Cadmium- and Flood-Induced Anoxia Stress in Pea Roots Measured by Electrical Impedance

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Electrical impedance measurement – complex resistance in the presence of alternating current – is a useful tool for the investigation of structural characteristics of solid materials but also for plant tissues. This measurement is easily done: only two electrodes are inserted into the plant tissue, so it can be considered as a non-invasive technique and it may be a successful method for detecting structural changes in plants caused by environmental stresses. The effects of flood and cadmium stress were investigated by electrical impedance measurement, because both of them cause structural changes in plant tissues. Apoplasmic resistance (*Ra*), symplasmic resistance (*Rs*), and membrane capacitance (*Cm*) of pea roots were calculated. In the first five days of flood treatment, the *Rs* and *Cm* values of roots decreased drastically. In case of cadmium treatment, the *Rs* and *Cm* values of roots showed an increasing tendency supposedly as a consequence of the enhanced membrane rigidity, the thickened cell walls and decreased growth phenomena caused by the heavy metal. There also was a remarkable difference in cadmium accumulation patterns and in the changes of the calculated parameters amongst anoxic and aerated seedlings. This initial work revealed that the development of stress caused by two environental stress agents, cadmium and flood, can be followed by electrical impedance measurement.

Key words: Cadmium, Electrical Impedance Spectroscopy

Introduction

Electrical impedance is the complex resistance in the presence of alternating current, and can be a useful tool for the investigation of structural characteristics of solid materials and also animal and plant tissues (Grimnes and Martinsen, 2000). The resistance and the capacitance of different cellular structures are represented by the elements of linear equivalent circuits (Hayden *et al.*, 1968; Zhang and Willison, 1991; Privé and Zhang, 1996).

Different models have been created in order to approximate cellular compartments: the Hayden model, in which the apoplasmic (*Ra*) and the symplasmic (*Rs*) resistance are calculated (Hayden *et al.*, 1968); the modified Hayden model also considers the cell membrane capacitance (Vozáry *et al.*, 1999); and the "double shell model" includes the interior resistance of vacuoles and the plasma membrane capacitance as well (Zhang and Willison, 1991).

Several stress-induced alterations have already been followed in plants by electrical impedance spectroscopy (EIS). During dehydration of potato (*Solanum tuberosum* L.) and carrot (*Daucus carota*) pieces, the ratio between *Rs* and *Ra* decreases (Toyoda *et al.*, 1994). Cold acclimation was found to enhance the impedance of alfalfa

Abbreviations: *Ra*, apoplasmic resistance; *Rs*, symplasmic resistance; *Cm*, membrane capacitance; *T*, tracheae number; *VC*, vascular cylinder diameter; *TxVC*, tracheae number · vascular cylinder diameter; EIS, electrical impedance spectroscopy.

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(*Medicago sativa* L.) (Hayden *et al.*, 1968). Greenham *et al.* (1972) found correlation between the phase angle data and rate of aging of the plasma membrane in the control and calcium- and phosphorous-deficient subterranean clover (*Trifolium subterraneum* L.). Membrane injuries caused by temperature extremities can also be followed by EIS (Repo *et al.*, 2000). A linear correlation was found between intracellular resistance and frost hardening capability of Scots pine (*Pinus sylvestris* L.) (Repo *et al.*, 2000). Prediction of mechanical destruction (Vozáry *et al.*, 1999), drying of apple slices (Mészáros *et al.*, 2005), and germinability of soybean and snap bean seed are also possible with EIS (Vozáry *et al.*, 2007). The magnitude of environmental stress factors originating from climatic changes or from polluting agents has increased, of which heavy metal stress is among the most harmful ones (Tuba and Csintalan, 1992). Cadmium initiates serious structural and functional disorders in plants (Tuba and Csintalan, 1992). It enters the root passively through the cortical tissue, and actively by H^+-ATPase (Costa and Morel, 1994) through ion channels of nutrients, such as Ca^{2+} (Greger and Bertell, 1992), and reaches the root xylem by apoplasmic and/or symplasmic transport (Salt and Wagner, 1993). In the presence of cadmium cell division does not stop but enhances the rigidity (*i.e.* decreases of elasticity) of root cell walls (Degenhardt and Gimmler, 2000) that inhibits cell expansion (Prasad, 1995). Cadmium lowers the content of unsaturated fatty acids in cell membranes of tomato plants (Ouariti *et al.*, 1997), so cell and vacuolar membranes become rigid (Ros *et al.*, 1992).

