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Effects of intermittent-direct-electric-current (IDC) on polyphenols and antioxidant activity in radish (*Raphanus sativus* L.) during growth

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(Received June 6, 2009)

Summary

In the present study the effects of intermittent-direct-electric-current (IDC; for one hour per day) on phenolic compounds and antioxidant activity in radish (*Raphanus sativus* L.) was studied. The radish plants were cultivated in rock wool substrate in combination with drip irrigation. Two stainless steel plates were added on the rock wool substrate and acted as electrodes to supply the currents to the plants. Three different IDC treatments (200 mA, 600 mA, and 1000 mA) were applied during growth period and passed horizontally through the nutrient solution as well as through the tissue of the radish plants. After 16 days of growth the radish plants were randomly harvested, divided into three segments (root, tuber, shoot) and were used for the determination of total phenolic content, anthocyanins, and antioxidative activity.

This new technology increased the health-promoting phytochemical compounds in radish segments with no sign of damage. In radish tubers total phenol content, anthocyanins, and antioxidant activity increased with increasing IDC. The same was observed for the total phenol content in radish roots. However in contrast, the phenolic compounds of radish shoots remained unaffected by IDC. In conclusion IDC can serve as a general mean to stimulate the synthesis of phenolic compounds and associated antioxidant activity in radish tubers.

Introduction

Various medical studies suggest a positive correlation between diets rich in vegetables and fruit and a reduced occurrence of chronic diseases such as cancer, atherosclerosis, cardiac dysfunctions, hypertension and neurodegenerative disorders (EBERHARDT et al., 2000; BAGCHI et al., 2003; LAMBERT and YANG, 2003; BOYER and LIU, 2004). The most important elicitor of most chronic diseases is associated with free radical reactions (MARTINEZ-CAYUELA, 1995; ARUOMA, 1998). Fruit and vegetables contain numerous different secondary plant compounds, many of which possess antioxidant properties. Based on the chemical structure secondary plant compounds such as carotenoids as well as phenolic compounds, e.g. anthocyanins and hydroxycinnamic acid have a free-radical binding character (BAZZANO et al., 2002). It is supposed that these may protect against such free radical reactions and affect health outcomes (PRIOR and CAO, 2000). However, secondary plant compounds (e.g. phenolic compounds) assume a wide range of function in plants. They play important roles in the interaction of plants with their environment (EVERS, 1994) and regulate stress situations (SCHREINER, 2005). For example phenolic compounds protect plants against herbivores (SUMMERS and FELTON, 1994), have fungicidal properties (KOESE et al., 1994; PADMAVATI et al., 1997), and protect against an increased radiation intensity, especially UV-B exposure (KAKANI et al., 2003). Increased UV-B radiation causes stress in plants and influences the secondary metabolism. In this context, plants res-

pond with an accumulation of various phytochemicals, e.g. phenolic compounds (DIXON and PAIVA, 1995; SCHREINER et al., 2009). Another kind of environmental stress are magnetic fields (NILSEN and ORCUTT, 1996). During the evolution plants have developed pathways for electrical signal transmission to respond rapidly to environmental stress factors. In contrast to chemical signals such as hormones, electrical signals are able to rapidly transmit information over long distances between plant tissues and organs (FROMM, 2006). Therefore, the relationship between electricity and biological processes in plants has been the subject of numerous investigations over the last 300 years (VOLKOV, 2006). LEMSTRÖM (1904) and BLACKMAN (1924a, 1924b) found a pronounced influence of atmospheric electricity (by positive and negative air ions) on plant growth of cereals. In contrast, ELKIEY et al. (1985) found an effect only at high concentration of negative ions, while positive ions had no significant effect, and COLLINS et al. (1929) found no evidence that air ions affect plant growth.

