Chilling Resistance of *Phaseolus vulgaris* and *Brassica oleracea* under a High-Intensity Electric Field

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An electric field may have different effects on plant metabolism depending upon its application style and density, and environmental conditions. The effects of an electric field, low temperature, and their combinations on tissue vitality and some physiological variables regarding antioxidant responses of "bean" (*Phaseolus vulgaris* L. cv. Gina) and "cole" (*Brassica oleracea* L. cv. Acephale) leaves were studied. Fifteen-day-old seedlings were exposed to an electric field (100 kV m^{-1}) for 10 or 40 min prior to cold treatment. In both plant leaves, cold application caused statistically significant increments in total soluble protein levels and selected antioxidant enzyme activities such as catalase, peroxidase and superoxide dismutase activities. However, tissue vitality and H_2O_2 levels did not change in "cole", while tissue vitality decreased and H_2O_2 levels increased in "bean". Electric field application itself did not cause any significant changes in "bean" and "cole" leaves. On the other hand, 40 min electric field application increased the deteriorative effect of cold in both plant species, while 10 min electric field augmented the chilling resistance by increasing the tissue vitality and antioxidant enzyme activities resulting in decreased H_2O_2 levels.

Key words: Brassica oleracea, Chilling Resistance, Electric Field, *Phaseolus vulgaris*

Introduction

Cold stress is known to be one of the major problems among factors limiting the growth and productivity of plants. Various significant changes occur in cellular membranes and metabolic functions when plants are injured by cold stress. In cold-sensitive plants, cold induces a phase transition of cellular membranes from liquid-crystalline to solid gel, resulting in an increase in membrane permeability and changes in the activities of some membrane-bound enzymes (Lyons, 1973). In addition, similar to other stresses, reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals are also produced during response to cold stress (Wise and Naylor, 1987; Okuda *et al*., 1991). ROS are highly reactive and, in the absence of any protective mechanism, they can seriously disrupt the normal metabolism through oxidative damage to lipids, proteins and other macromolecules (Rout and Shaw, 2001). Antioxidative enzymes are the most important components of ROS-scavenging systems (Kang *et al*., 2003). Superoxide dismutase (SOD) is a major scavenger of superoxide, and its enzymatic action results in the formation of H_2O_2. The H_2O_2 produced is then scavenged by catalase (CAT) and a variety of peroxidases (POX) (Noctor and Foyer, 1998). A correlation between the intracellular antioxidant capacity and cold tolerance has been demonstrated in some plant species (Baek and Skinner, 2003; Janda *et al*., 2003). At low concentrations, ROS act as secondary messengers responsible for signal transduction from extracellular signaling molecules and their membrane receptors to the intracellular regulatory systems, yet when the increase in ROS levels is above a certain threshold, oxidative stress can be accompanied by processes that are harmful for cell survival, such as lipid peroxidation and oxidative modification of proteins and nucleic acids (Li *et al*., 2004). Plants should maintain the activity levels of antioxidant enzymes in order to accommodate oxidative stress. However, under severe stress conditions, the antioxidant capacity may not be sufficient to minimize the harmful effect of oxidative injury.

From the beginning of the evolution process, all living organisms have an experienced electric field (EF), which is generated by the ionosphere and the globe. EFs abound in our natural environ-

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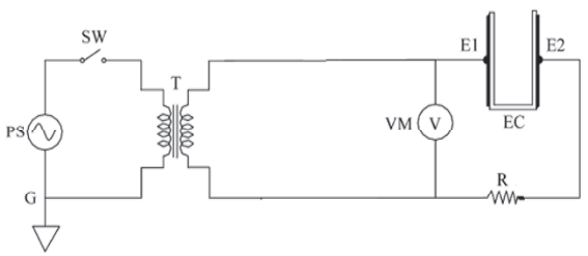
ment, in the living spaces of the developed society, in industrial factories, and in medical diagnostics as well as medical therapeutics (Isobe *et al.*, 1999). The impact of these EFs may be positive or negative on biological systems, depending upon the prevailing conditions where the event occurs. EFs sometimes perturb the structural organization of cells, involving enzymes and membranes, resulting in unusual metabolism and functions. Knowledge of the mechanisms of the action of an EF on various biological systems, like cells, tissues and organs, may be effectively used as a means regulating the biological activity and removing undesirable substances from the system. Comprehensive works have been done with plants on the stimulation or retardation of plant growth by EFs (Murr, 1963, 1965a; Bachman and Reichmanis, 1973). Plant damage due to an EF was observed in some cases (Murr, 1965b; Bachman and Reichmanis, 1973; Hart and Schottenfeld, 1979). An increase in plant respiration was reported by applying an EF of 5–10 kV m^{-1} (Sidaway and Asprey, 1968). Prolonged ripening was achieved by suppressing the respiration and retarding the ATP production using an EF (Prasad *et al.*, 1996).