The problem of flood (Tuba, 2005) on arable land is another serious environmental stress. Flood excludes air from soil causing anoxic conditions (*i.e.* total lack of soil oxygen), and plant respiration switches from aerobic to anaerobic, creating inefficient energy supply, which finally leads to nutrient deficiencies (Niki and Gladish, 2001).

A literature search revealed that EIS has already been successfully used for the detection of tissue structure changes caused by environmental stresses in plants. Till now there is not any work done on impedance spectra measured in plants exposed to either cadmium or flood stress. This encouraged us to follow these changes microscopically and determine the impedance of pea seedlings grown in cadmium-contaminated aerated and non-aerated hydroponics, as the simulation of flood stress. We expected an alteration of the resistance in both apoplasmic and symplasmic electrolyte systems and the membrane capacitance through which stress development can be followed by EIS that is a fast method carried out without grinding and processing of plant tissues.

Material and Methods

Plant material and growth conditions

Seeds of pea (*Pisum sativum* L. cv. 'Debreceni világos') were surface-sterilized in 3% (w/v) sodium hypochlorite, then rinsed several times, soaked and germinated. Three-day-old seedlings were transferred to half Hoagland solution: $Ca(NO_3)_2 \cdot 4\ H_2O$ (2.17 mM), KNO_3 (4.89 mM), KH_2PO_4 (1.5 mM), $MgSO_4 \cdot 7\ H_2O$ (0.134 mM), Na-FeEDTA (0.134 μM), H_3BO_4 (69.3 μM), $MnSO_4$ (13.6 μM), $ZnSO_4 \cdot 2\ H_2O$ (1.1 μM), $NaMoO_4 \cdot 2\ H_2O$ (0.15 μM), $CuSO_4 \cdot 5\ H_2O$ (0.48 μM) (Hoagland and Arnon, 1950), which was changed every third day. The seedlings were grown in a Conviron S10 type phytothrone (120 μmol m^{-2} s^{-1} light intensity, 22–23 °C, 85% relative humidity). The duration of light was 16 h/d.

Cadmium and anoxia treatments

Three-day-old seedlings, transferred to hydroponics, were treated with 0, 100 and 200 μM $CdCl_2$. Half of the containers of each cadmium treatment was constantly aerated and the other half was without aeration. Another batch of the three-day-old seedlings was transferred to hydroponics and treated with 100 μM and 200 μM $MgCl_2$ to investigate the effect of a non toxic, but also divalent metal ion. Each experiment was repeated three times and the averages are shown in graphs.

Morphological and light microscopical investigations

Morphological (root lengths) and tissue structure evaluations were carried out in parallel for each experiment, except in case of excess magnesium supply, where the treatment did not cause any observable difference in the morphology of seedlings or in the electrical impedance spectra. For light microscopical investigations, samples were collected from the epicotyl and the upper part of the primary root of the seedlings and fixed in 4% formaldehyde solution (in 0.1 M phos-

phate buffer, pH 7.2). After dehydration through a series of increasingly concentrated solutions of ethanol, as an intermediary solvent, benzene was used before paraffin embedding. Sections (10 micron) were prepared with a Leitz Wetzlar microtome and mounted on egg albumin covered with glass slides. Roots and epicotyls of 5 plants per treatment were sampled, from each of them 4 slides were prepared (25 sections per slides). Sections were stained with Bismarck brown and malachite green (Ruzin, 1999). Micrographs were taken with an Opton III photomicroscope.

Electrical impedance measurements

The impedance spectra of biological tissues can be modelled with equivalent circuits. In the simplest case, the measured impedance of tissue consisting of cells can be described with a lumped model containing discrete resistors and capacitors (Hayden *et al.*, 1968; Zhang and Willison, 1991). These resistors and capacitors can represent the resistance and capacitance of cell compartments, and the electrical behaviour of tissue can be explained using the properties of these models (Grimnes and Martinsen, 2000).