In further studies the influence of weak electromagnetic fields on growth and metabolism of plants were examined. MURAJI et al. (1998) found that a magnetic field, alternating at between 5 and 40 Hz (5 mT), causes a significant increase in the growth of primary roots in corn seedlings. Peak growth rate was achieved at 10 Hz. In the same manner SMITH et al. (1993) exposed seeds of radish to 60 Hz fields tuned to the ion cyclotron resonance frequencies for calcium and potassium ions. The results showed that calcium resonant tuning of the fields produced a significant delay in germination followed by rapid growth which resulted in significant increases in plant height and root weight.

Moreover, weak direct current (DC) of 1 or 2 μ A applied between an electrode at the top of the tobacco callus and another in the culture medium gave 5-fold increases in plantlet regeneration (RATHORE et al., 1988). Fresh weight and dry weight were significant increased when the callus was made negative to the medium. WARD (1996) investigated the influence of direct electric current (+6 V, -6 V, +12 V, -12 V) on tomato plants which were grown hydroponically and using wires as electrodes. The results showed that for all measurements treatment of 6 V downwards increased the total dry weight, while treatment of 6 V upwards led to an improved uptake of magnesium, calcium, and nitrogen. The highest uptake of calcium was achieved by the treatment (12 V upwards).

Most of the previous research results showed that electricity can stimulate physiological processes in plants. Pulsed electric fields may activate as well plant stimulus. YE et al. (2004) treated cell cultures of *Taxus chinensis* with a pulsed electric field (50 Hz, 10 V / m) and found that production levels of reactive oxygen species and phenolics were increased. However, information on changes in phenolic compounds and antioxidant activity in greenhouse crops as affected by small electric currents are scanty. Therefore, in the present study the effect of different intermittent-direct-electrical-currents (IDC) on phenolic compounds and antioxidant activity was investigated in different morphological compartments of radish plants. Furthermore, the aim of the study was to emphasis on the integration of electricity in greenhouse production, and thus to increase health-promoting phytochemicals in vegetables.

Material and methods

Plant material and experimental setup

Radish plants, *Raphanus sativus* L. cv 'Riesenbutter', were cultivated in rock wool substrate under climate chamber conditions. The temperature was maintained at 21.5 °C and the relative humidity was set at 70 %. Assimilation lighting was supplied by 5 Philips SONT-T AGRO 400 W high pressure sodium lamps, installed one meter above the plants, and connected for 16 hours per day. To investigate the effects of IDC on phenolic compounds and antioxidant activity in radish, the plants (n=72) were grown in two separate substrate rows each with 3 rock-wool cubes (one rock-wool cube: length = 100 cm; width = 20 cm; height = 8 cm). One of the two substrate rows was added left and right with stainless steel plates (length = 300 cm; width = 0.1 cm; height = 8 cm) which were fitted tightly on the rock-wool tubes and acted as electrodes to which a power supply has been connected (Fig. 1 picture climate chamber). Furthermore, the used circulating nutrient solution was a good electric conductor whereby the IDC passed horizontally through these as well as through the radish plants. The nutrient solution was given *via* drip irrigation one minute per hour and sixteen times per day (Tab. 1).

The experiment was conducted with 3 treatments of different electrical currents and with two replicates per treatment. In the first inves-

tigation an IDC of 200 mA was applied to radish plants during the growth period of 16 days, which was increased to 600 mA and to 1000 mA in the following investigations. In all cases the radish plants were subjected to IDC for one hour per day. Non-treated radish plants were used as control.

A laboratory power supply was used for a constant electric current during the growth period (Voltcraft, VLP 1303 pro; Hirschau, Germany). In addition, three temperature sensors were positioned in each of the two separate substrate rows to exclude a temperature effect, which could be caused by electric currents, on the phenolic compounds in radish. The temperature in the rock-wool cubes, the voltage, and the electrical current were recorded by a data analyzer (Fluke, Hydra 2620 A; Kassel, Germany) and relayed to a specific computer program, developed at Humboldt-Universität zu Berlin using visual basic 6.0.

