The Effect of Electric Field on Callus Induction with Rape Hypocotyls

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The influence of electric field treatment on dedifferentiation and calli formation on rape hypocotyls was investigated. Segments, 10 mm long, of the upper part of rape (*Brassica napus* L., cv. GórczaΔski) hypocotyls were stimulated by different combinations of voltage/time (1.5 V/120 h, 3 V/3 h, 10 V/15 min and 30 V/30 s) under *in vitro* conditions. With all electric field treatments, segments oriented with their apical part towards the cathode produced more calli as compared to control (non-treated with electric field). Under opposite orientation slight inhibition of callus growth was observed. As the strongest effect on callus growth was observed after treatment with 30 V/30 s, this electric field treatment was selected for following analyses: the incorporation of [^{14}C]-2,4-D (2,4-dichlorophenoxyacetic acid) and [^{14}C]-BAP (benzylaminopurine) from the culture medium, changes in ACC (1-aminocyclopropane-1-carboxylic acid) level and the redox activity in apical and bottom parts of hypocotyls during 18 d of culture.

In contrast to changes in fresh weight, electric field treatment (30 V/30 s) stimulated a higher accumulation of 2,4-D and BAP in basal parts of hypocotyls than in apical ones. Moreover, orienting the apical part towards the cathode resulted in lower uptake of hormones as compared with the opposite orientation. The ACC concentration increased, especially in the basal parts of hypocotyls, independently on electric field application. However, the highest level was observed after electric field treatment with orientation of the apical part towards the anode. The distribution of oxidative substances (measured as the amount of ferric ions) between the apical and bottom part of hypocotyls was not changed when the apical parts were oriented towards the cathode. Under these conditions a decrease in apical and an increase in basal parts was observed during culture. Opposite orientation influenced the redistribution of oxidative substances from the first day of electric field treatment. Based on these results we suggest that electric field action can be connected with its influence on specific concentration of oxidative substances and hormone distribution in cells.

Key words: Electric Field, Rape, Growth Regulators

Introduction

The physical properties of lipids and proteins, the main compounds of biomembranes, predetermine the membranes to originate an electric field (Chermomordic *et al.*, 1987; Tsong and Astumian, 1988). Endogenous currents occur in the form of transcellular potentials (Mycielska and Djamgoz, 2004). These electric fields are believed to play a significant role in biological processes (Le Saux *et al.*, 2001). Long-standing interest in the effect of electric fields was concentrated on animal systems (Robinson, 1985). However, analysis of its influence on biological molecules raises several problems. The main experimental problem is to apply a transmembrane electric potential of comparable magnitude as the one existing in physiological conditions. When the applied potential exceeds a critical value, mechanical instability and electroporation (pore formation) of membranes occur (Wothers *et al.*, 2001).

It was observed in animal cells that exogenous electric fields induce a variety of cellular responses. It can influence cell surface charge redistribution (Cho *et al.*, 1994), cytoskeletal reorganization (Cho *et al.*, 1996) and changes in the intracellular calcium ion concentration (Wallecczek, 1992). Because Ca^{2+} ions regulate numerous biological processes including signal transduction cascades, cell orientation and migration and cell differentiation and proliferation, changes in Ca^{2+} concentration have been hypothesized to mediate cellular effects induced by an exogenous electric field (Cho *et al.*, 1999).

