient solution in flasks was renewed every 48 h to avoid soluble oxygen deficiency or possible infection. Sampling was performed on the 8th, 12th and 17th days of growth in order to analyze possible weak MF- and EF-induced changes in the apoplastic and symplastic antioxidant systems in relation to the early leaf age. Control and application groups were kept at least 1 m away from each other to avoid any potential external influence. All samples were kept in well-controlled laboratory conditions of temperature (22 ± 2 °C) and illumination (16 h:8 h light/dark circle).

At harvest, roots and leaves of shallots were separated; the length of each part was measured with a 0.1 cm precision and weighed with 10<sup>-4</sup> g accuracy. Shoots and roots were dried at 80 °C for 48 h to determine dry biomass. Extraction of apoplastic and symplastic proteins was performed immediately at each time point. Samples required for ascorbate, glutathione, H<sub>2</sub>O<sub>2</sub> and malonyldialdehyde (MDA) determinations were weighed, frozen in liquid nitrogen and stored at -80 °C for further use.

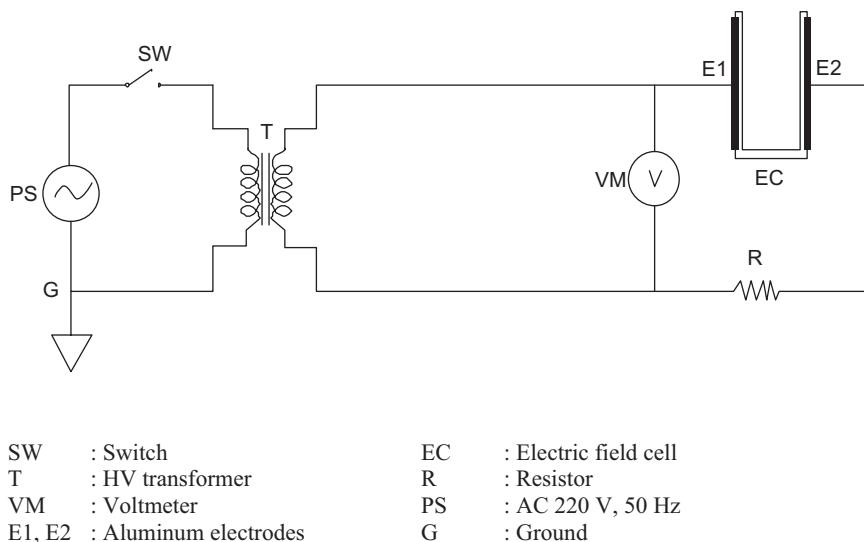
### Magnetic and electric field exposure

The body material of coils used for magnetic treatment was made of several layers of wood laminated and glued to each other. The coil dimension was 30 cm long with an inner radius of 17 cm; the outer radius of each was 28 cm and 24 cm, respectively. Each of the coils was located in a vertical position. The MF application was carried out in the coil at a vertical position of 6–26 cm above the coil bottom, where a uniform MF was obtained. The exposure magnitude of the MF did not at any point deviate more than 6% from the center value. A ventilation system around the coils was employed to avoid an overheating effect from the current in the coils. The temperature deviation inside the coils was negligible (23 ± 2 °C). The required current (0.426 A) and voltage (36 V) to generate MF was provided by power supplies (Global dual power supply Model no: 3521, Wilmington, USA). The number of turns of wire was 17,000 and the wire diameter was 1 mm. Static continuous MF in the axial center of the coils was measured as 7 mT with a gauss meter (F.W. Bell Gauss meter Model no: 5080, Delaware, USA).

The EF intensity was determined as the ratio of electric voltage charged on plates to the distance between them. The electric field was created between two parallel aluminum plates, whose diameters were 50 cm and distance between two plates was 75 cm. A 50 Hz, 15 kV DC voltage was applied to obtain EF intensity of 20 kV/m. A diagram of the experiment is shown in Fig. 1.

### Enzyme extraction

Apoplastic proteins from leaves were extracted as described previously (Vanacker et al., 1998) with some modifications. Harvested fresh leaves (6 g) were carefully cut with a sharp bistoury into 1 cm lengths and rinsed in 6 changes of distilled water to remove cellular proteins and epicuticular waxes from the cut ends. The leaves were then vacuum-infiltrated for 15 min in 20 mM ascorbic acid and 20 mM CaCl<sub>2</sub> solution. The leaves were blotted dry and placed vertically in a 20 ml syringe. The syringes were placed in centrifuge tubes. The apoplastic extract was collected from the bottom of the tubes after the leaves were centrifuged at 1500 × g for 20 min at 4 °C. Then apoplastic extract fluid was centrifuged twice at 1500 × g for 5 min at 4 °C to remove epicuticular waxes. After centrifugation, the supernatant was taken and proteins were precipitated from apoplastic supernatant by adding 1.5 times (v/v) MeOH containing 1% acetic acid and incubated the samples overnight at -20 °C. Then supernatant samples were centrifuged at 3500 × g for 20 min, protein pellets were washed with



**Fig. 1.** Set up for electric field treatment.

100% ice-cold EtOH and 70% ice-cold EtOH, and stored at  $-80^{\circ}\text{C}$  for further use of apoplastic enzyme activity determinations (Tasgin et al., 2006). Protein content of the apoplastic supernatant after protein precipitation was never detected over more than 7% of the precipitated proteins.