After 16 days of plant development, 12 radish plants per substrate row and treatment were randomly harvested and divided into three segments (root, tuber, shoot). The segments of radish plants were shock-frozen in liquid nitrogen, kept at -20 °C and afterwards freeze dried for 48 hours (Christ Alpha 1-4, Christ; Osterode, Germany). Dried samples were grinded and mixed to calculate the ratio of dry matter (DM) and water content, for subsequent extraction, and were used for the determination of total phenolic content, anthocyanins and antioxidative activity.

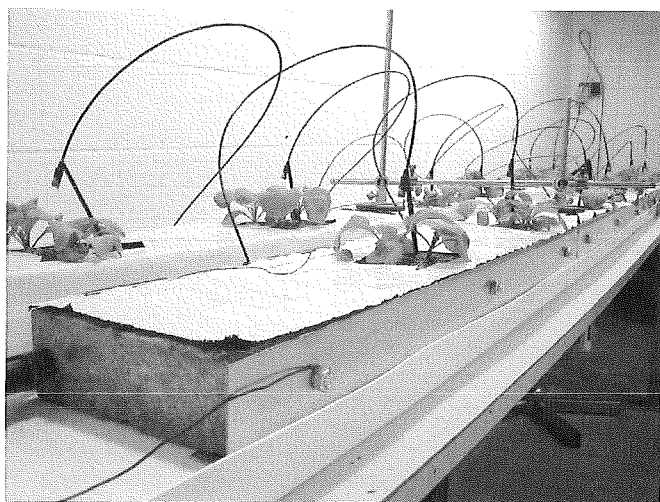


Fig. 1: Experimental setup in the climate chamber.

Tab. 1: Applied nutrient solution

| Mixture | [g / 100 L H ₂ O] |
|---|------------------------------|
| Macronutrient | |
| Ca(NO ₃) ₂ | 47.2 |
| KNO ₃ | 27.4 |
| Fe-EDDHMA (6% Fe) | 2.30 |
| MgSO ₄ | 42.0 |
| KNO ₃ | 27.4 |
| KH ₂ PO ₄ | 16.8 |
| Micronutrient | |
| Na ₂ B ₄ O ₇ | 0.095 |
| CuSO ₄ | 0.008 |
| MnSO ₄ * H ₂ O | 0.156 |
| (NH ₄) ₆ Mo ₇ O ₂₄ * 4H ₂ O | 0.010 |
| ZnSO ₄ 7*H ₂ O | 0.087 |
| electric conductivity = 2.0 dS/m; pH = 6 | |

Chemical analysis

Extraction of phenolic compounds

Three replications of the dried samples were used for the analysis of phenolic compounds in the plant segments and each extraction was determined in duplicate. The samples were extracted according to the method of CONNER et al. (2002) using acidified methanol (0.1 % (v/v) HCL-hydrochloric acid). An aliquot of 0.2 g of grinded shoots and tubers as well as 0.02 g of grinded roots were mixed with 5 ml acidified methanol, afterwards shaken and centrifuged for 15 minutes at 4000 rpm. The supernatant was collected in a 10 ml volumetric flask and the plant residue was extracted again with 5 ml acidified methanol and centrifuged for 15 minutes. The supernatants were collected in the same volumetric flask and filled up to a final volume of 10.0 ml. The extracts were used for the determination of the total phenol content in all segments of the radish plant and for the determination of anthocyanins and antioxidative activity, especially in the tuber of radish.

