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Effect of uranium on plant metabolism

Master thesis

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The presented work was carried out at the Department of Experimental Plant Biology, Faculty of Science, Charles University in Prague and at the Laboratory of Plant Biotechnologies, Institute of Experimental Botany AS CR, Prague.

I hereby declare that I completed this master thesis independently under the guidance of Doc. RNDr. Helena Lipavská, Ph.D. and RNDr., Mgr. Petr Soudek, Ph.D. It documents my own work if not explicitly otherwise mentioned. I have properly acknowledged and cited all sources used. The thesis is not subject of any other defending procedure.

Prague, August 15th 2013 ……

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„The Mother Earth provides us with food, provides us with air, provides us with water. We, the people, are going to have to put our thoughts together, to save our planet here. We've only got one water, one air, one Mother Earth."

Corbin Harney

Abstract

Nowadays, the environmental pollution by heavy metals is very serious problem all around the world. Radionuclides, including uranium, are heavy metals that cause both chemical and radioactive pollution. Naturally occurring uranium is not so dangerous for living organisms. Human activities, especially uranium ore mining and use of phosphate fertilizers, have increased its concentration in the environment with consequent contamination of soil, water and air. Compared to other countries, the Czech Republic is relatively rich in deposits of uranium ore. Extensive mining results in large contaminated areas, containing not only uranium but also other heavy metals and xenobiotics that need to be removed from the environment. One way how to decontaminate soils and waters is phytoremediation. This eco-friendly and cost-effective technique exploits the ability of plants to take up, translocate, transform and sequester xenobiotics. In order to provide functional phytoremediation, it is necessary to understand the mechanisms of plant responses to stress caused by xenobiotics. Therefore in my master thesis, I focused on the impact of uranium on physiological processes of uranium-stressed plants, with the emphasis on carbohydrate metabolism and antioxidative defense mechanism.

Key words

Antioxidative defence, carbohydrate, enzyme, heavy metals, horseradish (*Armoracia rusticana* Gaerth. Mey. et Scherb.), metabolism, phytoremediation, tobacco (*Nicotiana tabacum* L.), uranium.

Abstrakt

Znečištění životního prostředí těžkými kovy představuje v současné době velmi závažný problém celého světa. Radionuklidy, mezi něž patří uran, jsou těžké kovy způsobující jak chemické, tak radioaktivní znečištění. Přirozeně se vyskytující uran nepředstavuje pro živé organismy tak vysoké riziko. Až lidská činnost, hlavně těžba uranové rudy a používání fosfátových hnojiv, zvýšila jeho koncentraci v prostředí, a tím i rozsah znečištění. Specifikem České Republiky je výskyt bohatých nalezišť uranové rudy. Těžba po sobě zanechává veliké plochy kontaminované nejen uranem, ale i jinými těžkými kovy a xenobiotiky, které je potřeba z prostředí odstranit. Jednou z možností, jak půdu a vodu vyčistit, je fytoremediace. Tato ekologicky přívětivá a relativně levná technika využívá schopnosti rostlin přijímat, translokovat, transformovat a následně ukládat tyto látky. Pro plné využití této techniky je potřeba porozumět mechanismům obranné odpovědi rostlin na stres způsobený xenobiotiky. Proto jsem se v diplomové práci zaměřila na ovlivnění sacharidového metabolismu rostlin stresovaných uranem a jejich antioxidační mechanismus obrany.

Klíčová slova

Antioxidační obrana, enzym, fytoremediace, křen (*Armoracia rusticana* Gaerth. Mey. et Scherb.), sacharid, metabolismus, tabák (*Nicotiana tabacum* L.), těžký kov, uran.