Following collection of apoplastic proteins, the residual leaf material was pulverized in liquid nitrogen by means of a mortar and a pestle. For enzyme extracts, 1 g leaf was homogenized in 10 ml of extraction buffer (50 mM  $\text{KH}_2\text{PO}_4$ , pH 7.8 containing 2% soluble polyvinylpyrrolidone, 0.5 mM ascorbate and 1 mM EDTA). Homogenate was centrifuged at  $13,000 \times g$  for 40 min at  $4^{\circ}\text{C}$  and supernatant was centrifuged twice at  $1500 \times g$  for 5 min at  $4^{\circ}\text{C}$  to remove epicuticular waxes. Then supernatant was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further use as enzyme extract.

#### Determination of enzyme activities

The dried apoplastic protein pellets obtained from the leaves were dissolved in 0.2 M phosphate buffer (pH 6.5). Symplastic enzyme extract was thawed and used for protein level and enzyme activity determinations. Protein estimation of apoplastic and symplastic fluids was carried out using the method of Bradford (Bradford, 1976) using bovine serum albumin as standard.

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity was used to assess the contamination degree of apoplastic extract by cytoplasmic constituents. Activity was measured according to the protocol as described before (Kornberg and Horecker, 1955). The reduction of NADP at 340 nm was followed using an assay containing 66 mM potassium phosphate buffer (pH 7.6), 10 mM  $\text{MgCl}_2$ , 300  $\mu\text{M}$  NADP, 2 mM glucose-6-phosphate and 50  $\mu\text{l}$  extract. The activity of G6PDH was calculated using an extinction coefficient of  $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$  for NADPH at 340 nm.

Catalase (CAT, EC 1.11.1.6) activity was measured by monitoring the decrease in absorbance at 240 nm in 50 mM phosphate buffer (pH 7.5) containing 20 mM  $\text{H}_2\text{O}_2$ . The activity of CAT was calculated using an extinction coefficient of  $43.6 \text{ mM}^{-1} \text{ cm}^{-1}$  for  $\text{H}_2\text{O}_2$  at 240 nm (Beers and Sizer, 1952).

Unspecific peroxidase (GPOD, EC 1.11.1.7) activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM phosphate buffer (pH 5.5) containing 1 mM guaiacol and 0.5 mM  $\text{H}_2\text{O}_2$ . The activity of GPOD was calculated using an extinction coefficient of  $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$  for guaiacol at 470 nm (Upadhyaya et al., 1985).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated by recording the decrease in optical density of nitro-blue tetrazolium dye by the enzyme (Dhindsa et al., 1981). Three milliliters of the reaction mixture contained, 2  $\mu\text{M}$  riboflavin, 13 mM methionine, 75  $\mu\text{M}$  nitroblue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.1 ml from the apoplastic fraction. Reaction was started by adding 60  $\mu\text{l}$  from 100  $\mu\text{M}$  riboflavin solution and placing the tubes under two 30 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which yielded the maximal color, served as control. Reaction was stopped by switching off the light. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of enzyme activity was that amount of enzyme which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme.

Glutathione reductase (GR, EC 1.6.4.2) activity was determined following the oxidation of NADPH at 340 nm (Foyer and Halliwell, 1976). The assay mixture contained 25 mM sodium phosphate buffer (pH 7.8), 0.12 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and 0.1 ml enzyme extract in a final assay volume of 1 ml. Corrections were made for any NADPH oxidation in the absence of GSSG. The activity of GR was calculated using a molar extinction coefficient of  $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$  for NADPH at 340 nm.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined as detailed in Nakano and Asada (1981). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 1.2 mM  $\text{H}_2\text{O}_2$ , 0.1 mM EDTA and 0.1 ml enzyme extract in a final assay volume of 1 ml. Enzyme activity was calculated using a molar extinction coefficient of  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$  for ascorbate at 290 nm.