Analysis of the total phenol content

The total phenol content in the shoot, root and tuber extracts were determined using Folin-Ciocalteu method (SLINKARD and SINGLETON, 1977). First the extracted samples were diluted with distilled water (shoot 1:3; tuber 1:2; root 1:1). One ml of the diluted extracts was filled in a 10 ml tube and was supplemented with 5 ml of working solution (100 ml of 2 % [w/v] sodium carbonate, 800 mg potassium sodium-tartrate added with 2 ml of 0.5 % [w/v] copper sulphate), followed by the addition of 0.5 ml of Folin-Ciocalteu's phenol reagent diluted 1:3 with distilled water. Subsequently, the sample solutions were mixed with a vortex mixer developing a dark blue color during the reaction time. After a reaction time of one hour, the samples were transferred into glass cuvettes, and shortly thereafter the absorbance of the samples was measured spectrophotometrically at 765 nm (Spectrophotometer, Model 690, Gamma Analysen Technik GmbH; Bremerhaven-Lehe, Germany).

Gallic acid was used as a standard (Riedel-de Häen, 27645) and the total phenol content was expressed as milligram equivalents gallic acid per gram of dry matter (GAE mg / g DM).

Determination of anthocyanins

The content of anthocyanins in the tuber extracts was measured spectrophotometrically (Spectrophotometer, Model 690, Gamma Analysen Technik GmbH; Bremerhaven-Lehe, Germany) at a wavelength of 510 nm. The determination of anthocyanins was conducted following the pH differential method described by WROLSTAD (1976) and GIUSTI (1999). This method uses the colour changes of anthocyanins at a corresponding acidity (at pH 1.0, anthocyanins exist in the colored oxonium form whereas at pH 4.5 they are present predominantly in the colorless carbinol form). Therefore an aliquot of extracted sample was adjusted to pH 1.0 and another aliquot to pH 4.5 (1:59). The extinction coefficient and molecular weight of cyanidin-3-glycoside (26900 L/cm²/mol; 449.2 g/mol) were used for the calculations. The results were expressed as milligrams cyanidin-3-glycoside per gram dry matter (mg Cy-3 / g DM).

Determination of the Trolox Equivalent Antioxidant Capacity (TEAC)

The antioxidant activity in tuber extracts was monitored using the Trolox equivalent antioxidant capacity (TEAC) assay and was measured spectrophotometrically (Spektralphotometer, Model 690, Gamma Analysen Technik GmbH; Bremerhaven-Lehe, Germany) at 734 nm (modified by ROHN et al., 2004). A 500 µl aliquot of working solution ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt, 0.5 mmol/L in phosphate buffer, pH 7.2, Fluka 11557) was pipetted to 100 µl of the tuber extract, or instead the blank value of phosphate buffer, in glass cuvettes. To start the reaction, 200 µl of potassium persulphate (10 mmol / L in phosphate buffer, pH 7.2) were pipetted to a sequence of 8 samples (by duplicate) in a regular interval of 20 seconds. The mixture developed a dark green color during the incubation time of 6 minutes. After exactly 6 minutes, the cuvettes were measured spectrophotometrically in the same regular 20 seconds intervals. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard. In this context, the described procedure was also conducted for the Trolox calibration curve. Therefore, 10 different concentrations of Trolox, ranging from 0.05 to 4.5 mmol / L in phosphate buffer (pH 7.2), were used. Results were expressed as millimol Trolox per milligram dry matter (mmol Trolox / g DM).

Statistical analysis

The effects of IDC on polyphenols and antioxidant activity in radish were evaluated using analysis of variance with SPSS 11.5. Significant differences were calculated using *t*-test (*p* < 0.05). The mean variability was indicated by the standard deviation.

Results and discussion

In the present study the effect of IDC on polyphenols and antioxidant activity in radish was investigated during growth. High temperature is a source of environmental stress (EVERS, 1994) and influences the total phenol content in fruit (BERGQVIST et al., 2001). Therefore, it is important to set an equal temperature profile in the substrate of the control plants as well as in the substrate of IDC. The investigations have shown that the IDC treatments did not influence the temperature of the substrate (Fig. 2) and thus, excluded a temperature effect on the phenolic compounds in radish. Furthermore, the voltage increased with increasing IDC (Fig. 3) and increased from 4 V (200 mA) to 8 V (1000 mA). The voltage was constant during the respective IDC treatment and led to the conclusion that the circulating nutrient solution applied had been a good electric conductor. It was calculated that an applied IDC of 200 mA implicated an IDC of 104 µA / cm² stainless