In our earlier studies we observed the interaction of an external electric field with generative

development in plants (Macháčková *et al.*, 1990; Macháčková and Krekule, 1991; Filek *et al.*, 2002, 2003). The final effect in the number of flowering plants was dependent on voltage and time of exposition of tissues as well as on the electric field polarity. Inhibition of bud formation and flower induction was caused by direct current treatment with the cathode connected to the leaf tips in *Chenopodium rubrum* (Adamec *et al.*, 1989) or to the apical stem part in winter wheat and rape (Filek *et al.*, 2002, 2003) (the anode was in the nutrient solution around the roots). It was also described that an applied electric field induced a gradient of Ca^{2+} ions from apex to base in *Chenopodium rubrum* plants (Macháčková *et al.*, 1995). Experiments performed in various systems implicated calcium in the hormonal signal transduction chain (Hobbie *et al.*, 1994; Staxén *et al.*, 1999; Anil and Rao, 2000; Yang and Poovaiah, 2000; Pandey *et al.*, 2004). Moreover, electric field-induced changes in membrane potential can mediate the activation of plasma membrane hormone receptors (Barbier-Brygoo *et al.*, 1991; Astumian *et al.*, 1995). It was also documented that an increase in auxin concentration stimulates the activity of plasma membrane H^+-ATPase, which results in cell acidification (Hager *et al.*, 1991). The details of plant hormone action remain largely unknown, however, their participation in diverse aspects of plant growth and differentiation is well documented. Especially in *in vitro* experiments, the specific hormone composition of the medium is needed to induce both callus production on plant explants and embryogenesis.

The aim of presented experiments was to study the influence of an electric field on tissue dedifferentiation and hormone redistribution during callus formation on the segments of rape hypocotyl. At first the effect of voltage and time of the applied electric field on calli induction/inhibition was tested. In the conditions of specific electric field action the incorporation of [^{14}C]-2,4-D (2,4-dichlorophenoxyacetic acid) and [^{14}C]-BAP (benzylaminopurine) was detected. Both hormones are usually added to medium during rape *in vitro* cultures. Because an electric field as well as auxins can induce changes in membrane potential and ion channel activities, the redox activity was also detected. To assess the possible stress effect of the treatments with an electric field, the level of ACC (1-aminocyclopropane-1-carboxylic acid), precursor of ethylene, was measured.

Materials and Methods

Twenty seeds of the winter rape (*Brassica napus* L.) cultivar "Górczański" were sown in Magenta (Sigma) vessels on Murashige-Skoog (Murashige and Skoog, 1962) medium and grown under light condition 16 h day/8 h night (150 μmol m^{-2} s^{-1}) at 25/18 °C. After 7 d of culture segments of 10 mm were cut off from the upper part of hypocotyls (explants). These explants were placed into Magenta (Sigma) vessels equipped with two parallel silver electrodes (0.5 mm diameter, 50 mm long, distance between them: 50 mm) dipped in the medium. To induce dedifferentiation and callus induction, the medium was supplemented with 1 mg/l BAP and 0.5 mg/l 2,4-D. Explants were placed in parallel to lines of force of the electric field with the apical part toward the cathode or anode. The electric potential stimulation was applied after a 24 h culture as one direct electric current impulse. At the beginning explants were subjected for varying lengths of time to electric current of different voltages. Four different voltage/duration combinations were applied: 1.5 V/120 h, 3 V/3 h, 10 V/15 min and 30 V/30 s (see Fig. 1). Plants not stimulated by the electric field were used as control. Fresh weight of callus initiated on apical or bottom parts of hypocotyls was determined after 4 weeks of culture. On the basis of this experiment the electric field of 30 V lasting 30 s was chosen for further studies.

In the following studies fresh weights of apical and basal part of *in vitro* cultured segments of hypocotyls (hypocotyls were cut to two equal segments) were measured after 1, 4, 7, 11 and 18 d after electric field treatment (30 V/30 s). In some experiments the Murashige-Skoog medium was additionally supplemented with [^{14}C]-2,4-D or [^{14}C]-BAP (radioactivity of both hormones was 380 MBq $mmol^{-1}$). The samples from about 20 segments were collected in 5 replicates to measure fresh weight and hormone incorporation.

The accumulation of [^{14}C]-2,4-D and [^{14}C]-BAP in apical and basal parts of hypocotyls was measured after washing the tissue (3 times) in 0.6 M mannitol and carefully drying to remove the adsorbed substances. Then the tissue was homogenized in the Bray solution and the radioactivity was detected by a scintillation counter (Beckman LS 5801).