#### Quantitation of ascorbate and glutathione

Extraction of ascorbate and glutathione was accomplished as described previously (Noctor and Foyer, 1998). Fresh leaf material (0.5 g) was ground in liquid nitrogen and then extracted into 2 ml 0.2 N HCl. The homogenate was transferred into Eppendorf tubes and centrifuged at  $16,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . A 0.5 ml supernatant was neutralized with 50  $\mu\text{l}$   $\text{NaH}_2\text{PO}_4$  (0.2 M, pH 5.6) and 0.4 ml NaOH (0.2 M). The final pH was between 5 and 6. The levels of ascorbate and glutathione were measured using previously described enzyme-linked spectrophotometric methods (Queval and Noctor, 2007). The total ascorbate level was quantified

after conversion of dehydroascorbate to ascorbate by incubation of the neutralized supernatant with 1 mM dithiothreitol (DTT) in NaH<sub>2</sub>PO<sub>4</sub> buffer (0.1 M, pH 7.5) for 30 min. For ascorbate measurement, the initial absorbance of a 30 µl of supernatant (incubated with 1 mM DTT) was measured at 265 nm in NaH<sub>2</sub>PO<sub>4</sub> (0.1 M, pH 5.6), then re-measured over 3 min following the addition of Ascorbate Oxidase (0.5 U). An extinction coefficient of 12.6 mM<sup>-1</sup> cm<sup>-1</sup> for ascorbate at 265 nm was used for calculation. The method followed for glutathione measurement relies on the GR-dependent reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) monitored at 412 nm. The assay mixture used for glutathione measurement contains 100 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.8), 0.6 mM DTNB, 6 mM EDTA, 0.1 mM NADPH, 25 µl extract and 0.6 U GR. The change in absorbance at 412 nm was recorded for 5 min. Glutathione concentrations were calculated from a standard curve constructed using GSH over the range of 0–1 nmol ( $y = 1.143x - 0.0453$ ,  $R^2 = 0.993$ ).

#### Determination of the malonyldialdehyde and hydrogen peroxide levels

The thiobarbituric acid (TBA) test, which determines malonyldialdehyde (MDA) as an end product of lipid peroxidation, was employed to measure lipid peroxidation in the leaves of shallots. Briefly, 1 g of leaf sample was homogenized in 5 ml 80% ethanol solution with a mortar and pestle. The homogenate was centrifuged at 3000 × g for 20 min and 2 ml of supernatant was aliquoted into two Eppendorf tubes as 1 ml per tube. Then; 20% trichloroacetic acid (TCA) (w/v) solution including 0.01% (w/v) butylated hydroxytoluene and 0.65% TBA (w/v), or 1 ml 20% TCA solution including 0.01% (w/v) butylated hydroxytoluene was added into these aliquots and they were incubated at 95 °C for 20 min. The reaction was stopped by placing the reaction tubes in an ice bath for 5 min and then the samples were centrifuged at 3000 × g for 10 min. The absorbance of the supernatants was monitored at 532 nm for MDA compounds, 440 nm and 600 nm for correction of anthocyanin and sugar absorbance. The MDA equivalents were calculated using an extinction coefficient of 157 mM<sup>-1</sup> cm<sup>-1</sup> as described previously (Hodges et al., 1999).

For H<sub>2</sub>O<sub>2</sub> determination, 1 g of leaf sample was ground in liquid nitrogen and homogenized in 5 ml of 0.1% (w/v) TCA. The homogenate was centrifuged at 12,000 × g for 15 min. An aliquot (1 ml) of the supernatant was mixed with an equal volume of 10 mM potassium phosphate buffer (pH 7.0) (KH<sub>2</sub>PO<sub>4</sub>) and 1 ml of 1 M KI. The absorbance of the mixture was monitored at 390 nm. The content of H<sub>2</sub>O<sub>2</sub> was calculated by using a standard curve (Velikova et al., 2000).

Two independent experiments, with three replicates for each measurement, were performed. All data were expressed as the mean values ± standard deviation (SD). Statistical analysis was carried out from raw data using two tailed probability values of the student's *t*-test and the differences between treatments were expressed as significant at a level of  $P < 0.05$ , 0.01, or 0.001 significance criterion.

#### Results and discussion

The world's natural MF has been reported as 25–65 µT and EF has been calculated as 100–140 V/m in rural areas (Belyavskaya, 2004; Neamtu and Morariu, 2005). However, they can show dramatic increases in industrialized regions (Isobe et al., 1999). According to the report released in 2001 by the American Conference of Governmental Industrial Hygenists Organization, occupational threshold limit values for workers were defined as 25 kV/m EF and 10 mT MF (Belyavskaya, 2004). In this study, we wanted to assess the possible effects of weak static MF (7 mT) and

EF (20 kV/m) on the antioxidant status of plant leaves. The shallot plant was chosen due to the feasibility to extract apoplastic fluids for effective evaluation of apoplastic and symplastic antioxidant status in leaves in response to weak MF and EF applications.

