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CYTOKININS AND GROWTH RESPONSES OF MAIZE AND PEA PLANTS TO SALT STRESS

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Summary. Maize and pea plants were grown on a liquid nutrient medium containing NaCl (50, 100, 150 mM stress). Growth and developmental reactions and endogenous cytokinin level of control and stressed plants were compared. Zeatin and isopentenyl adenine and their ribosides were purified and characterized by several procedures including immunochemical techniques. Results are discussed in search of the significance of endogenous cytokinin alteration to some growth and developmental reactions of salt-stressed plants.

***Key words*:** adaptative response, cytokinins, growth, maize, pea, salt stress

***Abbreviations*:** z – *trans*-zeatin; ip – N^6-isopentenyl adenine; zr – *trans*-zeatin riboside; ipa – N^6-isopentenyl adenosine; ELISA – enzyme-linked immunosorbent assay; TLC – thin-layer chromatography; IgG – immunoglobulins

The present work influenced beneficially by Professor Dieter Klämbt is dedicated to his 65th birthday.
The authors

Introduction

Severe environmental factors, so called stresses, provoke a wide range of changes in physiology, growth and development of plants. Many of these changes are important for their adaptation and survival. There have been suggestions that the plant's adaptive responses may be modulated by phytohormones (Davies et al., 1986; Incoll

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and Jewer, 1987; Kuiper et al., 1990). Data for the variation of cytokinin level in plants grown at salt conditions might be not easily generalized. Different responses of cytokinin level depending on the plant organ studied, the duration of the treatment and the plant's resistance to salinity were described (Munns and Termaat, 1986; Incoll and Jewer, 1987; Kuiper et al., 1990). In most of these studies the cytokinin levels were measured by bioassays.

We performed a concurrent measure of growth and internal cytokinin content in order to see whether there is any correlation between them when maize and pea plants are subjected to salt stress. The dominating in higher plants cytokinins: *trans*-zeatin, N^6-isopentenyl adenine and their ribosides: *trans*-zeatin riboside and N^6-isopentenyl adenosine were determined by two immunochemical techniques: immunoaffinity chromatography and indirect competitive ELISA.

Materials and Methods

Growth of maize plants

Maize (*Zea mays* L., cv. Knezha) plants were grown on 50% strength Hoagland–Arnon nutrient solution at natural conditions in June/July. NaCl of 50, 100 and 150 mM was added to the nutrient solution when plants expanded their 4th leaf. The duration of salt treatment was 10–12 days. Growth was evaluated by length and biomass production of maize roots and shoots.

Cytokinin determination of maize plants

Cytokinins were extracted from fresh roots and leaves with 80% ethanol and extracts were hydrolysed with alkaline phosphatase, to digest conjugated cytokinins to ribosides. Several procedures were developed for purification and separation of cytokinins:

- partition with *n*-butanol, pH 9 followed by Sephadex LH-20-chromatography (10% ethanol as eluent) resulting in separation of zr + z from ipa + ip.
- DEAE-cellulose-chromatography followed by Sep Pac C_{18} cartridge-partition after Von Schwartzenberg et al. (1989). TLC on silikagel in *n*-butanol–ammonia–water (6:1:2) was used to separate ribosides from free bases.
- DEAE-cellulose-chromatography combined with immunoaffinity chromatography on Sepharose coupled with antibodies against zr or ipa by Hansen et al. (1989). The final step was TLC as mentioned above. In this way z + zr and ip + ipa were isolated and then ribosides from the respective bases were separated. Estimation of zr, z, ipa and ip was made by ELISA.

Amaranthus-betacyanin bioassay was applied for evaluation of cytokinin activity of maize roots. These results were used for comparison with ELISA-estimation.

Growth of pea plants

Pea plants (*Pisum sativum* L., cv. Ran-1) were grown on 50% strength of Hoagland–Arnon nutrient solution in climatic chamber at 23–25 °C, light intensity 100 μmol.m^{-2}.s^{-1} and a day/night photoperiod 14/10 hours. 11 days after seed soaking NaCl of 50, 100 and 150 mM was added to the nutrient solution. Depending on the duration and salt strength two series of plants were grown:

- plants exposed for 7 days to 50 and 100 mM NaCl;
- plants exposed for 2 and 5 days to 150 mM NaCl.

Growth of pea plants was evaluated by length and fresh weight of roots and shoots.