SW : Switch
T : HV transformer
VM : Voltmeter
E1, E2 : Aluminum electrodes
EC : Electric field cell
R : Resistor
PS : AC 220 V, 50 Hz
G : Ground

Fig. 1. Set-up for electric treatment.

But the underlying mechanism of this phenomenon is still poorly understood.

It is well-known that plant cold resistance can be influenced by exogenous application of substances (*e.g.*, phytohormones) and that EF application can markedly influence the plant growth. Yet, the effect of EF application on chilling resistance has never been tested. The aim of this paper is to describe the physiological effects of an EF on chilling resistance in "bean" and "cole" seedlings.

Material and Methods

Growth conditions

Seeds used in this study were supplied from East Anatolia Agricultural Research Center, Erzurum, Turkey. Cold-sensitive "bean" (*Phaseolus vulgaris* L. cv. Gina) and cold-tolerant "cole" (*Brassica oleracea* L. cv. Acephale) plants were used. Before sowing, seeds were surface-sterilized for 10 min with water/bleach (10:1, commercial NaOCl) solution and then washed five times with distilled water. Plants were grown in pots filled with sand in a growth chamber under controlled environmental conditions for 15 d (22/20 ºC day/night temperature, 70% relative humidity, and a photon flux density of 400 μmol m^{-2} s^{-1} photosynthetic active radiation at a 16-h photoperiod). In order to supply plants with nutrient and element requirement, pots were routinely watered with Hoagland solution. Fifteen-day-old "bean" and one-month-old "cole" seedlings were treated with an 100 kV m^{-1} 50-Hz EF, for 10 or 40 min, which was created between two parallel aluminum plates, whose diameter was 50 cm and distance was 20 cm (Fig. 1). The EF intensity was determined as the ratio of electric voltage between the two plates to the distance between them. After EF application, plants were either transferred into a growth chamber with 5/2 ºC day/night temperature, 55%

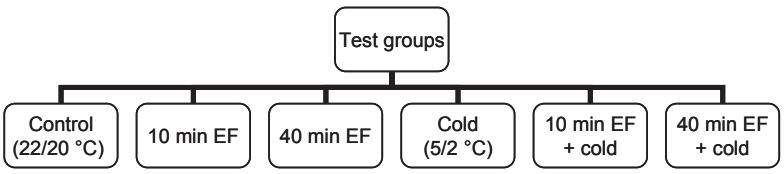


Fig. 2. Experimental set-up for "bean" and "cole" seedlings. Sampling was done on the 3rd and 6th days after application(s).

relative humidity, and a photon flux density of 400 μmol m^{-2} s^{-1} photosynthetic active radiation at a 14-h photoperiod, or a growth chamber with 22/20 ºC day/night temperature, 70% relative humidity, and a photon flux density of 400 μmol m^{-2} s^{-1} photosynthetic active radiation at a 16-h photoperiod. Plants were then harvested on 3rd and 6th days after EF application. Test groups are shown in Fig. 2.

Determination of H_2O_2 content

Tissue material (500 mg) was ground with 5 ml of 0.1% (w/v) trichloroacetic acid (TCA) and sand in a mortar on an ice-bath. The homogenate was centrifuged at 12,000 × *g* for 15 min. An aliquot (0.5 ml) of the supernatant was mixed with an equal volume of 10 mM potassium phosphate (KH_2PO_4) buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the mixture was monitored at 390 nm. The H_2O_2 content was calculated using a standard curve (Velikova *et al.*, 2000).

Enzyme activity assays

For enzyme activity measurements, 500 mg of pooled leaf tissue were frozen in liquid nitrogen, pulverized, and added to 3 ml of extraction buffer containing 50 mM KH_2PO_4 (pH 7.8), 0.1 mM EDTA, and 2% (w/v) soluble polyvinylpyrrolidone-40. The suspension was centrifuged (13,000 × *g*, 4 ºC, 40 min), and the total protein concentration of the supernatant was determined according to Bradford (1976). The activities of CAT (EC 1.11.1.6), SOD (EC 1.15.1.1), and POX (EC 1.11.1.7) were determined according to previously published protocols (Beers and Sizer, 1952; Dhindsa *et al.,* 1981; Upadhyaya *et al*., 1985).