Plants have the ability to adjust their metabolism according to changing environmental conditions. They accelerate their metabolism and grow faster when optimal conditions develop. However, when a stress condition arises, plants generally decelerate their metabolism and limit their growth (Atici and Nalbantoglu, 2003). In this study, root and leaf length increased in response to MF but these effects were not observed in response to EF application (Table 1). Moreover, weak MF induced sprouting approximately one day earlier than other groups. Emergence of the first leaf was observed on 6th day of the incubation. Root and leaf dry biomass increased in response to EF and MF applications. Increases were found to be relatively higher under EF application (Table 1). In plants, the reactive oxygen species (ROS) production level increases under stress conditions or at some growth stages (e.g., germination, early growth, senescence). At certain levels, increases in ROS production imply either increased metabolic activity or possible redox imbalance depending on changes in the levels of oxidative stress markers such as lipid peroxidation and protein carbonylation (Mittler, 2002). Our results showed that H<sub>2</sub>O<sub>2</sub> levels decreased, but the level of MDA compounds, which are end products of lipid peroxidation, remained unchanged depending on leaf age (Fig. 2a and b). Younger leaves had a higher H<sub>2</sub>O<sub>2</sub> but approximately the same MDA compound levels, which reflects the fact that they have a higher metabolic activity than older ones. An increase in ROS levels, to some extent, was reported as an indicator of metabolic activity in plants (Mittler, 2002). Moreover, H<sub>2</sub>O<sub>2</sub> and MDA levels did not show a considerable change in MF applied leaves, but both increased significantly in EF applied leaves when compared to their respective controls (Fig. 2a and b). These results show that 7 mT MF application does not, but 20 kV/m EF application may, form a stress factor on shallot growth.

Under stress conditions or increased metabolic activities at some growth stages, plants generally increase the activity of one or more antioxidant molecules, and the elevated activity levels usually correlate with increased stress tolerance (Mittler, 2002). Therefore, resistance to stress or the holding maximum growth rate under prevailing environmental conditions is related to a plant's antioxidant capacity, which counteracts redox imbalance by scavenging overproduced ROS. In addition, many studies have suggested that enzyme systems localized at the cell surface or apoplast are important sources of superoxide (O<sub>2</sub><sup>−</sup>) and H<sub>2</sub>O<sub>2</sub> production (Tasgin et al., 2006). The antioxidant enzymes in apoplast spaces of plants have important roles in the removal of ROS under both normal and stress conditions (Cakmak and Atici, 2009; Patykowski and Urbanej, 2003). However, a possible correlation between apoplastic and symplastic antioxidant pool is not well documented, and to our knowledge, there is no study reported thus far on the evaluation of EF or MF effects on apoplastic and symplastic antioxidant systems in plants. Our results showed that the soluble protein level in symplastic areas slightly increased in response to MF (Fig. 3a). On the other hand, 12 days of MF application caused more than a two-fold increase in protein levels and a statistically important decrease in protein level was observed in apoplastic washing fluid at the end of 17 days of EF application (Fig. 3b). These results show that different enzymatic regulation patterns may exist in apoplastic and symplastic areas of shallot leaves in response to MF and EF applications.

Before starting enzymatic activity determinations, we measured G6PDH activity to examine whether there was contamination of symplastic fluid into apoplastic areas. Activity of G6PDH in apoplastic washing fluid of all leaf samples was below the detection limits while symplastic G6PDH activity increased on 12th and 17th days of

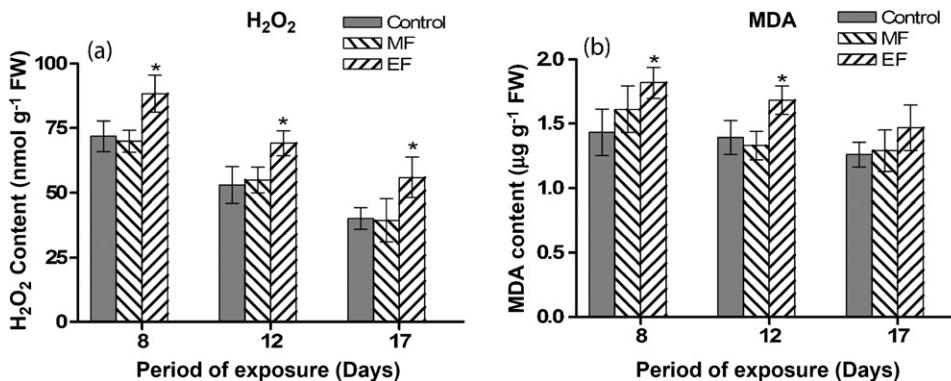
**Table 1**

Changes in the length and dry biomass of shallot root and leaves under EF and MF conditions.