Cytokinin determination of pea plants

Cytokinins were extracted from lyophilised roots and shoots with 80% ethanol. Extracts were purified by two different procedures:

- DEAE-cellulose chromatography followed by Sep Pak C_{18} -partition;
- DEAE-cellulose chromatography followed by anti-zr-IgG-Sepharose- or anti-ipa-IgG-Sepharose-column.

TLC was used for separating cytokinin bases from their ribosides. Estimation of zr, z, ipa and ip was made by ELISA.

ELISA

Cytokinins were measured by means of indirect competitive ELISA after Von Schwartzenberg (1989) with several modifications made in our laboratory.

Polyclonal antibodies against albumin conjugates with t-zr and ipa were raised in rabbits, New Zealand. This work started in the laboratory of D. Klämbt (Institute of Botany, Bonn) in 1990 and continued in our Institute in collaboration with I. Todorov (Institute of Molecular Biology, Sofia). The collaborations resulted in preparation of high specific IgG against *trans*-zr/z and ipa/ip which were used for ELISA and immunoaffinity chromatography.

Anti-t-zr-IgG showed a level of cross-reactivity with z to 55% and reacted very slightly with ipA/ip (< 0.3%) and other similar structures (dihydrozeatin, adenine/adenosine, benzyladenine etc). Anti-ipA-IgG reacted with ip to 45% and had low cross-reactivity with zr/z (< 0.1%) and the compounds mentioned above.

The calibration curves for zr/z and ipA/ip and results of immunoassays were calculated by the programme developed by Ts. Tsonev (Institute of Plant Physiology,

Sofia) using the formulae of percentage calculation of maximum binding and of log-logit transformation.

Results

Growth was retarded by exposure of maize plants to salinized nutrient solution. The growth reduction increased with the increase of salt concentration (Fig.1).

We determined the endogenous cytokinins in roots and leaves of control plants and plants treated at 100 mM NaCl for 10–12 days. At these conditions shoot growth was about half of that of control plants. Roots were less affected by salt treatment – the reduction of fresh biomass was up to 30%. Determined by the procedure including Sephadex LH-20 chromatography and *Amaranthus* bioassay the activity of z- and ip-type cytokinins of treated roots did not differ significantly from the respective control activity (Fig. 2). After enzyme treatment with alkaline phosphatase the cytokinin activities of both control and treated roots increased. There was a hint for more conjugated cytokinins in salt-treated roots (data not shown). The tendencies found by bioassay were confirmed after employing the other separation procedures. Thus, regardless of nearly 40% growth reduction of stressed maize plants, their root (zr+z)- and (ipa+ip)-levels were nearly the same as the control ones (Fig. 3A). The conjugated z- and ip-cytokinins, sensitive to alkaline phosphatase, seemed to increase at salt stress (Fig. 3B).

The effect of salinity on cytokinin level in leaves was variable in the different experiments. Nevertheless, the predominant cytokinins in maize leaves were of zeatin-type (Fig. 4A). Zeatin content was not influenced in NaCl-treated leaves but increased in some cases (Fig. 4B).

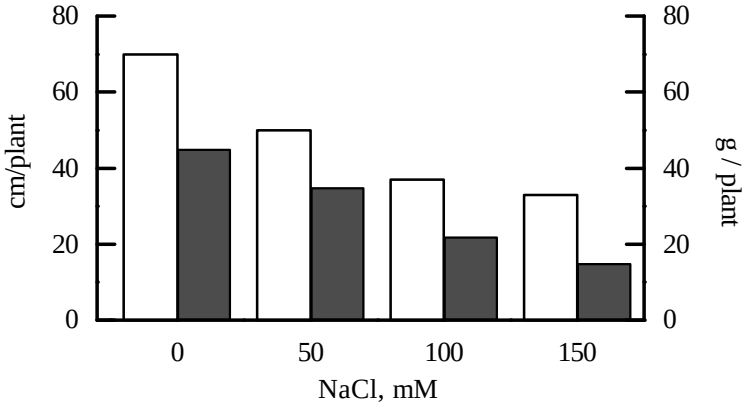

Fig. 1. Effect of different NaCl-concentrations on growth of maize plants. Length, white bars; Biomass, shadow bars