The impedance spectra of roots and stem tissues were measured. Electrodes (copper covered with gold) were inserted into the stem 2 cm above the basis along the longitudinal axis or into the roots 2 cm below the basis along the longitudinal axis. The distance between the two electrodes was 2 mm. The size of electrodes was 0.35 mm in diameter and 5 mm in length. The magnitude (Z) and the phase angle (φ) of the complex impedance were measured in the frequency range 800 Hz to 1 MHz at frequency points of 100 by using a precision LCR meter type HP 4284A. The measuring device was calibrated by using OPEN and SHORT corrections. The input voltage level of the sine signal was 1 V. The corrected impedance spectra were approached with the modified Hayden model (Fig. 1) using the complex least square method. The resistance of extracellular space (apoplasm, *Ra*), the resistance of intracellular space (symplasm, *Rs*), and the cell membrane impedance (*Zm*) were evaluated. *Zm* can be expressed as follows:

$$Zm = \frac{\cos \psi + j \sin \psi}{Cm\omega},$$

where $j = \sqrt{-1}$, $\omega = 2\pi f$, *Cm* is the cell membrane capacitance, and ψ is a constant phase angle (Vozáry *et al.*, 1999).

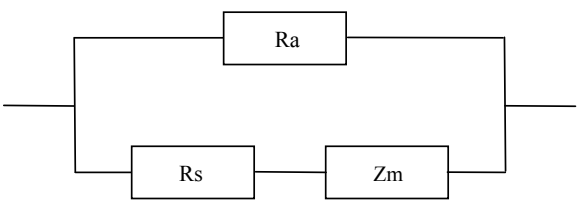

Fig. 1. Modified Hayden model, where *Ra* means the apoplasmic resistance, *Rs* means the symplasmic resistance, and *Zm* means the cell membrane impedance.

Cadmium content measurements

0.2 g dried (105 °C, 24 h) roots were ground in an 1:1 mix of hydrogen peroxide and nitric acid, and left at room temperature overnight. Next day samples were boiled in teflon bombs for 30 min, then filtered and diluted with distilled water to 10 ml (Horváth *et al.*, 1996). The concentration of cadmium ions was measured by an ICAP-61E type plasma emission spectrometer.

Statistical analysis

The values are expressed as the mean ± SD of three independent replicates of each experiment. In each experiment the impedance was measured on 15–20 seedlings in each of the different treatments. The significance for the differences of the impedance parameters between control, cadmium- and flood-treated plants was determined by ANOVA ($p > 0.05$) using the SPSS 7.0 program.

Results

Changes in the cadmium content

The plants were able to accumulate the maximum amount of cadmium according to their potential and the applied concentration all the time, since the cadmium content of the solution was constantly in excess. The cadmium content of all the treated seedlings, except the controls, rose constantly proportional to the applied cadmium concentration throughout the duration of the experiment (Fig. 2). The rate of accumulation was an order of magnitude higher in roots (Fig. 2A) than in shoots (Fig. 2B). It should be noted that the cadmium content of aerated roots treated with 100 μM cadmium was approximately three-fold higher than in the non-aerated roots.

The 200 μM cadmium concentration resulted in an even higher accumulation than the 100 μM treatment, but there was no statistically significant difference between the extent of accumulation with or without aeration. Since the majority of cadmium remained in the roots (Prasad, 1995), only the changes occurring in the roots will be discussed below.

Morphological changes of pea roots

Measurement of root length, as a sensitive cadmium stress-indicating parameter (Lima *et al.*, 2006), showed that after cadmium treatment the length of roots (Fig. 3A) remained constant (*i.e.* there was no growth). It was observed that the lengths of roots of aerated seedlings were always