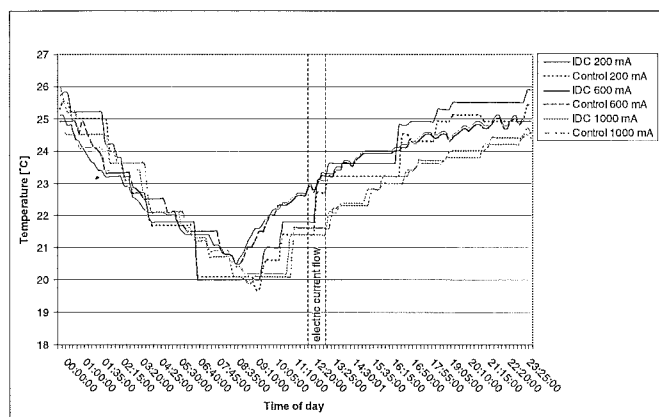


Fig. 2: Temperature profile in the rock wool substrate influenced by an IDC of 200 mA, 600 mA, and 1000 mA.

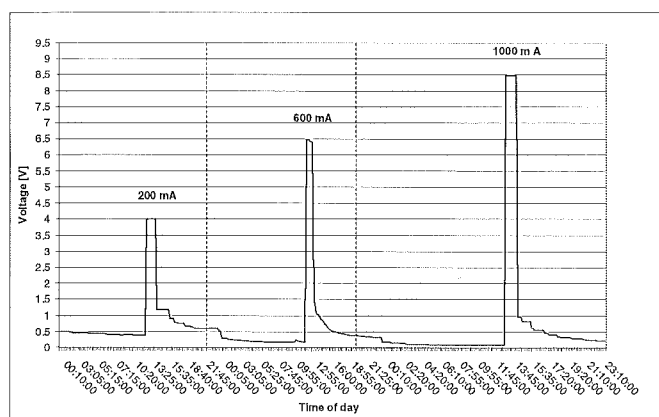


Fig. 3: Average voltage profile (16 days) influenced by an applied IDC of 200 mA, 600 mA, and 1000 mA.

steel plate and increased to 250 µA / cm² (600 mA) and 417 µA / cm² (1000 mA) in the following IDC treatments. From this it is concluded that a weak IDC passed horizontally through the nutrient solution as well as through the tissue of the radish plants. The results obtained are consistent with other studies on the application of electrical current (HERDE et al., 1995; WARD, 1996).

At the end of each IDC treatment period, the plants were healthy with no signs of damage. The IDC treatments did not influence the germination rate, plant growth, and their direction of growth (data not shown). In contrast, SMITH et al. (1993) found that magnetic fields (24 h per day) delayed the germination of radish plants, however, plant growth proceeded thereafter rapidly. MINA et al. (1991) applied a transverse positive direct electrical current from a point source to tobacco cells and found that cells re-polarized transversely with a greater proportion of its transcellular currents flowing in the direction of the current applied.

The total phenol content (4.7 - 5.9 mg / g DM), anthocyanin content (0.62 - 0.74 mg Cy-3 / g DM), and antioxidant activity (0.016 - 0.022 mmol Trolox / g DM) in radish tuber was consistent with results reported in the literature (GIUSTI and WROLSTAD, 1996; PELLEGRINI et al., 2003; BACCHIOCCA et al., 2006). The biosynthesis of phenolic compounds was affected by all IDC treatments. An IDC of 200 mA resulted in a significant increase of total phenol content in roots, but no significant differences were found in shoots and tubers of radish plants (Fig. 4). The results changed with increasing IDC as presented in Fig. 5 and 6. The biosynthesis of phenolic compounds in roots and