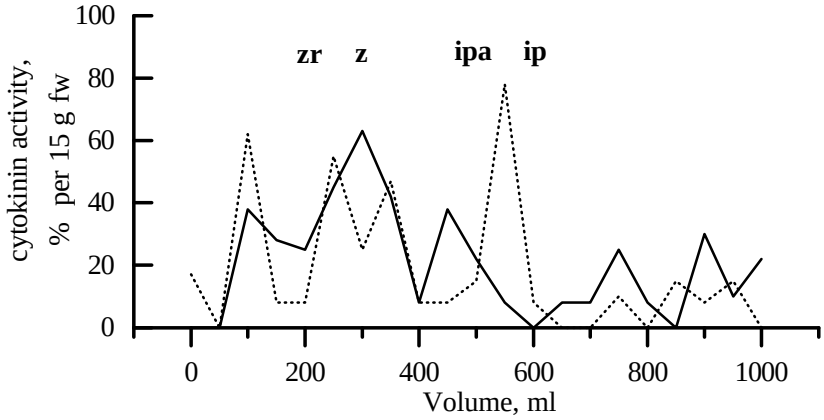

Fig. 2. Betacyanin bioassay of control (solid line) and NaCl-treated (broken line) maize roots after Sephadex LH-20 chromatography. zr, z, ipa, ip - standards

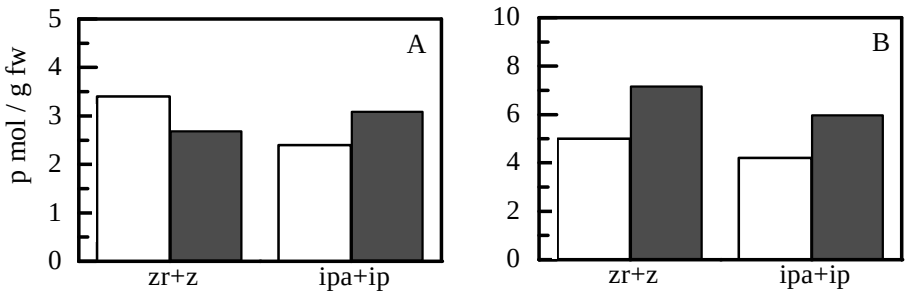

Fig. 3. Content of cytokinins in control (white bars) and NaCl-treated (shadow bars) maize roots before (A) and after (B) enzyme treatment

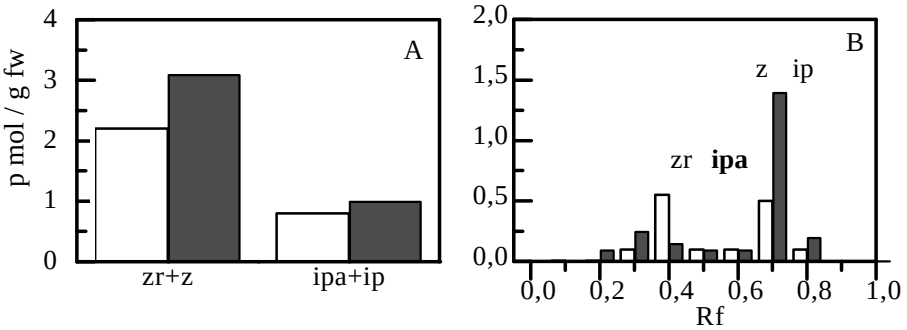

Fig. 4. Content of zr+z and ipa+ip in control (white bars) and NaCl-treated (shadow bars) maize leaves purified by immunoaffinity chromatography (A) and TLC (B)

After exposure to increasing salt concentrations pea shoot growth, measured by length and fresh weight, decreased. The growth reduction was obvious at NaCl-concentration higher than 50 mM (Fig. 5). Root growth was stimulated at 50 mM NaCl and was much more reduced at 100 and 150 mM stress.

We also noticed an effect of NaCl-treatment upon pea development. 100 and 150 mM NaCl-treated plants formed flower buds earlier than the other plants. Control and 50 mM NaCl-treated plants showed similar development.

The cytokinin content of plants grown at lower salt levels for 7 days was near to the content of control plants (Fig. 6). Z-type cytokinins increased slightly in roots of 50 mM NaCl-treated plants. Both, zr + z and ipa + ip were less in roots at 100 mM NaCl (Fig. 6A). In shoots of salt-treated plants ip-type cytokinins increased with the increase in salt concentration (Fig. 6B).

