The aim of our study was to find, establish and verify new methods for early indication of stress evoked by organic compounds in plants. The level of endogenous phytohormones in pea plants (*Pisum sativum* L. cv. Garde) cultivated *in vitro* in the presence of polycyclic aromatic hydrocarbon (PAH) fluoranthene (FLT) was detected. The selected concentrations of FLT simulate low (0.1 mg/L) and high (1 and 5 mg/L) environmental loading. With the increasing loading of the environment by toxic compounds (e.g., PAHs) it is necessary to develop and use new methods for the early indication of stress. The effect of PAHs treatment on the vegetation cover has not yet been studied at the level of *in vitro* cultures. The use of *in vitro* cultures can be suitable for the study of influence of xenobiotics, with regard to their spatial and financial demands. The apical segments of pea plants were cultivated on Murashige-Skoog (MS) cultivation medium. MS medium was enriched with 0.1 mg/L indole-3-acetic acid (IAA) or combination of IAA and 0.1 mg/L N6-benzylaminopurin (BA). The production of gaseous hormone ethylene was analysed after 1, 2, 3, 7, 14 and 21 days of cultivation by GC-FID. In 21-day-old plants the level of cytokinins was analysed after purification of methanolic extracts using P- and DEAE-cellulose columns and C18 Sep-pak cartridge by reversed-phase HPLC separation to individual fraction of cytokinins. Their levels were determined using the ELISA method. In 21-day-old plants the level of ABA was analysed by RIA method with monoclonal antibody MAC 252. Radioligand $^3$H-ABA was used. Radioactivity was calculated using software Securia Packard. The results were processed with software STATISTICA 6. The content of endogenous phytohormones (ethylene, cytokinins (DHZR, iP, BA, BAR, mT, mTR) and ABA) significantly increased with increasing FLT concentration (1 and 5 mg/L) in the environment. This fact indicates that it could be a precious tool for the study of toxic effects of other environmental contaminants.

**Key words:** fluoranthene, phytohormone, pea plants, *in vitro*

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INTRODUCTION

The development of human activities and industrialization has led to an increased accumulation of organic compounds in the environment (Zhou et al., 2006). Polycyclic aromatic hydrocarbons (PAHs) constitute an important group of micro-pollutant, which can be found in air, soil, water, vegetation, ice and sediments (Wang et al., 2005). Most of PAHs are by-products of the incomplete combustion of organic matter and thus can originate from both natural and anthropogenic sources. Several of them are carcinogenic and/or mutagenic in animals, as well as phytotoxic (Meudec et al., 2006). Their fate is determined by their physico-chemical properties. Crucial position of plants, especially cultivated plants, in the food chain provides a potential exposure route for these chemicals to higher tropic levels.

The plant ability of PAHs uptake, translocation, transformation and accumulation is one of the determination factors for phytotoxicity of these compounds. The biochemical and physiological changes of plants can be detected earlier than the morphological changes and can be used as an early bioindicator. A number of environmental factors cause changes not only in processes of energetic metabolism, but also in another regulation mechanisms involved in the growth and development of plants. Especially changes in phytohormone levels controlled by the respective hormone system are essential steps in acclimation of the plant to a stress (Mok and Mok, 2001). The increase or decrease in concentration of a hormone is achieved by changes in the levels of gene expression leading to synthesis of the enzymes involved in its biosynthesis, or by regulation the enzymes that degrade or deactivate the compound (Vaseva-Gemisheva et al., 2005). These changes, caused by environmental factors, could be also used for the early indication of stress.

With the increasing loading of the environment by toxic compounds (e.g. PAHs) it is necessary to developed and use new methods for the early indication of stress. The use of in vitro cultures can be suitable for the study of influence of xenobiotics, with the regard to their spatial and financial demands.

The objective of the present study was to evaluate the effect of one of the most frequent polycyclic aromatic hydrocarbon fluoranthene on the level of endogenous phytohormones in pea plants cultivated in vitro. The effect of FLT simulating low (0.1 mg/L) and high (1 and 5 mg/L) environmental loadings. The production of ethylene was analysed after 1, 2, 3, 7, 14 and 21 days of cultivation. In 21-day-old plant the content of cytokinins and abscisic acid was determined.

MATERIAL AND METHODS

The used plant material was pea plants (Pisum sativum L. cv. Garde). Apical segments (1 cm) of shoot were cultivated on the Murashige-Skoog (MS) cultivation medium (Murashige and Skoog, 1958) in controlled conditions (PAR 40 µmol/m²/s, photoperiod 16/8, 25±1°C). MS medium was enriched with the growth regulators indole-3-acetic acid (IAA, 0.1 mg/L) or combination of IAA and N6-benzylaminopurine (BA, 0.1 mg/L). Fluoranthene (Supelco, USA), was added into the MS medium in the concentration 0.1, 1 and 5 mg/L.
Production of ethylene was determined after 1, 2, 3, 7, 14, and 21 days of cultivation. The contents of ethylene was analysed in the flame-ionisation detector gas chromatograph (GC-FID) (GC800 Series with 50 m capillary column Al$_2$O$_3$ „S“ 15 µm, ID 0.53 mm, Fisons Instruments, Italy). Temperatures of the packing, column and detector for the assessment of gas hydrocarbons were 230°C, 40°C and 200°C, respectively (Fišerová et al., 2001).

The plant material (1 g of fresh matter) was homogenised in liquid nitrogen and extracted with Bieleski solution (Bieleski et al., 1964). The content of endogenous cytokinins (CKs) was analysed after purification of methanolic extracts, i.e., using P- and DEAE-cellulose columns and C18 Sep-pak cartridges (Macháčková et al., 1993) by reversed-phase HPLC (Ecom, Czech Republic; capillary column LUNA C18 (2), 5 µm, 250x2 mm, PHENOMEX) separation to individual fraction of CKs (trans-zeatin, dihydrozeatin, isopentanyladenine, benzyladenine, meta-topolin and their ribosides). Their contents were determined using the ELISA method (Strnad, 1996).