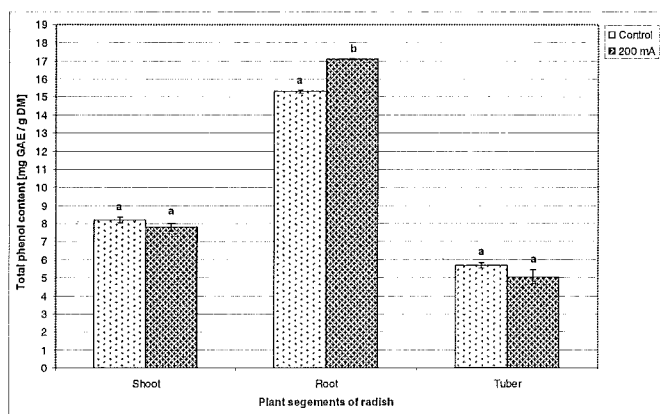


Fig. 4: Effects of IDC treatment (200 mA) on the total phenol content of different plant segments of radish (n=72). Different letters indicate significant differences $p < 0.05$ (*t*-test).

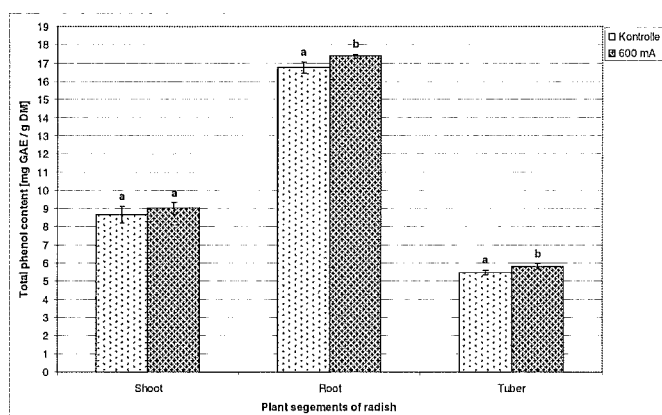


Fig. 5: Effects of IDC treatment (600 mA) on the total phenol content of different plant segments of radish (n=72). Different letters indicate significant differences $p < 0.05$ (*t*-test).

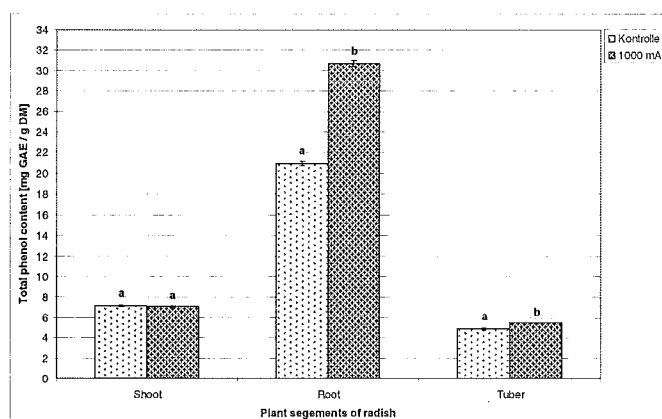


Fig. 6: Effects of IDC treatment (1000 mA) on the total phenol content of different plant segments of radish (n=72). Different letters indicate significant differences $p < 0.05$ (*t*-test).

tubers was promoted by the IDC treatments of 600 mA and 1000 mA. In both cases, the amount of total phenols in roots and tubers significantly increased. An IDC treatment of 600 mA increased the