For detection of redox activity, hypocotyl segments were cut to apical and basal parts and incubated for 15 min in 1 mM Tris-HCl [tris(hydoxy-

methyl)aminomethane hydrochloride] (pH 7.2) containing 0.5 mM $CaCl_2$ and 50 mM KCl. Redox reaction was initiated by introducing 1 mM $K_3Fe(CN)_6$ and followed as the decrease in absorbance at λ = 420 nm (Federico and Giartosio, 1983).

ACC was extracted from the apical and basal parts of cultured hypocotyl segments with boiling 80% (v/v) ethanol. The extract was centrifuged at 10,000 × *g* for 15 min and the supernatant was evaporated to dryness in vacuum at 35 °C. The dry residue was dissolved in distilled water and ACC was oxidized to produce ethylene according to Lizada and Yang (1979). The ethylene level was determined by gas chromatography (Hewlett-Packard 5890) with a flame ionisation detector and an alumina capillary column (30 m length, 0.53 mm diameter).

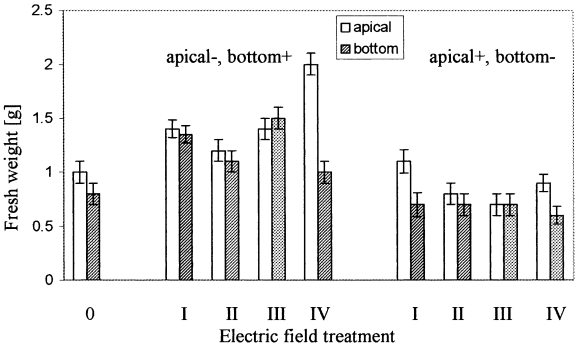


Fig. 1. Calli formation (in g of fresh weight) on apical and bottom part of rape hypocotyls after 4 weeks culture under *in vitro* conditions. Hypocotyls were treated by electric field: I, 1.5 V/120 h; II, 3 V/3 h; III, 10 V/15 min; IV, 30 V/30 s; and oriented with the apical part towards cathode (−) or anode (+). 0, control (without electric field application). For more details see Materials and Methods. Each value is a mean ± SD (n = 3).

Results

Under *in vitro* conditions rape hypocotyl segments induced calli on both apical and basal parts (Fig. 1). After electric field treatment, explants oriented with their apical part towards the cathode produced calli more effectively after 4 weeks of culture at any combination of voltage/time (Fig. 1). Under opposite orientation slight inhibition of calli as compared to control (non-treated with electric field) was observed. Moreover, independently of the used polarity, higher fresh mass of callus was generally determined on the apical part of hypocotyl segments. The strongest stimulation and/or inhibition of callus growth on the apical and the basal part, respectively, was observed after treatment with 30 V/30 s. Thus, this electric field treatment was selected for further experiments.

Calli developed at the apical and the basal part of segments cultured without electric treatment showed differences in their fresh mass (Fig. 2). In spite of a continuous increase of mass at both parts of hypocotyls during 18 d of culture, a significantly higher increase at the apical part in comparison to the basal one was observed. On day 18, callus cells at the apical part appeared, while at the basal part callus appeared by 2–3 d later.

Electric field treatment (30 V/30 s) increased the fresh mass of hypocotyls in comparison to control only when the apical parts were oriented towards the cathode. The increase was evident starting on day 4 of culture and was visible only at the apical part. In the opposite orientation, the fresh mass was lower in both hypocotyl segments in comparison to control.