Abbreviations

ABTS	2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ABTS-PX	peroxidise converting ABTS substrate
APX	ascorbate peroxidase
ATSDR	Agency for Toxic Substances and Disease Registry
Bq	Becquerel
BSA	bovine serum albumin
CAT	catalase
CAT1	peroxisomal catalase
CDNB	1-chloro-2,4-dinitrobenzene
CSD1	cytoplasmic copper/zinc superoxide dismutase
CSD2	plastidic copper/zinc superoxide dismutase
DTE	dithioerythritol
DTT	dithiothreitol
EDDS	[S, S]-ethylenediamine disuccinic acid
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol-bis(aminoethyl ether)-*N,N´*-tetraacetic acid
FSD1	plastidic iron superoxide dismutase
GPX	peroxidase converting guaiacol substrate (guaiacol peroxidase)
GR	glutathione reductase
GSH	reduced glutathione
GST	glutathione S-transferase
Hepes	4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid
HPLC	high-performance liquid chromatography
IAEA	International Atomic Energy Agency
IEB	Institute of Experimental Botany
IT	invertase
LOX1	cytosolic lipoxygenase
LOX2	plastidic lipoxygenase
MIPase	*myo*-inositol monophosphate
MES	2-(*N*-morpholino)ethanesulfonic acid
NAD^+	nicotinamide adenine dinucleotide
NADPH-oxidase	nicotinamide adenine dinucleotide phosphate oxidase
NLMWOA	natural low molecular weight organic acid
OFFR	oxidized fructan free radical
PF	phytoremediation factor
PHT1;4	high afinity phosphate transporter gene
pNBC	p-nitrobenzoyl chloride
PVP	polyvinylpyrrolidone
PX	peroxidase
RBOHD	isozyme of NADPH-oxidase
RFO	rafinose family oligosaccharide
ROS	reactive oxygen species
SOD	superoxide dismutase
SPS	sucrose phosphate synthase

SPX	syringaldazine peroxidase
SS	sucrose synthase
TF	transfer factor
Tris	tris(hydroxymethyl)-aminomethane
UDP	uridine diphosphate
UDPGD	uridine diphosphate-glucose dehydrogenase
WHO	World Health Organization

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1 Introduction

Uranium is a radionuclide that occurs naturally all around the world. It is commonly found in rock, soil, water, and in very low concentrations also in air and organisms. Uranium is the 51st element in order of abundance in the Earth's crust. The uranium contamination of surface soils and waters has resulted from the development of nuclear industry, which involves mining, milling and fabrication of various uranium products. In addition, use of phosphate fertilizers contributes to the higher uranium concentrations in the environment, which are usually toxic for plants and other biota. Selection of appropriate techniques for the remediation of soils and waters contaminated with uranium and other xenobiotics belong to main goal of many research laboratories worldwide. Phytoremediation utilizes the ability of plant to accumulate hazardous toxic metals for decontamination of soils, waters and the ambient environment (Cunningham et al., 1995; Salt et al., 1995). Better understanding of plant responses to stress induced by uranium exposure is necessary prerequisite for phytoremediation.

Generally, the heavy metals are taken up by plants; their accumulation in plant tissues can result in growth inhibition, structure damages and changes in plant metabolism (Cuypers et al., 2002; Jha and Dubey, 2004; Zelko et al., 2008). The alterations in carbohydrates levels were frequently observed under heavy metal stress (Moya et al., 1993; Choundhury et al., 2010). Sugars can serve as an important osmoprotectans and effective scavengers of reactive oxygen species, and therefore may play an important role in the reduction of damages caused by heavy metal stress. Moreover, sugars are signaling molecules involved in regulation of plant stress responses under various unfavourable conditions. Metal toxicity can cause a redox imbalance in cells and induces the increase of the reactive oxygen species accumulation which results in activation the plant antioxidative defense mechanisms consisting of antioxidative enzymes and metabolites (Schutzendubel and Polle, 2002). Although there are several studies focused on uranium accumulation by plants and few sets of non-systematic data on selected physiological responses exist (Aery and Jain, 1998; Vanhoudt et al., 2008; Viehweger and Geipel, 2010), any complex study of

the effects of uranium on structure and metabolism of particular plant species is completely missing.

This master thesis is focused on two main aspects of the plant responses to uranium stress, i.e. carbohydrate metabolism and antioxidative defense mechanism. Prior to the application of phytoremediation in field conditions, it is necessary to carry out laboratory-scale studies, for which plant model systems are required. Hydroponically cultivated tobacco (*Nicotiana tabacum* L.) and *in vitro* hairy root culture of horseradish (*Armoracia rusticana* Gaerth. Mey. et Scherb.) were chosen due to their well-studied characteristics. Tobacco has been widely used for study of both structural and biochemical features of plant stress responses (Lyubenova et al., 2009). Based on the results from previous experiments performed at the IEB, I used tobacco cultivar La Burley 21 that seemed to be highly tolerant to uranium exposure. The suitability of this choice was recently verified by Stojanovic et al. (2012), who classified this cultivar as an uranium hyperaccumulator. Contents and spectra of carbohydrates and changes in activities of enzymes involved in antioxidative defense are studied in tobacco plants. Horseradish was chosen for experiments due to its well developed root system. *In vitro* cultures of roots are useful for studying the direct interaction of heavy metals with plant tissues (Sanita di Toppi and Gabrielli, 1999), therefore I decided to use the hairy root culture of horseradish. In addition, horseradish is native to Euroasia and thus it is suitable species for phytoremediation techniques in our climatic conditions. Nevertheless, studies are needed on behavior of heavy metals in plant tissues of horseradish as a representative of crop plants for a potential food chain contamination. The hairy root culture was used to examine changes in carbohydrate contents and activities of enzymes involved in sucrose breakdown under uranium toxicity.