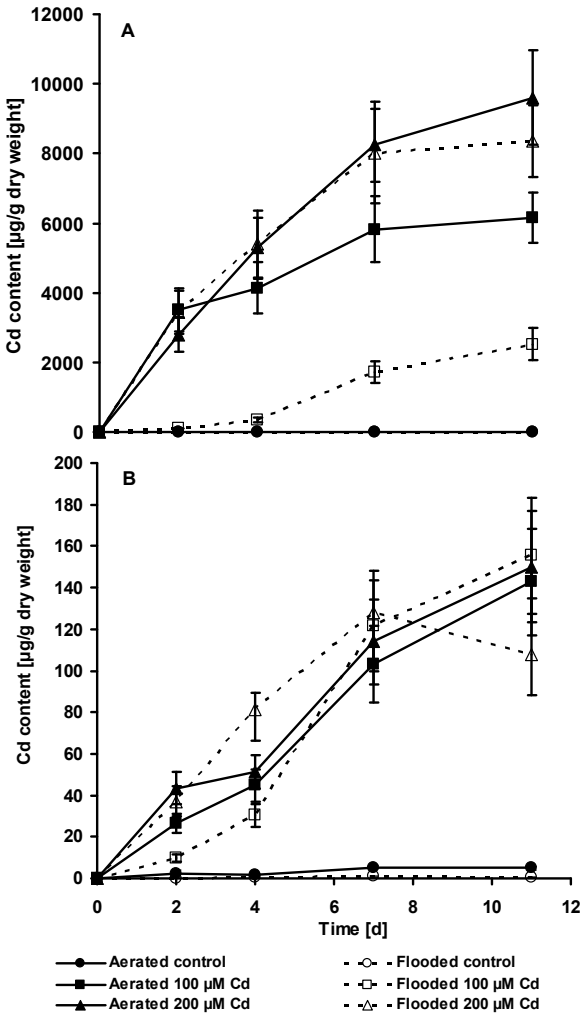

Fig. 2. Cadmium content of (A) roots and (B) shoots of pea seedlings. Three-day-old seedlings were transferred to half Hoagland solution and were treated with different cadmium concentrations with or without aeration. 0, 2, 4, 7, 11 days indicate the sampling dates after the cadmium treatment of aerated and non-aerated (flood-treated) seedlings. Error bars represent SD (n = 15–20).

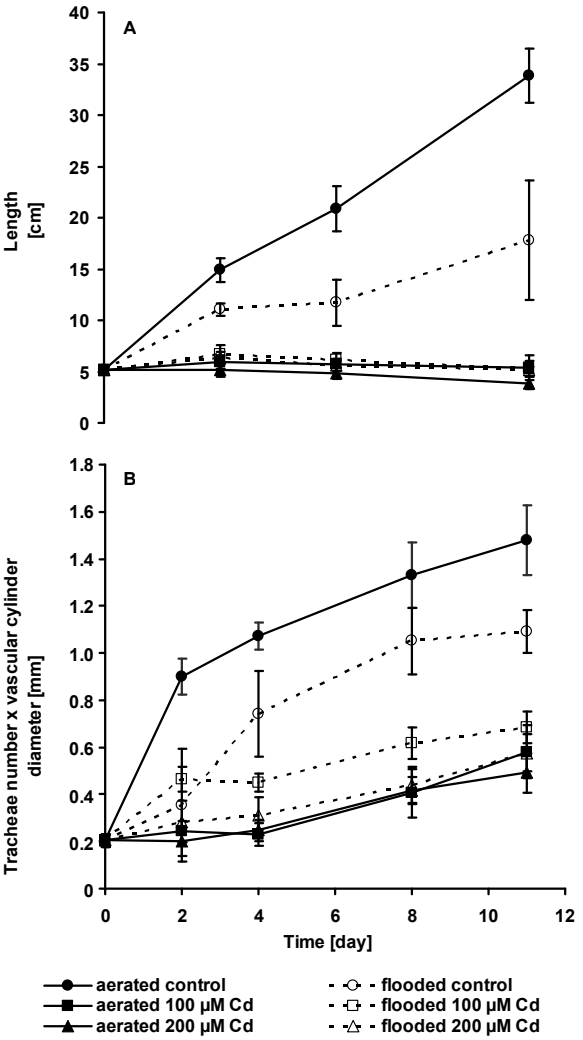

Fig. 3. (A) root length and (B) root tracheae number · vascular cylinder diameter of pea seedlings. Error bars represent SD (n = 15–20).

longer than of those without aeration. The aerated control roots were the longest, more than twice as long as the control roots and approximately seven times longer than the cadmium-treated roots.