Tetrazolium vitality test (TTC)

The TTC vitality test was achieved according to a method advanced by Chin *et al.* (2001) from Towill and Manzur (1975). Ten leaf discs (10 mm diameter) were randomly punched from the leaves below the shoot apex of each plant. The leaf discs were floated in a test tube (10 × 15 mm indicate the diameter and length of the tube) in 4 ml of 0.6% TTC in 50 mM KH_2PO_4 buffer (pH 7.0) containing 0.005% Triton X-100. They were then vacuum-infiltrated with the TTC solution by a single cycle of vacuum at 0.8 atm with occasional patting until they sank to the bottom. The tubes were then positioned at 45 ºC in a roller and rotated (5 rpm) at 25 ºC in the darkness for 24 h to allow the production of triphenylformazan (TPF). They were prepared for quantitative analysis of TPF using a refinement of the French and Parkin (1993) method where water was substituted by ethanol. First the leaf discs were rinsed with water, ground in 2 ml water, and then homogenized. The homogenate was vortexed with 4 ml of *n*-hexane and centrifuged at 700 × *g* for 5 min. Most of the tissue residues congregated at the *n*-hexane/water interface. The absorbance of the *n*-hexane fraction was then measured at 492 nm using a double beam spectrophotometer (Shimadzu UV-Vis 1240) for TPF quantification.

Statistical analysis

All experiments were performed at least three times with two replicates. Data were analyzed by two-way analysis of variance (ANOVA), and means were compared by Duncan's multiple range test using SPSS 16.0 for Microsoft Windows (SPSS, Inc., Chicago, IL, USA) at 0.05 level of significance.

Results and Discussion

In the present study we describe the effect of an EF on total protein content, tissue vitality, and physiological variables such as H_2O_2 and enzymatic antioxidants (CAT, POX, and SOD) activity levels in cold-sensitive "bean" and -tolerant "cole" plant leaves under control and cold conditions. Additionally, in order to determine the influence duration of the cold and EF treatments, all these parameters were followed on the 3rd and 6th days after both treatments.

There are lots of papers published regarding acceleration of germination and plant growth in response to EF application (Brayman and Miller, 1990; Nechitailo and Gordeev, 2001; Cao *et al*., 2004). It was reported by Cramariuc *et al*. (2005) that some low and medium levels of EF application enhance plant growth. However, unlike what we focused on, most of the EF studies were performed with long-term EF application. Even if there were some fluctuations, our results showed that EF application itself did not show statistically significant variations in protein quantity, tissue vitality, H_2O_2 levels, and antioxidant enzyme activities of both plant leaves in general (Figs. 3

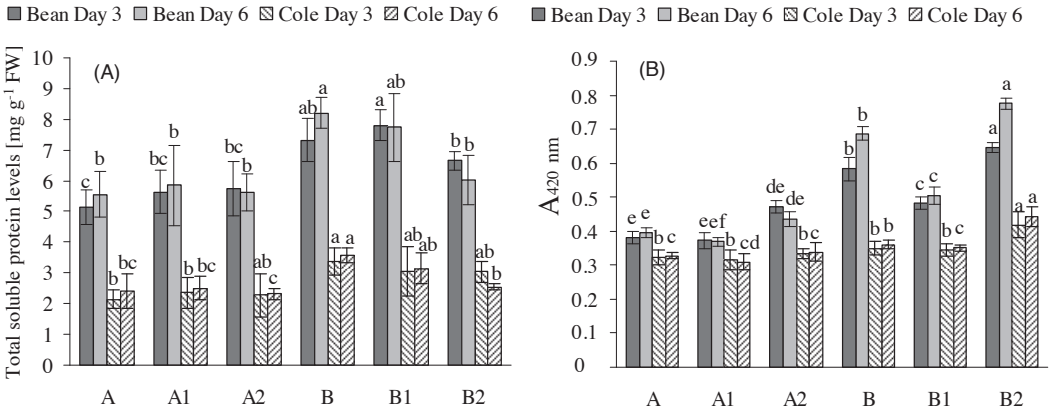


Fig. 3. (A) Total soluble protein and (B) TPF (triphenylformazan) formation levels in "bean" and "cole" leaves in response to different applications. Increased absorbance levels at 420 nm indicate decreased tissue vitality. Data are presented as means ± SE from at least three independent experimental replicates. Different letters in the same group indicate statistically significant differences ($P < 0.05$). Groups: A, control (22/18 ºC); A1, 10 min EF application; A2, 40 min EF application; B, cold (5/2 ºC); B1, 10 min EF plus cold application; B2, 40 min EF plus cold application.

and 4). Considering that we harvested plants after 3 and 6 d of EF application, even if there were some important changes in these biochemical parameters, this time period might be enough for plants to turn their metabolism into normal.