In 150 mM NaCl-treated plants the level of both types of cytokinins increased significantly during the period of 5 days (Fig. 7B). A period of 2 days seemed to be sufficient to establish significant difference in cytokinin content (Fig.7A).

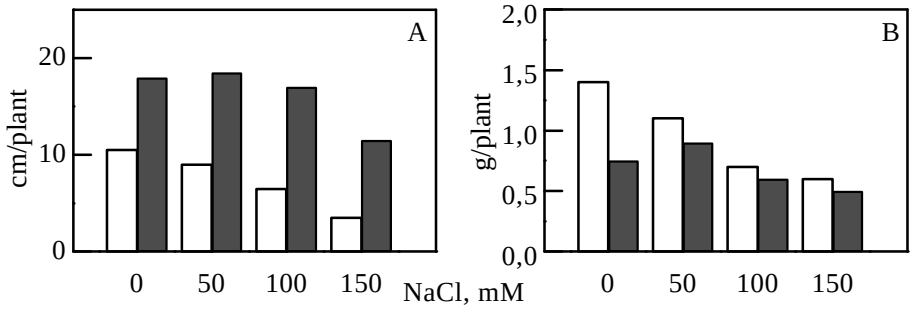

Fig. 5. Growth (length, A, and biomass, B) of pea shoots (white bars) and roots (shadow bars) depending on NaCl-concentrations

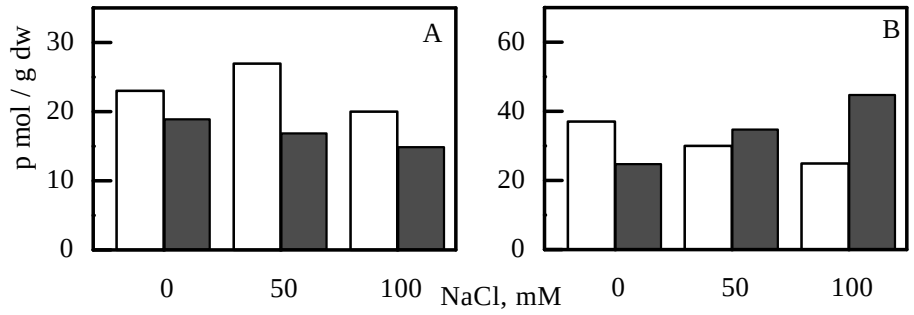

Fig. 6. Content of cytokinins in roots (A) and shoots (B) of pea plants grown on different NaCl-concentrations. zr + z, white bars; ipa + ip, shadow bars

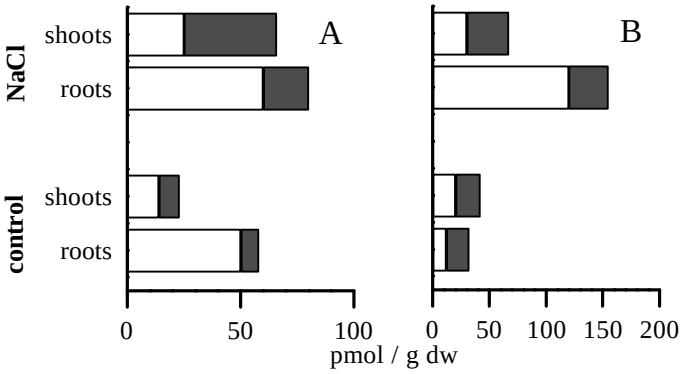

Fig. 7. Content of immunoaffinity-purified zr+z and ipa+ip in control (white bars) and 150 mM NaCl-treated (shadow bars) pea plants. Duration of treatment: A, 2 days and B, 5 days

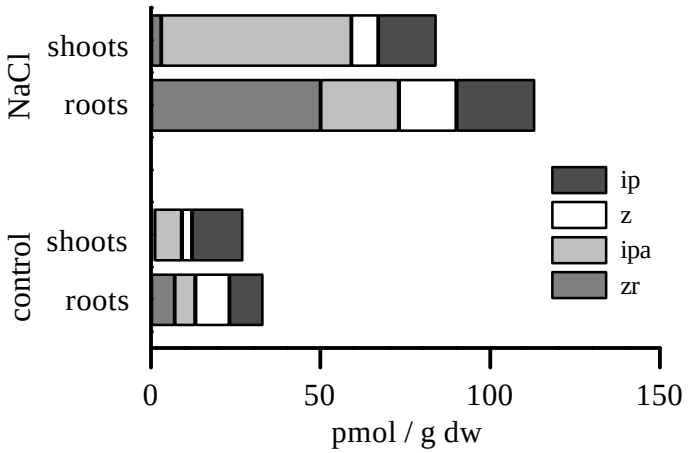

Fig. 8. Levels of z and ip and their ribosides in control and 150 mM NaCl-treated pea plants

To distinguish cytokinin ribosides from their bases the immunoaffinity chromatography was followed by TLC. These results indicate a significant rise of cytokinin ribosides in 150 mM NaCl-treated plants (Fig. 8).