total phenol content in roots (3.8 %) and in tubers (6.2 %) compared to the control plants. The highest total phenol content was determined in radish roots treated with 1000 mA IDC and drastically increased (46 %) compared to the non-treated plants. However, in radish tubers the total phenol content increased by 12.8 % and was 2-fold higher than the 600 mA IDC treatment. No IDC treatment affected the total phenol content in radish shoots. The outcome of this result is that IDC influenced the secondary metabolism resulting in an accumulation of various phenolic compounds especially in plant segments which were positioned in the current flow. In an other electric current study YE et al. (2004) treated cell cultures of *Taxus chinensis* with a pulsed electric field (50 Hz, 10 V / m) and found an intracellular accumulation of a bioactive secondary metabolite (taxuyunnanine C) which was increased by 30 % without loss of biomass. Production levels of reactive oxygen species and phenolics were increased too, whereas cell capacitance was decreased. Inaba et al. (1995) investigated the effects of a direct electrical current on the ethylene biosynthesis in fruit segments of cucumber. The ethylene production was rapidly induced by application of 3 mA (1 h) and was greater with a stronger current. Furthermore, in previous studies BRATTON and HENRY (1977) found that an applied direct electrical current increased the content of indol acetic acid, a phytohormone from the group of auxins, in the petiole and leaves of tomato plants.

All these investigations used a different type of electrical stimulus than that of our study but they revealed an effect on the primary and secondary metabolism, which also supports our observation on the stimulation of the biosynthesis of phenolic compounds in radish. Assumingly, the change in secondary metabolism is a stimulus response to IDC and is initiated when the plant recognises a stimulus at the cellular level (GASPAR et al., 2002). Second messengers (e.g. Ca^{2+}) are the transducer of information from membranes to metabolism (NILSEN and ORCUTT, 1996). Calcium as a divalent cation is an intracellular messenger in the cytosol and enters plant cells through Ca^{2+} -permeable ion channels in their plasma membranes (WHITE, 2000). It might be possible that IDC increased the membrane permeability for Ca^{2+} which has a large impact on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). A rapid influx of Ca^{2+} through cation channels generates perturbations in the $[Ca^{2+}]_{cyt}$ and initiates cellular responses (SANDERS et al., 2002). To respond appropriately to a $[Ca^{2+}]_{cyt}$ perturbation, a cell has to activate a unique combination of Ca^{2+} -binding proteins, such as calmodulin (CAM), calcineurin B-like proteins (CBLs) and Ca^{2+} -dependent protein kinases (CDPKs) (FOX and GUERINOT, 1998). ALLWOOD et al. (1999) found in French bean CDPK phosphorylated phenylalanine ammonia-lyase (PAL) which was associated with stress signalling processes (CHENG et al., 2001). It is known that PAL is a key step enzyme in the biosynthesis of phenolic compounds. HEINZMANN and SEITZ (1974) found a high correlation between anthocyanin synthesis and activity of PAL in callus cells of *Daucus carota*. Thus, it is assumed that the phenolic compounds in radish segments were increased by an increase in PAL activity being promoted by IDC in this context.

A significant increase of anthocyanin content in radish tubers was found in the IDC treatments of 600 mA and 1000 mA (Fig. 7). In both cases, the anthocyanins were increased by approximately 28 % compared to the control plants and could not be further increased by a higher IDC. The correlation between total phenol content and anthocyanins revealed a high correlation ($r = 0.74$, $p \leq 0.05$) in respect to an IDC treatment of 600 mA, however it was higher at a higher IDC of 1000 mA ($r = 0.906$, $p \leq 0.01$).

The antioxidant activity as measured by the TEAC assay is presented in Fig. 8. Based on the fact that only high IDC of 600 mA and 1000 mA increased the total phenol and anthocyanin contents in radish tubers, the antioxidant activity in tuber extracts was studied only for these treatments. The highest increase of antioxidant activity