Parameters	Control			7 mT MF			20 kV/m EF		
	Day 8	Day 12	Day 17	Day 8	Day 12	Day 17	Day 8	Day 12	Day 17
Leaf length (cm)	4.63 ± 0.21	9.28 ± 0.71	14.11 ± 1.27	6.12 ± 0.81*	12.4 ± 1.15*	17.8 ± 2.1*	4.1 ± 0.67	10.6 ± 0.22	14.26 ± 2.25
Root length (cm)	3.52 ± 0.68	3.91 ± 0.22	4.42 ± 0.34	4.12 ± 0.68	4.7 ± 0.43*	5.58 ± 0.41*	3.65 ± 0.21	3.36 ± 0.85	4.71 ± 0.44
Leaf dry biomass (%)	5.71 ± 0.44	5.87 ± 0.55	6.37 ± 0.21	6.15 ± 0.22	5.81 ± 0.62	7.38 ± 0.38*	6.72 ± 0.48*	6.95 ± 0.29*	7.65 ± 0.36*
Root dry biomass (%)	6.24 ± 0.36	6.46 ± 0.25	6.85 ± 0.62	6.62 ± 0.51	7.2 ± 0.47	6.87 ± 1.07	7.18 ± 0.27*	7.64 ± 0.51*	8.42 ± 0.46*

Data are means ± SE of at least six separate measurements.

\* A significant difference from the control in t-test at  $P < 0.05$  in the same day.



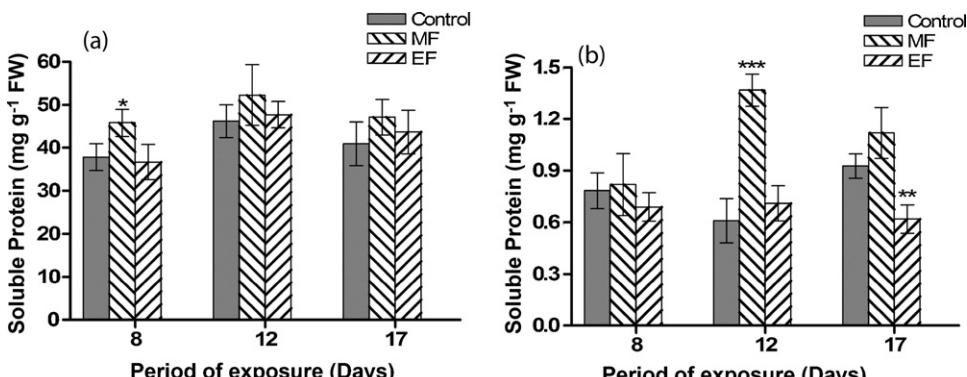
**Fig. 2.** Changes in (a) cellular  $\text{H}_2\text{O}_2$  and (b) MDA levels in shallot leaves in response to weak static MF and EF applications. Data are means ± SE of at least six separate measurements. Asterisk in the same column denote a significant difference from the control in t-test at  $P < 0.05$ .

MF and EF applications (Fig. 5a). Apoplastic washing fluid isolated from shallot leaves was found to contain CAT, GPOD, APX, and SOD, but not GR. This is consistent with previous reports (Patykowski and Urbanek, 2003).