Cold application itself caused increases in protein quantity, tissue vitality, H_2O_2 levels, POX and SOD antioxidant enzyme activities in "bean" leaves (Figs. 3 and 4). While there were increases in TPF levels, which indicates a decrease in tissue vitality, increased protein levels, antioxidant enzyme activities, and H_2O_2 levels showed that cold application itself had deteriorative effects on "bean" which is cold-sensitive. In "cole" leaves, cold application itself caused increases in protein quantity, CAT, POX, and SOD enzyme activities, while there was no significant difference in TPF formation (Figs. 3 and 4). In addition, there was no significant difference in the H_2O_2 level on the 3rd day but an increase on the 6th day (Fig. 4). Especially increases in antioxidant enzyme activities were strikingly higher in "cole" leaves in comparison to those of "bean" leaves showing the fact that "cole" is more effective in adjusting its defense mechanisms to cold stress rather than "bean". This is why, we chose these plants to observe the physiological effects of EF application on chilling resistance. Low-temperature injury can occur in all plants, but the mechanisms and types of damage vary considerably. Regardless of the fact that whether a plant is cold-sensitive or -tolerant, from carbon metabolism to respiration and antioxidant responses, overall physiological changes occur in plant metabolism during cold exposure, and, depending on plant species, all these changes differ from plant to plant.

When we had applied an EF for 10 min prior to cold application, we observed increases in CAT, POX, and SOD enzyme activities (Fig. 4), H_2O_2 levels (Fig. 4) and TPF formation (Fig. 3) in both "bean" and "cole" leaves in comparison to the solely cold applied group (cold control). Mostly these effects were higher in "bean" leaves compared to "cole". However, exposing plants for 40 min to an EF prior to cold application showed that, together with an increment in SOD activity, H_2O_2 levels and TPF formation, there were decreases in total soluble protein quantity, CAT and POX enzyme activities in "bean" leaves compared to the cold control group (Figs. 3 and 4). In "cole" leaves, compared to respective cold controls, we did not see significant differences in the antioxidant enzyme activities but increased levels of TPF formation and H_2O_2,and a decrease in total soluble protein levels when we had applied an EF for 40 min in advance of cold application. Intriguingly, these results show that 100 kV m^{-1} EF application for 10 min is not a stress factor but possibly an activating power (as some growth regulators) encouraging stimulated metabolic func-