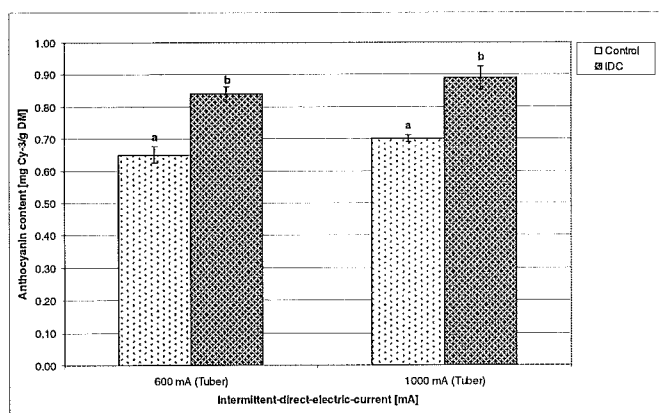


Fig. 7: IDC induced changes of anthocyanin content of radish tuber (n=72). Different letters indicate significant differences $p < 0.05$ (*t*-test).

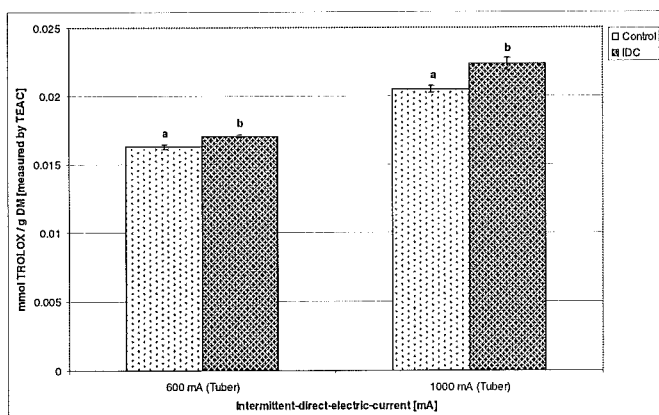


Fig. 8: IDC induced changes of antioxidant activity in radish tuber (n=72). Different letters indicate significant differences $p < 0.05$ (*t*-test).

(9.1 %) was measured at 1000 mA and was significantly increased in comparison to the control plants. However, the treatment with 600 mA IDC promoted the antioxidant activity to a slightly lower extent (4.5 %). A strong correlation between antioxidant activity and the total phenol content as well as anthocyanins were found: total phenol content at 600 mA IDC ($r = 0.97$, $p \leq 0.01$), anthocyanins at 600 mA ($r = 0.84$, $p \leq 0.05$); total phenol content at 1000 mA IDC ($r = 0.97$, $p \leq 0.01$), anthocyanins at 1000 mA ($r = 0.92$, $p \leq 0.01$). It is possible that the anthocyanins might have affected the antioxidative activity, however, other substances than anthocyanins, e.g. other phenolic compounds or glucosinonates might have a greater impact on the antioxidant activity in radishes. That is due to the nearly same differences of anthocyanins between the different IDC treatments and the non-treated plants. The radish tuber consists of different phenolic compounds such as hydroxycinnamic acid (e.g. p-coumaric acid, caffeic acid, and ferulic acid) and flavonols e.g. kaempferol (HERRMANN, 1976; BILYK and SAPERS, 1985). RICE-EVANS et al. (1996) found a lower antioxidant activity of these phenolic compounds in comparison to anthocyanins (cyanidin). However, the sum of all mentioned phenolic compounds increased the antioxidant activity and thus, phenolic compounds were promoted by an increasing IDC.

In further studies the emphasis will also be focussed on the analysis of micronutrients in plant segments as it is known from the literature that micronutrients are influenced by electrolysis of metal electrodes (RAJDA, 1986).

Conclusion

The results of the present study demonstrate that the synthesis of phenolic compounds and associated antioxidant activity of radish tubers can be stimulated by an intermittent-direct-electric-current during the growth period. This new technology increased the health-promoting phytochemical compounds in radish segments and produced plants with functional properties revealing no sign of damage. However, the phenolic compounds in radish shoots remained unaffected by IDC. In further studies the effects of IDC on the secondary metabolism will be investigated in more detail.

Acknowledgements

The authors thank particularly Wolfgang Pfeifer for his valuable support during experiments.

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