All investigated tissues took up [^{14}C]-2,4-D and [^{14}C]-BAP (Fig. 3). Accumulation of 2,4-D was lower on the first day of culture and then it systematically increased (Fig. 3A). Higher uptake of 2,4-D was detected in control segments in comparison to those treated by the electric field. BAP accumulated at a high degree already at the begin-

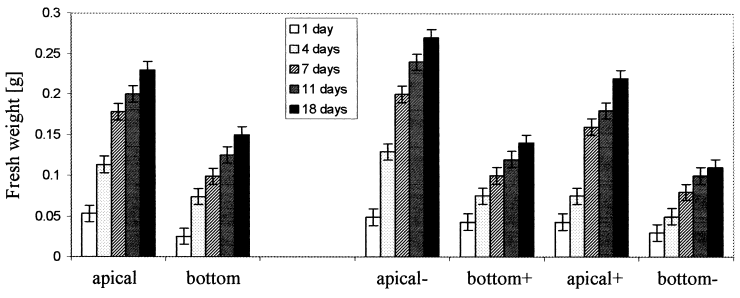
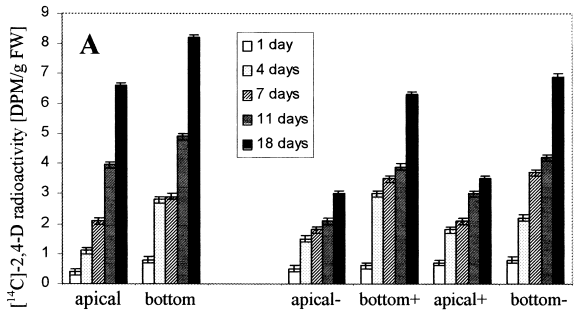


Fig. 2. Changes in fresh weight (g) of apical and bottom part of rape hypocotyls during 18 d of culture without and after electric field treatment (30 V/30 s). Hypocotyls were oriented with the apical part towards cathode (−) or anode (+). Each value is a mean ± SD (n = 5).



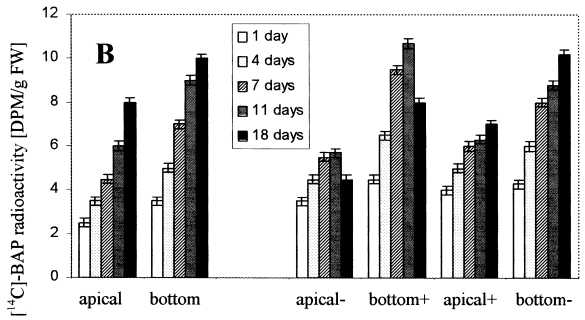


Fig. 3. Accumulation of hormones (DPM g^{-1} FW): [^{14}C]-2,4-D (A) and [^{14}C]-BAP (B) in apical and bottom part of hypocotyls during 18 days of culture without and after electric field treatment (30 V/30 s) with the apical part towards cathode (–) or anode (+). Each value is a mean ± SD (n = 5).

ning of culture especially after electric treatment (Fig. 3B). Moreover, on contrary to fresh mass, higher levels of both hormones were found in basal parts than in apical ones. In addition, orienting the apical part towards the cathode resulted in lower or comparable accumulation of hormones as compared with the opposite orientation.

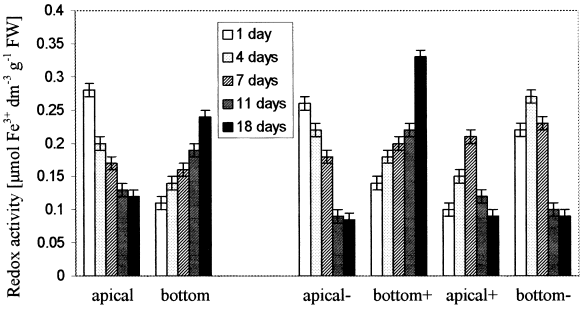


Fig. 4. Changes of redox activity (μmol Fe^{3+} dm^{-3} g^{-1} FW) in apical and bottom part of hypocotyls during 18 d of culture without and after electric field treatment (30 V/30 s). Hypocotyls were oriented with the apical part towards cathode (–) or anode (+). Each value is a mean ± SD (n = 4).