The level of endogenous ABA was analyzed by RIA method with monoclonal antibody MAC 252 (Quarrie et al., 1988). Radioligand $^3$H-ABA was used (Amersham Pharmacia Biotech, specific activity 1.9 TBq/mmol). Radioactivity was calculated using software Securia Packard.

For a statistical evaluation of results, the software STATISTICA 6 (StatSoft, Inc.®, USA) was used. The obtained results are means of six repetitions of ethylene and four repetitions of cytokinins and ABA. The significance of the differences in average values between the treatments was evaluated by means of the analysis of variance of simple classification after preceding verification of normality and homogeneity of the variance (ANOVA, $P=0.05$) or by the non-parametric Kruskal – Wallis test. A detailed evaluation of the variance was carried out using the Tukey contrast or Scheffé test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Organic pollutants, which are taken up by plants, can affect a number of biochemical and physiological processes that contribute to production of biomass both quantitatively and qualitatively. Growth and differentiation processes in plants are controlled primarily by a system of endogenous growth regulators. Similar interactions between the effect of PAHs and growth regulation using plant hormones were explored only partially. Estimation of the growth rate on the base of biomass increments thus represents a measure for assessing of the effect of FLT on plants. The biomass production is a reliable external indicator of the internal affection of plant metabolism.

Ethylene production (nL/L) is a result of the occurrence of stressors in the environment. The results of this experiment indicate a high response of ethylene as a stress indicator even in the case of PAHs-like organic pollutants. Its increasing production reflected the level of environmental load by FLT. Higher values of produced ethylene, which were recorded in all treatments (IAA, IAA+BA and all combinations with 0.1, 1 and 5 mg/L FLT) during the first 48 hours of the experiment, were caused by stress resulting from the presence
of all these compounds in the medium (Fig. 1). Ethylene is oxidized in a few steps into CO$_2$ (Adams and Yang, 1979).

*Fig. 1 The influence of fluoranthene on ethylene production during 21 days of cultivation.*

Changes recorded in production of biomass in plants exposed to FLT could be related to the changes in their endogenous levels of cytokinins (ng/g FW). Endogenous cytokinins, i.e. dihydrozeatin riboside (DHZR), isopentanlyadenine (iP), benzyladenine (BA), benzyladenine riboside (BAR), *meta*-topolin (mT) and *meta*-topolin riboside (mTR) were estimated as well (Tab. 1). As compared with control samples (IAA, IAA+BA), the content of all these cytokinins increased with increasing concentrations of FLT (1 a 5 mg/L) in the environment. Higher levels of cytokinins were detected in plants cultivated in a medium enriched by BA. The lowest levels of cytokinins were observed in plants treated by IAA+FLT 0.1 and IAA+BA+FLT 0.1. Lower concentration of the same stressor could inhibit synthesis of cytokinins. This fact could be caused partly by their reduced biosynthesis and partly by the control of enzymes, which participate in the degradation or deactivation of these compounds.

The phytohormone abscisic acid (ABA) plays regulatory roles in a most of physiological processes throughout plant growth and development (Yang et al., 2007). The content of ABA (ng/g FW) increased with increasing concentrations of FLT (0.1, 1 and 5 mg/L) in the environment (Fig. 2). An increase in ABA content has been reported in many of higher plant species which have been exposed to water stress, nitrogen deficiency, chilling, excessive radiation, heat stress, salinity, low relative humidity, air pollutants, wounding, pathogen attack, etc. (Finkelstein and Gibson, 2002; Hronková et al., 2003). Higher level of ABA was detected in plants cultivated in a medium enriched by IAA+BA. In accord with Korableva et al. (2002) we determined a rise in ethylene production accompanied by increased synthesis of ABA.
Tab. 1 Content of endogenous cytokinins detected in the fresh mass of 21-day-old pea plants cultivated in vitro in medium containing IAA or IAA+BA in combination with fluoranthene treatment (0.1, 1 and 5 mg/L); dihydrozeatin riboside (DHZR), isopentanlyadenine (iP), benzyladenine (BA), benzyladenine riboside (BAR), meta-topolin (mT) and meta.topolin riboside (mTR).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Content of cytokinins (ng/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHZR</td>
</tr>
<tr>
<td>IAA</td>
<td>0.474 ± 0.036&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>IAA+FLT (0.1)</td>
<td>0.091 ± 0.029&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>IAA+FLT (1)</td>
<td>0.613 ± 0.055&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IAA+FLT (5)</td>
<td>0.700 ± 0.049&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>IAA+BA</td>
<td>0.543 ± 0.058&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IAA+BA+FLT (0.1)</td>
<td>0.220 ± 0.008&lt;sup&gt;ae&lt;/sup&gt;</td>
</tr>
<tr>
<td>IAA+BA+FLT (1)</td>
<td>0.928 ± 0.088&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>IAA+BA+FLT (5)</td>
<td>1.081 ± 0.180&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different letters show differences between values on P = 0.05.
CONCLUSION

Investigated parameters (production of ethylene, content of cytokinins and abscisic acid) responded sensitively to the occurrence of stressors in the environment. This demonstrated a negative effect of an important polycyclic aromatic hydrocarbon fluoranthene on physiological processes taking place in plants. Organic pollutants occurring in the environment and taken up by plants affected significantly also production of growth regulators. The effect of PAHs-treatment on the vegetation cover has not yet been studied at the level of in vitro cultures. Our results indicate that it could be a precious tool for the study of toxic effects of other contaminants.

REFERENCES


