PhD Theses

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The selection of parameters applicable for the early phase of heavy metal induced stress detection in plants

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1. Introduction and objectives

1.1 The actuality and importance of the topic

Environmental pollution has been tremendously increasing on Earth due to human activity, thus investigation of the detrimental effects is highly important as well as the naturalization and elimination of them. The contaminating agents released into the biosphere affect plants in more or less extent. Since polluting agents accumulate in plants, reaching human body through food chain and may cause poisoning, it is an important to recognize, investigate and elucidate the factors affecting plants and changes that these factors induce in plants.

Nowadays more scientific areas deal with the detection of environmental pollution (chemistry, biochemistry, physics, biophysics, molecular biology, genetics) moreover with the limitation and elimination of the detrimental effects. Thus it is reasonable to conduct an investigation using combined methods of different scientific areas in order to gain a more holistic picture of the subject of the investigation.

As a result of the increasing industrial activity, the number and the amount of poisoning agents increases continuously. These substances naturally occur in all parts of the biosphere, but since plants grow in soil, the poisoning agents of the soil stand in the focus of our research. Nowadays, the heavy metals, especially their presence in the soil is the greatest problem in this regard. Those heavy metals that interact with plants can be essential, meaning to be necessary for plant productivity, and can be non-essential meaning to be toxic for plant even in the slightest concentration. The two element categories mentioned above are different in terms of their effect, thus during plant physiological investigations it is important to compare their effects and distinguish among them. Nickel is an essential element, that causes toxic symptoms when in excess in plants. Cadmium however is one of the most toxic non essential element that gets into

the environment and into plants through mining and agricultural activities (farmyard manure, fertilizer, soil amending substances, plant protecting chemicals, sewage sludge, car tyres). As for the physiological effects of cadmium, its accumulation in plants cause serious metabolic thus developmental disorders that may result is less production.

The heavy metal tolerance of plants is greater than that of animals, so in case of nickel and cadmium poisoning, the amount accumulated may not be toxic for the plant, but when it reaches the animal body in a certain amount can be toxic.

Flooding occur more and more often nowadays that leads to the total or partial extrusion of oxygen from soils that has a detrimental effect on the plant. All the agricultural plants need oxygen in their root region. When plants do not get oxygen in sufficient amount, first they get into a declining state then perish as the result of the negative processes induced in oxygen deficient soil, such as surplus of carbon dioxide and toxic material. Taken notice of all the above mentioned facts, it is obvious that the investigation of stress factors and their effects is an important task of plant physiology.

It is reasonable to distinguish among different concentrations and among the occurrence of the reactions. The classical plant physiological measuring methods (chlorophyll content measurement, elemental composition measurement) are good tools of plant stress physiology, since they are suitable for the characterization of the physiological state of plants.

These methods usually require the grinding and processing of plants that makes the method invasive, so one plant can be investigated only once. Contrarily, the consecutive in vivo investigation of the same individual would lead to a more sophisticated picture about the reactions of a plant for a stress agent.

Besides, it is also important that the investigation cause the least possible .injury on plants, since injury itself could be interpreted as a stressor, thus this fact may question the punctuality of the measurements.

In modern plant stress physiological research, non invasive investigation methods (fluorescence induction, SPAD measurement, photosynthetic activity measurement, stomatal conductance measurement) are widely used.

Electrical impedance is the complex resistance in the presence of alternating current, and can be a useful tool for the investigation of structural characteristics plant tissues, without causing injury to the plant, since only two short and thin electrodes are inserted into the plant tissue. It is assumed that the methods mentioned above are capable of characterizing the physiological state of the plant, moreover with the help of which the early phase of stress evolution maybe more precisely described. This may also be important in terms of basic research and also the results provide practical guidance for plant genetics, phytoremediation and other areas of environmental protection.

1.2 The aim of the research

During my doctoral work I intended to carry out the followings:

1. Determining the constitutive amounts of five organic acids (oxalic acid, citric acid, malonic acid, succinic acid and fumaric acid) in barely seedlings.
2. Do the amount of the above mentioned organic acids change as a result of cadmium and nickel stress in the early (0-24 hours) and later (0-7 days) phase of stress evolution?

3. Determining the activity of GPX and APX enzymes as a result of cadmium and nickel stress in the early (0-24 hours) and later (0-7 days) phase of stress evolution.
4. Comparison of the changes of organic acid levels and antioxidative enzyme activities caused by nickel and cadmium, naming the differences between the two metal type (essential, non-essential), finding the most sensitive enzyme and organic acid in the early (0-24 hours) and later (0-7 days) phase of stress evolution.
5. Characterizing the effects of cadmium treatments in pea seedlings under different growing conditions (aerated and anoxic hydroponics) with electrical impedance measurement thus finding the ideal growing condition for carrying out EIS measurements.
6. Is there any effect of a physiologically neutral ion (magnesium) on the parameters of the electrical impedance measurement?
7. Is electrical impedance measurement suitable for the detection of cadmium stress?