The amount of oxidative substances measured as the level of oxidized ferric ions was higher in the apical part of hypocotyl segments than in the basal one on day 1 of culture of control tissues (Fig. 4). During culture the level of the oxidized form in the apical part decreased and in the basal one increased reaching the same level of the oxidized and reduced form on day 7. Orientation of the apical part towards the cathode did not influence the direction of migration of the oxidized form, however, it decreased the concentration of the oxidized form in the apical part and increased it in the basal part on day 18 of culture. In the opposite orientation with the apical part towards the anode, changes in distribution of the oxidized form were observed already on day 1: a higher level of the oxidized form was detected in the basal part than in the apical one. On day 18 in both parts of hypocotyl segments comparative amounts of the oxidized substances were detected (Fig. 4).

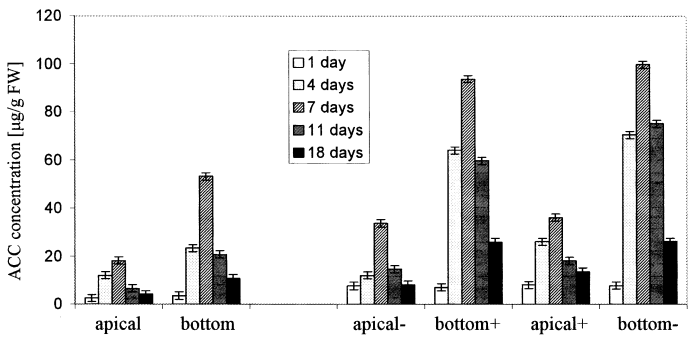


Fig. 5. Changes in ACC concentration (μg g^{-1} FW) in apical and bottom part of hypocotyls during 18 d of culture without and after electric field treatment (30 V/30 s). Hypocotyls were oriented with the apical part towards cathode (–) or anode (+). Each value is a mean ± SD (n = 4).

The lowest level of ACC was observed in control tissues and the highest after electric field treatment with orientation of the apical part towards the anode. However, the ACC concentration in all investigated tissues was higher in the basal than in the apical part. During culture the level of ACC increased to day 7 and afterwards it decreased (Fig. 5).

Discussion

Tissue dedifferentiation and calli formation appear especially on the young, wounded parts of plants and also on apical tissues (Caswell *et al.*, 2000). Thus, the segments of rape hypocotyls were very suitable material to study the effect of electric field on induction/inhibition of calli production.

In control tissues, calli were initiated on both parts of explants after 4 weeks of *in vitro* culture. However, calli formed at the apical part had higher fresh mass. Differences in the response of both parts of explants can be explained by their differences in totipotency (Cassells and Cury, 2001) and also by preferential assimilate (sugar) transport to the apical part. High concentration of sucrose was shown to induce an increase in cells number in *in vitro* cultures (Anbazhagan and Ganapathi, 1999). However, the main substances involved in regulation of plant growth and differentiation are hormones. Both exogenous and endogenous auxins are closely involved in the process of callus formation and embryogenesis (Michalczuk *et al.*, 1992; Padmanabhan *et al.*, 2001). According to the theory of auxin polar transport in stems, auxin anions are translocated towards the bottom, positively charged part of the stem. Higher accumulation of [^{14}C]-2,4-D in the basal part of explants than in the apical ones observed in our experiments is in accordance with this theory. It may also be hypothesised that in the upper part of the hypocotyl the exogenous auxin was accumulated in a lower degree because of its higher endogenous level. It seems that a specific hormone level (endo- + exogenous) is needed for induction of redifferentiation of rape hypocotyl segments. Differences in the sensitivity to auxin between the apical and basal part of segments also have to be taken into account.