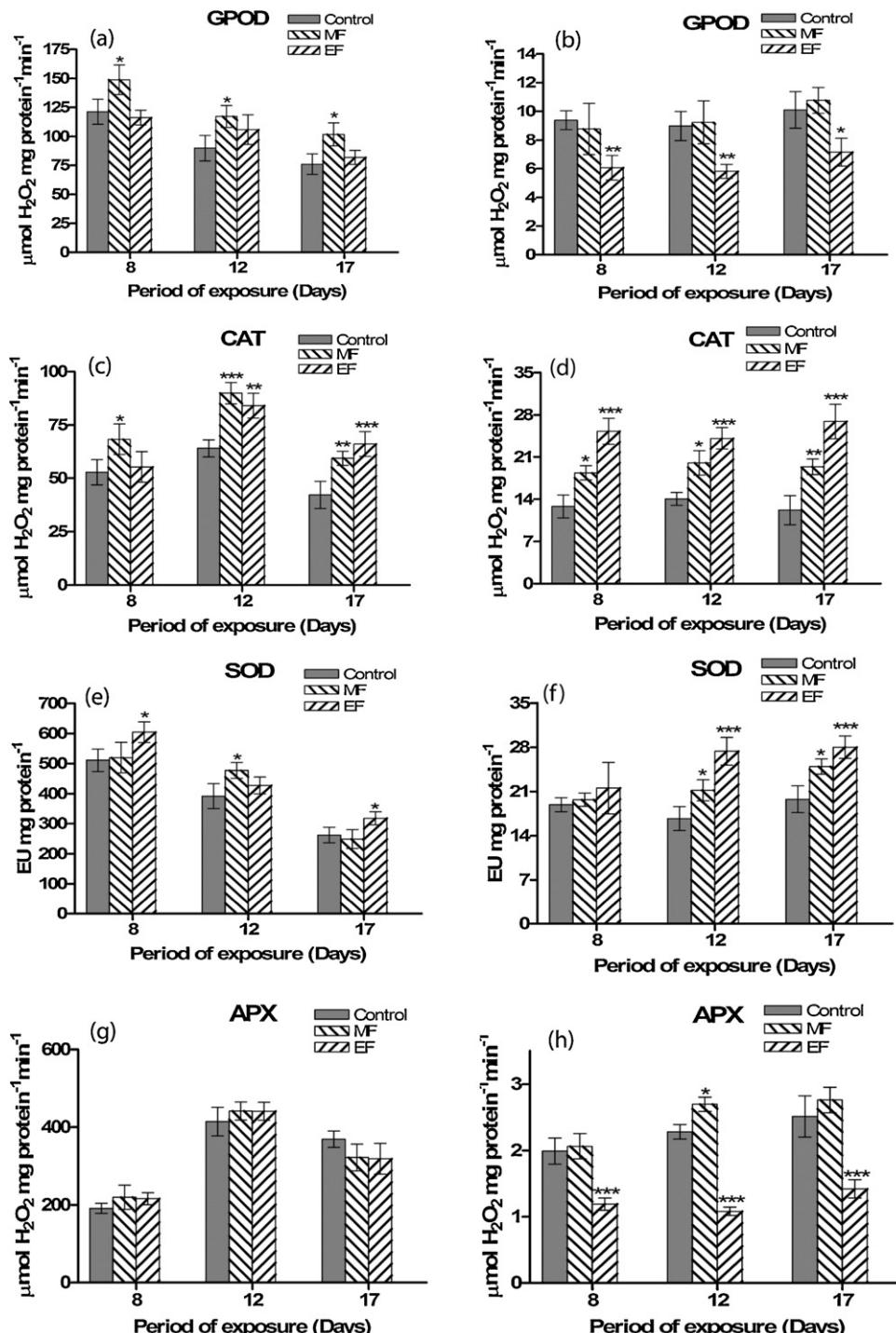
In this study, symplastic GPOD activity increased while no significant change was observed in apoplastic areas in response to MF during all days studied (Fig. 4a and b). On the other hand, symplastic GPOD activity did not change, but apoplastic GPOD activity decreased in response to EF application (Fig. 4a and b). Unspecific PODs protect cells against damaging effects of  $\text{H}_2\text{O}_2$  during an oxidative-burst response which occurs as a result of cellular redox changes. Apoplastic PODs are bound to cell wall polymers by ionic or covalent bonds, and were reported to be easily released from the cell wall into the apoplast and play a critical role in regulating the wall stiffening process (De Pinto and De Gara, 2004), and many other functions related to their ROS scavenging activity (Xue et al., 2008) under normal and stress conditions. Our results show that EF application may impede cell wall lignification process by affecting

chemical composition of apoplastic GPOD and some other related enzymes bound to cell wall polymers. Symplastic GPOD activity decreased, but apoplastic GPOD activity did not show a significant quantitative change depending on leaf age in any of the groups studied. The functional significance of such changes in cell wall properties under the influence of MF and EF are worthy of detailed investigation.

Remarkably, both CAT and SOD activities in apoplastic and symplastic areas increased in response to MF and EF applications (Fig. 4c-f). However, increases in enzyme activities were found to be higher in response to EF. Of the antioxidant enzymes, SOD catalyzes the conversion of two superoxide molecules to hydrogen peroxide and oxygen, and hydrogen peroxide is eliminated mainly by CAT. Increased CAT and SOD activities have been related to increased metabolic activity, cold tolerance (Cakmak and Atici, 2009; Clare et al., 1984), freezing tolerance (Cakmak et al., 2010b; McKersie et al., 1993), and salt stress tolerance (Yazici et al., 2007). In this study, apoplastic SOD activity did not change but symplastic



**Fig. 3.** Changes in symplastic (a) and apoplastic (b) protein levels in shallot leaves in response to weak static MF and EF applications. Data are means ± SE of at least six separate measurements. Values followed by different symbols (\*, \*\*, and \*\*\* ) in the same column indicate significant difference from the control ( ${}^*P < 0.05$ ,  ${}^{**}P < 0.01$ , or  ${}^{***}P < 0.001$ ).