2. Materials and methods

Barley seedlings (*Hordeum vulgare* L. 'Triangel') were soaked and germinated in a Conviron S10 chamber (20 °C; 120 µM $m^{-2} \cdot s^{-1}$ light intensity; 12-12 h light/dark period) then grown on half - Hoagland solution for five days. The growing solution was changed in every two days. 10 days old seedling were treated with $CdCl_2$ and $NiCl_2$ (cadmium: 10 µM, 50 µM, 100 µM, 300 µM; nickel: 100 µM, 500 µM, 1000 µM), then sampled in the 0,1,4,7 days for organic acid, antioxidative enzyme activity, fluorescence induction and SPAD measurements. For the electrical impedance measurement pea seedlings (*Pisum sativum* L. cv. 'Debreceni világos') were used. After germination the seedlings were grown in Conviron S10 chambers under 16 hours, 120 µM $m^{-2} \cdot s^{-1}$ light intensity, on 22-23 °C, 58 % relative humidity. The temperature of the chamber during the 8 hours of dark period was 20 °C. Seedlings were watered with distilled water. Three days old seedlings were put on half Hoagland solution. Half of the plants were aerated with Maxima (Hagen- Germany D-25488 Holm) aquarium pumps. Three days old seedlings were treated with 0; 100; 200 µM cadmium in the growing solution and grown for 7 more days. Samples were taken on the 0, 1., 4., 7. days of treatment and cadmium content, morphological analysis and electrical impedance measurements were carried out. The values of the graphs are the average (±S.D) of three independent experiments 15-20 seedlings were used for sampling. Significance was determined with ANOVA ($p>0,05$) analysis, Duncan test, SPSS 7.0 programme.

3. Results

In the first part of my work I intended to elucidate the time course dynamics of stress effect evolution. According to my results the changes of organic acid levels and antioxidative enzyme (GPX, APX) activities react more sensitively for the effects of cadmium and nickel stress than fluorescence induction and SPAD measurements. The were no significant changes in case of the latter two. Contrarily, APX showed intensive activity rise three hours after nickel exposion and cadmium treatment also enhanced APX activity: in roots after 9 hours of treatment and in leaves after 24 hours. GPX activity however rose as a result of both metal treatments: in roots after 9 hours, in leaves after 3 hours of exposion.

As for the organic acids, in roots after 12 hours of cadmium treatment resulted in decrease of fumaric acid level- the first acid that reacted the treatment. In leaves only malic acid rose after the third day of cadmium stress, all the other acid levels showed decrease.

Nickel however caused more intensive changes: in roots malic acid, in shoots oxalic acid quantities rose as early as 3 hours after nickel treatment.

These results may be informative in the early detection of heavy metal stress, moreover the quantitative changes indicated the different physiological effects of the two metals.

The results of the second part of my work showed that the changes of the parameters of electrical impedance measurement are also suitable for the detection and following tissue structure changes caused by cadmium in seedlings. Also the combined effect of cadmium and anoxia was also detectable with this method. Moreover it fulfills an important criterion: provides fast information about the physiological state of plant tissues without processing, and it can be carried out in young seedlings, thus with the help of this method it is possible to gain information about the effects of stressors before the occurrence of visible symptoms.

The measurement is fast and can be easily carried out that makes it promising in plant physiological and plant stress physiological investigations. Propriate and careful aeration became inevitable during the hydroponic plant growing, because the lack of aeration did not only result in tipical flooding symptoms, but affected the uptake of cadmium in a disadvantageous manner. According to the results of the experiment, the parameters (Ra, Rs, Cm) calculated from the measured plant impedance are suitable for the detection, distinction and monitoring the changes of tissue structure caused by cadmium and flooding even in the early phase of stress evolution.

Overall, electrical impedance, antioxidative enzyme activity and organic acid level measurements are suitable for the following the dynamics of heavy metal accumulation and early tress detection even when classical plant stress detection methods do not provide information about the state of stress.

4. Theses

1. The constitutive levels of five organic acids in barley seedlings and the growth related organic acid decrease was determined.
2. The effect of cadmium was shown in decrease of organic acids (12. hour: fumaric acid, 24. hour: citric acid, malic acid and oxaloacetic acid) in roots. In leaves an overall rise was detected after 12 hours.
3. Nickel initiate a rise of organic acids even in lower concentrations: in roots malic acid, in leaves oxaloacetic acid level rose after 3. hours of treatment. This makes organic acid level measurement suitable for following heavy metal accumulation in the early phase of stress.
4. GPX and APX enzymes provide information about ROS binding capacity of the whole plant cell. GPX activity rose in roots (9. hour) and in leaves (3. hour). APX however rose after 3 hours of treatment in both plant organs for nickel and later in the first day for cadmium. The changes of activities are good indicators of heavy metal caused early oxidative stress.
5. The changes of the parameters of electrical impedance are suitable for the distinction of different growing conditions.
6. The physiological effect of cadmium on pea seedlings was characterized by the rise of the parameters (Ra, Rs, Cm) of electrical impedance measurement.
7. It was concluded that electrical impedance measurement is a suitable method for the detection and the following of structural changes caused by cadmium poisoning in the early phase of stress evolution.

5 Conclusions and proposals

Before the changes of organic acid levels, the activity of the enzymes catalising their synthesis should also rise. In order to prove this, it may be important to investigate the activity of malate-dehydrogenase and succinyl-CoA enzymes of citrate cycle under the same experimental setup.

Phytochelatin synthase enzyme activity measurement, or the determination of the amount of synthetised phytochelatins would also enlighten the processes of early stress evolution especially for cadmium.

Smaller time scale measurement, for example hourly, of GPX would be advisable in order to determine the very start of activity rise.

measuring of other constituents of antioxidative enzyme system (CAT, GR, SOD) would be useful to investigate for the two metals under the same conditions.

The electrical impedance measurement should also be optimised for essential heavy metal as well. Pin electrodes would be useful to substitute for contact electrodes, thus the method would become fully non-invasive.

By the first sampling all the investigated parameters changed drastically, that is why it would be advisable to carry out samplings more frequently, but definitely after one day of cadmium treatment.

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