Results of the microscopical investigations

The cross sections were made in order to show the changes of the vascular cylinder of the root over time. The state of the vascular cylinder can be characterized by the number of tracheae and size of the vascular cylinder; so the product of the tracheae number (*T*) and the diameter of the vascular cylinder (*VC*) was calculated as an informative parameter of the vascular cylinder (Fig. 3B). The total area of tracheae elements of the cross sections in flooded pea seedlings (with or without aeration) was three times larger than in the cross sections of cadmium-treated seedlings. The inhibition of the development of tracheae elements and the whole vascular cylinder also showed a concentration dependency; thus the seedlings treated with 200 μM cadmium had the lowest value of the *TxVC* parameter.

Changes in the electrical impedance pattern

The *Ra*, *Rs* and *Cm* parameters were calculated from the locus curves (*R-X*) (Fig. 4). The symplasmic and apoplasmic resistances give information about the inner and outer cell status, respectively, while the membrane capacitance provides an insight into the membrane status of the cell.

Since it is known that the ion content alone also has an effect on impedance parameters, first, we intended to investigate the effects of a non-toxic, but also divalent metal ion (magnesium), applied in the same concentration, on the *Rs* and *Ra* values (Table I).

As it is clearly shown in the graphs the *Rs* and *Ra* values of 100 μM and 200 μM magnesium-treated seedlings did not show pronounced differences from those of the controls.

The *Rs* of roots of hydroponically grown seedlings decreased continuously to one fifth of the starting value over the period of our experiment (Table I). The *Rs* values of cadmium-treated seedlings were notably higher than those of the flooded seedlings either with or without aeration. The changes of the *Rs* values showed a concentration dependency, since the seedlings, which were treated with 100 and 200 μM cadmium, had higher *Rs* values compared to the controls, and the 200 μM treatment resulted in the most pronounced changes (Table I).

The *Rs* values of the 200 μM Cd-treated, aerated roots were consistently about 1.5 times higher than the *Rs* values of the flooded and 200 μM Cd-treated seedlings throughout the experiment.

The *Ra* of pea seedlings showed a decrease with time, and all values decreased to the half of their original value by the end of the experiment. The *Ra* values of all the cadmium-treated seedlings were higher than those of the flooded ones (Table I).

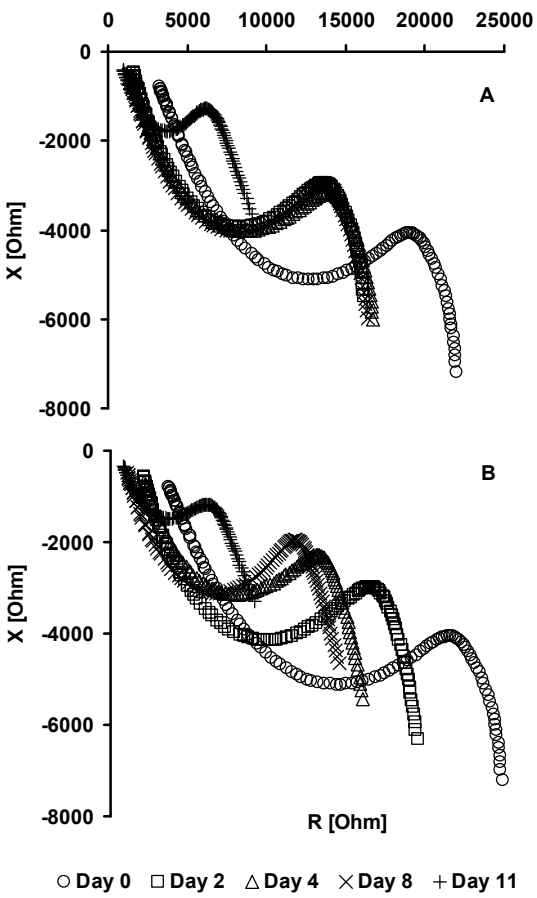

Fig. 4. Locus (*R-X*) curves of (A) aerated and (B) flooded controls. Real (*R*) and imaginary (*X*) parts of the measured impedance (*Z*). The R-X curves are illustrations of typical curves derived from the measured impedance spectra, from which the apoplasmic resistance, the symplasmic resistance and the membrane capacitance were calculated.