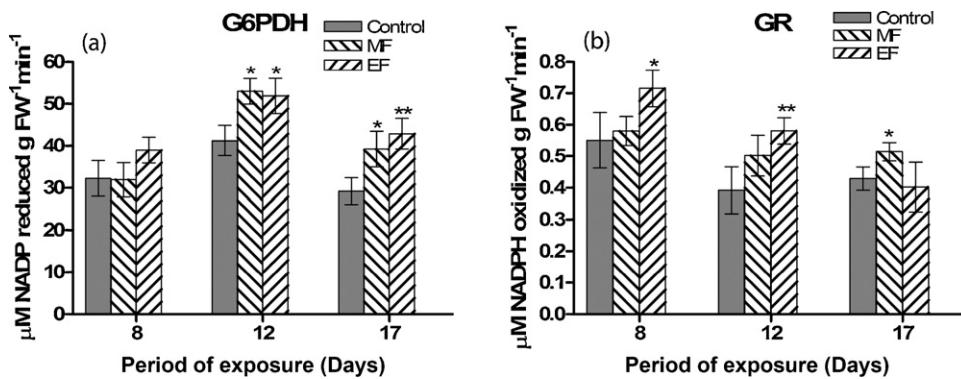


**Fig. 4.** Changes in symplastic (a, c, e, and g) and apoplastic (b, d, f, and h) antioxidant enzyme activities in shallot leaves in response to weak static MF and EF applications. (a and b) GPOD, unspecific peroxidases; (c and d) CAT, catalase; (e and f) SOD, superoxide dismutase; (g and h) APX, ascorbate peroxidase. Data are means  $\pm$  SE of at least six separate measurements. Values followed by different symbols (\*, \*\*, and \*\*\* in the same column indicate significant difference from the control ( $*P < 0.05$ ,  $**P < 0.01$ , or  $***P < 0.001$ ).

SOD activity decreased quantitatively depending on leaf age in all groups studied (Fig. 4e and f). Indeed, full function of this enzyme is not well documented. It is therefore difficult to assess the significance of the decrease in the activity of this enzyme in the symplast depending on the age of the leaf. Apoplastic SOD has been associated with cell wall lignification (Kukavica et al., 2009). Thus, a possible conclusion from our results might be that some of the symplastic SOD molecules might be transferred to the apoplastic area in order to help cell wall strengthening depending on leaf age.

Increased apoplastic SOD activity in response to EF application supports this hypothesis, as apoplastic SOD was also implicated in the perception and signaling of oxidative stress (Foyer et al., 1997).

Symplastic APX activity did not change in response to MF and EF applications (Fig. 4g and h), but symplastic GR activity increased during 12 days of EF application while there was no significant change in response to MF (Fig. 5b). On the other hand, apoplastic APX activity sharply decreased in EF applied leaves but it either increased or remained unaffected in MF applied leaves. To our



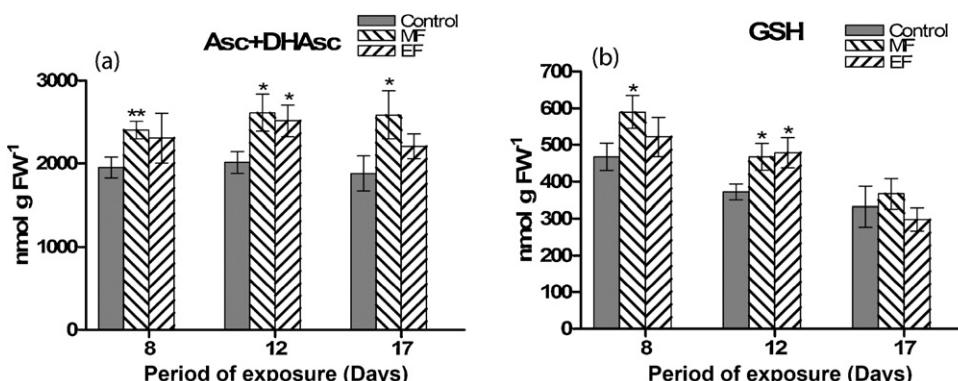
**Fig. 5.** Changes in (a) glucose-6-phosphate dehydrogenase (G6PDH) and (b) glutathione reductase (GR) enzyme activities isolated from residual leaf extract after apoplastic fluid separation in shallot leaves in response to weak static MF and EF applications. Data are means  $\pm$  SE of at least six separate measurements. Values followed by different symbols (\*) or (\*\*) in the same column indicate significant difference from the control (\* $P < 0.05$  or \*\* $P < 0.01$ ).