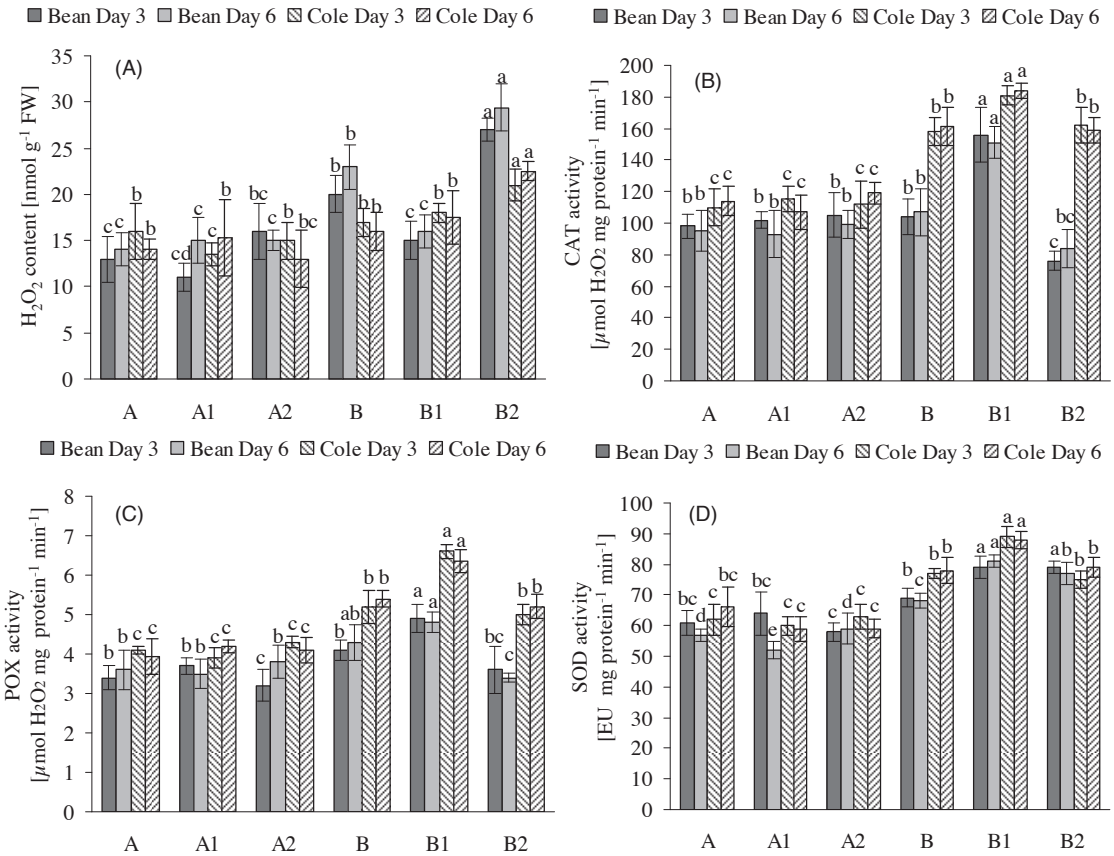


Fig. 4. Changes in (A) hydrogen peroxide levels, (B) catalase, (C) guaiacol peroxidase, and (D) superoxide dismutase activities in "bean" and "cole" leaves in response to different applications. Data are presented as means ± SE from at least three independent experimental replicates. Different letters in the same group indicate statistically significant differences ($P < 0.05$). For the superoxide dismutase assay, one enzyme unit is defined as the quantity of superoxide dismutase required to produce 50% inhibition of nitroblue tetrazolium photoreduction (Dhindsa *et al.*, 1981). Groups: A, control (22/18 ºC); A1, 10 min EF application; A2, 40 min EF application; B, cold (5/2 ºC); B1, 10 min EF plus cold application; B2, 40 min EF plus cold application.

tions in plants. Moon and Chung (2000) applied different intensities of an EF changing from 4 to 12 kV cm^{-1} onto tomato seeds during 15, 30, 45 and 60 s and followed differences in germination and early growth of tomato seeds. They reported that, in general, EF application increases the germination ratio and early growth up to 12 kV cm^{-1} and 60 s while 12 kV cm^{-1} EF application over 60 s a effects germination and early growth negatively. Here we report that 100 kV m^{-1} EF application for 10 min augments the chilling resistance of plants while a 40-min application increases the deteriorative effect of coldness.

Cell membranes were reorganized biochemically (Matos *et al.*, 2007; Rodriguez-Vargas *et al.*, 2007) and physically (Son *et al.*, 2005; Wei *et al.*, 2006) during cold acclimation. On the other hand, as reported by some researchers (Ming, 1988; Kurinobu and Okazaki, 1995), electric and/or magnetic field applications have different effects on plant metabolism by affecting biochemical functions which eliminate free radicals and by enhancing specific enzymes and proteins syntheses. It has also been reported by some researchers (Ho *et al.*, 1995; Angersbach *et al.*, 2000) that EF application, with increasing application time and density, causes changes in structures of lipids and proteins of membranes and cell performances, changes tissue and organs performances consequently, and changes vital functions of organisms

negatively. One very recent study performed by Wang *et al.* (2009) regarded the stimulation of early growth of rice by using high-voltage EFs in the range of 250 to 450 kV m^{-1}. Applying a 300-kV m^{-1} EF for 30 min right before germination, they observed elevated activities of antioxidant enzymes (SOD, POX, and CAT), which improved the penetrating capability of the membrane, and adjusted the level of lipid peroxidation and lowered the content of malonedialdehyde. They concluded that a high-voltage EF could elevate the aged rice seeds' vigor and improve the membrane system of aged rice seedlings; the treatment for 55 min was more effective than that for 30 min. When we applied a 100 kV m^{-1} EF, either for 10 or 40 min, we did not observe any significant changes. Therefore, we propose that applying a 100 kV m^{-1} EF for a very short time, like 10 min, before chilling treatment augments the chilling resistance by elevating the enzymatic antioxidant activation levels and tissue vitality, thus resulting in less H_2O_2 formation especially in cold-sensitive species.

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