Table I. The effect of flood, cadmium and magnesium treatment on the *Ra*, *Rs* and *Cm* values of pea seedlings. Values designated by the same lower case letters did not differ significantly from one another in the Duncan multiple comparison test at the $p < 0.05$ level.

Treatment	*Ra*			*Rs*			*Cm*		
	Day 0	Day 4	Day 11	Day 0	Day 4	Day 11	Day 0	Day 4	Day 11
Aerated control	25.121±3.785a	20.052±1.972c	11.591±1.914a	2.780±0.285a	1.123±0.167a	0.651±0.059a	10.685±1.051a	2.691±0.171a	1.286±0.118a
Flooded control		17.302±2.910b	11.660±1.965a		1.089±0.157a	0.471±0.078b		3.054±0.343b	1.118±0.171b
Aerated 100 μM Cd^{2+}		17.630±1.90b	12.726±1.838b		1.235±0.176a	0.998±0.152c		5.395±0.359e	2.421±0.223c
Flooded 100 μM Cd^{2+}		14.226±2.095a	13.902±2.642c		1.235±0.157a	0.906±0.142d		3.628±0.171c	2.575±0.153d
Aerated 200 μM Cd^{2+}		20.029±1.373c	13.747±1.742c		2.206±0.152b	2.458±0.042e		7.027±0.221f	9.093±0.293e
Flooded 200 μM Cd^{2+}		14.654±1.487a	14.305±1.134c		1.064±0.052a	1.285±0.190f		4.994±0.453d	3.735±0.342f

Treatment	*Ra*			*Rs*			*Cm*		
	Day 0	Day 4	Day 11	Day 0	Day 4	Day 11	Day 0	Day 4	Day 11
Flooded control	21.380±1.457a	19.667±0.577c	13.343±0.385a	2.440±0.266a	1.580±0.223a	1.400±0.187a	10.249±0.451a	3.110±0.190a	1.431±0.172a
Flooded 100 μM Mg^{2+}		14.953±0.318b	13.423±0.365a		1.623±0.375a	0.997±0.127a		2.847±0.180a	1.248±0.126a
Flooded 200 μM Mg^{2+}		12.087±0.346a	12.173±0.336a		1.257±0.182a	1.013±0.192a		3.550±0.337a	1.486±0.214a

The *Cm* of roots showed a drastic decrease during the first five days of the treatments; thereafter there was a difference between the control and the cadmium-treated plants: the decrease in the *Cm* values of flooded control plants continued until the final experimental day, whereas in the cadmium-treated ones, the decrease ceased or even a slight increase occurred (Table I).

The seedlings which were treated with cadmium had higher capacitance values than the flooded controls. The values appeared to be dependent on the applied cadmium concentration.

Statistical analysis confirmed the observed tendencies (Table I) and showed that the effect of the treatments was detectable from the fourth day of the experiment.

Discussion

The *Rs*, *Ra* and *Cm* values of roots of pea seedlings decreased as the result of flood stress. This may be the consequence of several flood-induced mechanisms. Water gets into plant roots mainly via the apoplasmic route, but it can also enter cells through aquaporins – cell membrane protein channels that allow the faster passage of water (Martinoia *et al*., 2000). The latter mechanism allows to increase the amount of water rapidly in flooded pea root tissues and this in turn increases tghe ion mobility, which is one of the main factors affecting plant tissue impedance (Vozáry *et al.*, 1999). Moreover, the natural growth phenomena of pea seedlings lead to the formation of tracheae, which may be considered as electrical conductors, and an increase in their diameter. If the cross section of an electrical conductor increases, the resistance becomes lower. The formation of tracheae elements from tracheids occurs through degradation of the longitudinal cell walls as well as of their cell membranes. That may be the reason why membrane capacitance drastically decreased during the experiment.