knowledge, there is no study thus far reported on effects of MF and EF on apoplastic antioxidant status, but researchers reported different results of MF and EF effects on cellular antioxidant enzyme activities in plants. It has been reported that weak static MFs (10 and 30 mT for 5 days, 5 h each day) increased SOD but decreased CAT and APX enzyme activities in tobacco cell lines (Sahebjamei et al., 2007). Supported with increased level of lipid peroxidation, these authors concluded that weak MF could have deteriorative effect on antioxidant defense system of plant cells. On the other hand, magnetically (180 mT) pretreated lentil seeds grew faster and appeared as more resistant to drought with the increased SOD and APX activities (Shabranghi and Majd, 2009). A comprehensive study was reported on the stimulation of germination and early growth of rice by using high-voltage EFs in the range of 250–450 kV/m (Wang et al., 2009). They observed induced activities of antioxidant enzymes (SOD, APX, and CAT), and lowered malonyldialdehyde content in response to a 300 kV/m EF for 30 min right before germination. They concluded that a high-voltage EF could elevate the aged rice seeds' vigor and improve the membrane system of aged rice seedlings. In addition, our previous investigation (Cakmak et al., 2010b) showed that short term (10 and 40 min) EF application with a magnitude of 100 kV/m does not have a significant effect on antioxidant enzyme activities under normal growth conditions. However, 10 min EF right before cold application could augment chilling resistance of cold-sensitive bean species with increased CAT and SOD activities. In this study, we observed significant increases of oxidative stress markers ( $H_2O_2$  and lipid peroxidation levels) in response to EF application but not to MF (Fig. 2a and b). Moreover, increased CAT and SOD activities were followed by decreased APX activity in

the apoplastic area of EF applied shallot leaves. However, apoplastic APX activity either increased or remained unaffected by MF (Fig. 4). Both CAT and APX are involved in scavenging  $H_2O_2$  and they have distinct affinity levels for  $H_2O_2$ . Catalase has been reported as a primary enzyme that effectively eliminates the bulk of  $H_2O_2$  while APX can scavenge low levels of  $H_2O_2$  that is not removed by CAT as it has higher affinity for  $H_2O_2$  compared to CAT (Dat et al., 2001; Ghanati et al., 2005). In addition, similar changes in apoplastic APX and POD activities (Fig. 4) in response to MF and EF show that peroxidases are important elements of the apoplast taking on the task of sensing and signaling environmental changes.

In this study, increases in GR and G6PDH activities were more pronounced in EF applied leaves than MF applied ones in general (Fig. 5a and b). Glucose-6-phosphate dehydrogenase is the first enzyme of the pentose phosphate pathway. Thus, an increase in this enzyme activity supported with increased GR activity may indicate that ascorbate–glutathione pathway works faster in EF applied leaves. In this case, EF applied leaves are expected to have higher levels of ascorbate and glutathione. However, increases in ascorbate and glutathione levels were more pronounced in MF applied leaves than EF applied ones (Fig. 6a and b). Such effects remain to be investigated in *A. ascalonicum*.

In conclusion, the data presented in this paper indicate that weak MF promote growth, possibly by increasing antioxidant system activity, but EF has some negative effects on shallot growth despite a lack of visible symptoms of injury. An increase in growth in response to MF, change in metabolic activity depending on leaf age, and slight oxidative stress caused by EF are directly related to collaboration between apoplastic and symplastic antioxidant



**Fig. 6.** Changes in (a) total ascorbate (Asc + DHAsc) and (b) glutathione (GSH) content of shallot leaves in response to weak static MF and EF applications. Data are means  $\pm$  SE of at least six separate measurements. Values followed by different symbols (\*) and (\*\*) in the same column indicate significant difference from the control (\* $P < 0.05$  or \*\* $P < 0.01$ ).

activity of leaf cells. Differential activity levels of apoplastic and symplastic ROS scavengers in response to MF and EF showed that the apoplastic area is as important as the symplastic area for sensing and overcoming a stress factor. Shifts in antioxidant status of the apoplast and symplast contribute to redox regulation and help plants adapt to a new environment. Lastly, weak MF applications may be involved in antioxidant-mediated reactions in apoplast resulting in overcoming of possible redox imbalance. Thus, weak MF can be used as an effective means for augmenting plant resistance to different stress factors. To uncover possible practical applications of weak EF and MF in agriculture, more research on the effects of weak MF and EF applications on growth and biochemical response in plants is necessary. Our ongoing studies are focused on the potential importance of apoplastic antioxidants in mediating the redox state of plant cells at different growth stages.

## Acknowledgement

This work was supported by grants from the Research Fund of Ataturk University (Grant no: BAP-2009/233, Grant no: 2009/384) and The Scientific and Technological Research Council of Turkey (TUBITAK, grant no 2218).

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