Discussion

There is general agreement that cytokinin biogenesis is located in all dividing tissues throughout the plant (Hansen et al., 1984; Chen et al., 1985; Munns and Termaat, 1986; Incoll and Jewer, 1987). Roots have been suggested as main site of cytokinin

production. The meristematic tissues in the root tips and cambia regions are placed closely to the xylem. This proximity provides an easy transfer of cytokinins from the zone of biogenesis into the transpiration stream. The activity for dividing is affected by environment (Turner, 1986; Incoll and Jewer, 1987). At the same time the levels of endogenous cytokinins should be affected too. Up to certain limits plants are able to respond with morphological and functional changes which ensure their adaptation to the changing environment. It was suggested that cytokinin supply is of decisive importance for the appearance of adaptive reactions (Turner, 1986).

We noticed that pea plants grown at 50 mM NaCl showed slightly increased root growth and maintained shoot growth. Maintenance of shoot growth may be expected if adaptation as osmotic adjustment of roots occurs (Turner, 1986). The internal cytokinin concentration of salt-treated pea plants did not change dramatically, and zeatin-type cytokinins of roots increased. The latter fact could be considered as a result of higher root meristematic activity.

In our experiments maize root growth at 100 mM NaCl was less affected than shoot growth at the same salt concentration. Maize roots are known to have lower osmotic potential at water stress than maize leaves (Turner, 1986). Peterson et al. (1988) found that maize root growth was inhibited by 30% at 75 mM NaCl for 19 days but root apices of control and salt-treated plants did not differ significantly in fresh weight and cell number. We found similar growth reduction of maize plants after 10 days salinity. Root internal cytokinins were not greatly affected by salinity but there was an increase of the level of cytokinin nucleotides. This may be a signal to shoots for some changes in root cytokinin production. In the leaves of 5-week-old control plants we determined more z-type than ip-type cytokinins and predominant zeatin content in leaves of salt-treated plants. High content of zr + z was determined in both nodes and stems near to the apex of 8-week-old maize plants (Hansen et al., 1984). The authors suggested that leaves and shoot apices may be additional cytokinin sources. The significance of cytokinin response of maize plants to salinity in our experiments is difficult to be discussed in details now. We need more information from plants of prolonged experiments.

In pea plants grown on salinity higher than 100 mM NaCl a rapid development of flowers was noticed. This developmental response is caused by drought. It reflects the ability of pea plants to respond to stress by shortening ontogenesis. There is strong suggestion that this adaptative reaction is regulated by plant growth substances (Turner, 1986). At the conditions of our experiments pea plants grown at 150 mM NaCl formed reproductive organs earlier than 100 mM NaCl-treated plants. We found an increase of ip-type in shoots treated at lower salt concentration (Fig. 6) and of ribosylated cytokinins in whole pea plants grown at 150 mM NaCl (Fig. 8). It is well known that high cytokinin concentration promoted bud formation in callus tissue. The pea plant was the model system for demonstrating the effectiveness of high exogenous concentrations of N^6- substituted adenines in increasing lateral bud growth

of shoots (Skoog and Abdul Ghani, 1981). Our results indicate that high endogenous cytokinin level might be the decisive factor influencing pea plants to early bud formation and flowering at defined stress conditions.

The rise of ribosylated cytokinins is a response also found in other plants surviving at different suboptimal environmental conditions. For example, tomato leaves at salt stress (Walker and Dumbroff, 1981), bean plants stressed at Al-toxic level (Massot et al., 1994), spruce needles damaged by air-born pollution (Von Schwartzenberg et al., 1989), contained high cytokinin riboside levels. The biochemical background of the increase of cytokinin ribosides in plants grown at unfavourable environment is totally unknown and open to research.

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