Additionally, sudden flooding induces the growth of vacuoles, which are also part of the electrical conductor system of the cells, and this can also contribute to decreased resistance and capacitance values (Niki and Gladish, 2001). Tissue impedance is closely related to both cellular ionic mobility (Vozáry *et al.*, 1999) and the diameter of electrical conductors. The changes in these two factors will also lead to a decrease of

symplasmic resistance and membrane capacitance values in the roots.

The magnesium treatment did not cause any difference in the impedance parameters. This indicates that the changes in symplasmic and apoplasmic resistance and membrane capacitance parameters were induced by the toxic effects of cadmium rather than the effect of increased ion content.

The results of the microscopic investigation and cadmium content determination showed that cadmium gets into the roots of pea seedlings easily and rapidly, causing almost complete inhibition of growth, which is one of the most obvious effects of heavy metal stress (Kabata-Pendias and Pendias, 1984). This inhibitory effect is realized not through blocking of the cell division, but through inhibition of the cell expansion (Prasad, 1995). Cadmium binds to cell wall pectins causing stronger bonding between cell wall components (Prasad, 1995), which in turn leads to a more compact tissue structure. From an electrical point of view, a compact tissue structure means decreased diameters of electrical conductors, which, contrary to the effect of water, results in the observed higher resistance and capacitance values. As the graph of root lengths (Fig. 3) shows the roots of cadmium-treated plants remained the same initial size or even slightly decreased in length during the experiment, because their growth was impeded by the heavy metal. As a consequence the inhibition of the development of root tissue structure, thus the elements of the vascular system, cannot evolve, and consequently degradation of longitudinal cell walls and cell membranes of the tracheids is not able to occur.

The composition of lipid membranes changes drastically due to cadmium stress: the amount of unsaturated fatty acids decreases in the membranes and cadmium can bind to several membrane proteins and inhibit their function (Ouariti *et al.*, 1997; Ros *et al.*, 1992), so that the whole membrane structure becomes rigid. This rigidity contributes to the increased membrane capacitance of cadmium-treated seedlings. Cadmium also greatly affects the water status of plants through degradation of root tip cells; water uptake becomes inhibited (Costa and Morel, 1994). The depressed water uptake and lower cellular water content lead to decreased ion mobility, which is also another factor resulting in increased resistance values. Most of the cadmium accumulates in roots, so its detrimental effects occur mostly in root tissues (Prasad, 1995). The cadmium-treated and also aerated seedlings showed higher symplasmic resistance and membrane capacitance values, which resulted from a combined effect of the treatments. It is known that flood-induced anoxia causes inhibition in the ion uptake, which is visible in the data on cadmium content, where the flooded, anoxic plants had less cadmium in their root tissue compared to the aerated ones. Since cadmium uses the same energy-requiring ion channels as calcium (Greger and Bertell, 1992) for its entrance into root tissue cadmium treatment causes more serious damages in aerated seedlings than in flooded ones, where the lack of an efficient energy supply partly inhibits the uptake of cadmium. The above-mentioned combinatorial effect of flooding and cadmium treatment was clear and statistically significant by the end of the experiment. This finding draws attention to the importance of aeration in experiments carried out using hydroponics, that are designed to study the elemental (either essential or toxic) uptake, utilization or toxicity, since, without aeration, anoxia may induce disorders in the normal ion uptake and mislead the results.

Considering all these observations we conclude that parameters calculated from the measured impedance spectra constitute a non-invasive method by which it is possible to follow the changes of pea seedlings' tissue structure caused by cadmium, as well as by simulated flooding. The interactive effects of the two stressors are also possible to distinguish. In addition, they fulfil important criteria, such as the measurement being carried out rapidly, as it was earlier reported in cold acclimation investigations (Repo *et al.*, 2000), and not requiring the processing of plant tissues. Furthermore electrical impedance measurements can be done on relatively young seedlings, so that the effects of stress agents can be detected even in the early stages of the development before visible symptoms occurr. In this developmental stage, highly sensitive environmental stress detecting methods, for example fluorescence induction measurements cannot be realized because of the lack of fully opened leaves. The method of electrical impedance measurement can be suitable for the detection of cadmium- and flood-induced plant tissue structure changes through the alteration of *Ra*, *Rs* and *Cm* parameters even in the very early stage of stress evolution.

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