All investigated electric field treatments increased calli formation on both apical and basal parts, but only in the case of explants being oriented with the upper part towards the cathode. Opposite electric field orientation inhibited calli production, especially on the basal parts of explants. Comparison between the increase in fresh mass and decrease of 2,4-D accumulation during cultivation indicates correlation between these two factors. It may be assumed that an external electric field stimulates callus growth when its polarity favours the natural translocation of auxin along the hypocotyl. The opposite polarity counteracted natural movement of auxin in the stem resulting in accumulation of auxin in the apical part of the explant.

Accumulation of exogenous cytokinins showed a similar trend as that of auxin. Elliot and Weston (1993) reported, that a higher cytokinin level might increase cell division rate and sucrose accumulation. The participation of cytokinins in recallusing of pigeon pea cultures was suggested by Anbazhagan and Ganapathi (1999). In our experiment, higher accumulation of exogenous BAP in the basal parts of explants could be connected with its lower endogenous level, similar as in the case of auxins. However, generally lower radioactivity of both hormones in hypocotyls treated with an electric field suggests that in these conditions not only natural redistribution plays a role in callus formation.

Treatment with an electric field can act as a stress factor and preparation of explants for *in vitro* culture is also connected with stress factors like wounding of tissues, which leads to oxidative stress. As production of ethylene and of its precursor ACC is strongly linked to oxidative stress (Pell *et al.*, 1997), we determined the levels of ACC in our segments. Observed differences in the ACC level in apical and basal parts of control tissues can be connected with differences in the intensity of stress. Thus, it seems that the basal part is stressed in a higher degree than the apical one. Both parts of explants seem to be adapted to stress conditions during 7 d of culture. After this time, ACC level significantly decreased. Electric field treatment, as an additive stress factor, increased the ACC level; making the apical part negative was less effective than the opposite polarity. ACC is known to interact with auxin and cytokinins (Jia *et al.*, 1996; Kim *et al.*, 2001) and thus an increase in the ACC concentration in stress conditions can inhibit hormone accumulation and their transport from medium.

Redistribution of substances between the apical and bottom part was confirmed by measurements

of redox activity. In control tissues, a lower level of oxidized (Fe^{3+}) ions than in the apical one was observed in the basal part. It can suggest the presence of higher level of free electrons in the basal parts on the first day of culture. An electric field mimicking natural tissue polarization, it means upper part towards the cathode, inhibited the electron transport to the apical part and stimulated the translocation to the bottom. In the opposite orientation electrons are translocated towards the apical parts which results in reduction of Fe^{3+} to Fe^{2+} (decrease of Fe^{3+} concentration). A higher level of the oxidized form (Fe^{3+}) registered in basal explant parts confirms lower concentration of electrons in this tissue. It seems that the change of natural polarization on the first day of culture is a crucial factor for growth and differentiation of cells, disregarding the following translocation of oxidized/reduced forms during further culture.

The most effective electric field treatment for callus induction was 30 V/30 s. The time of the impuls (30 s) seems to be too short to induce a permanent change in the level of electrons as well as of exogenous and endogenous hormones. But this treatment can change the distribution of Ca^{2+} ions by both passive flux and activation/deactivation of ion chanells (Cho *et al.*, 1999). The electric signal could also affect the membrane potential and mediate specific changes in plasma membrane transport and enzymatic systems and thus influence redistribution of some substances (Robertson and Astumian, 1992). The relation between extracellular electric fields and changes in membrane potential has been described by Gross (1988). However, by far not all steps of electric field action leading to physiological responses are recognized. On the basis of presented results we can suggest that electric field-induced membrane hyper-/depolarization and changes in membrane structure and function can affect the formation of oxidizing agents and increase the Ca^{2+} efflux. Ca^{2+} ions are involved in many signal mechanisms including responses to hormones, *e.g.* in regulation of auxin transport (Price *et al.*, 1994). An increased ethylene level is a factor which changes the auxin economy, especially in stress conditions (Michaelli *et al.*, 1999). In spite of principles of its action are not yet clear, treatment with an electric field offers a variety of possibilities for investigation of physiological processes (Groves and Boxer, 1995).

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