This master thesis was conducted in cooperation with the team of Dr. Soudek from the Institute of Experimental Botany AS CR (IEB). His team has extensive experiences with heavy metal contamination and remediation for a long time and this cooperation, therefore, offers good background for my study. The thesis is also connected with another master thesis that is focused on anatomical and structural changes in the roots of cultivated plants exposed to uranium.

Objectives

The main aim of the thesis is to contribute to understanding the mechanisms of model plant responses to stress caused by uranium exposure, and thus provide the basis for further use of phytoremediation techniques.

Partial objectives

- To evaluate responses of hydroponically cultivated tobacco (*Nicotiana tabacum* L.) cv. La Burley 21 to stress caused by uranium exposure.
    - Assessment of changes in carbohydrate contents of uranium-stressed tobacco plant cultivated in hydroponic medium with different supplements.
    - Assessment of selected antioxidative enzymes activities in uranium-stressed tobacco plant cultivated in medium with different supplements.
- To evaluate responses of the hairy root culture of horseradish (*Armoracia rusticana* Gaerth. Mey. et Scherb.) cultivated *in vitro* to stress caused by uranium exposure.
    - Assessment of changes in carbohydrate contents of the hairy roots treated by uranium ions.
    - Assessment of activities of enzymes involved in sucrose breakdown in the hairy roots treated by uranium ions.

2 Literature survey

2.1. General information

2.1.1. Historical information about uranium

First known use of uranium can be dated back to 79 A.D. in Italy. Yellow-coloured glasses founded near Naples contained more than 1% uranium oxide. However, discovery of the element is attributed to German chemist Martin Heinrich Klaproth in 1789. He had been analyzing pitchblende from the pits of George Wagsfort, Germany and of Joachimsthal, Bohemia. By dissolving the mineral in nitric acid and neutralizing the solution with sodium hydroxide, he obtained yellow powder (Klaproth, 1789). Klaproth assumed that the yellow powder was a yet-undiscovered element. He named this new element after the planet Uranus, which was discovered eight years earlier. In reality, the yellow powder was oxide of uranium. The pure element was first isolated in 1841 by Eugene-Melchior Peligot, who reduced anhydrous chloride with potassium (Peligot, 1842). The radioactive properties of uranium were discovered by Antoine Becquerel in 1896.

2.1.2. Chemical and physical characteristics of uranium

Uranium is a silver-white, lustrous and dense radioactive metal, a member of the actinide series of the periodic table. According to its high density (19 g cm^{-3}), uranium belongs to heavy metals. Elemental uranium has an atomic number of 92 and an atomic weight of 238.0289 g mol^{-1}. Uranium can exist in the +3, +4, +5 and +6 oxidation states, but the most common are +4 and +6 oxidation states. Species in +6 oxidized states exist under oxidizing and mildly reducing environment and are strongly adsorbed by soils, forming stable complexes with many ligands, e.g. carbonate, phosphate or sulphate ions. In comparison, species in +4 oxidized states are stable under reducing conditions. In these forms, uranium ions have a strong tendency to bind to organic matter and to iron, and are therefore relatively immobile.

Natural uranium contains three radioactive isotopes ^{234}U, ^{235}U and ^{238}U. The dominant isotope by mass is ^{238}U (99.27 %), it has half-life of about 4.5×10^{9} years (Sheppard et al., 2005). Other characteristics of radio-isotopes are summarized in the

Table I. Each uranium isotope is a member of naturally occurring radioactive decay chain. ^{238}U is the parent isotope of the Uranium decay series, where ^{234}U is a decay product of ^{238}U, while ^{235}U is the parent isotope of the Actinium decay series. Every series ends with stable isotopes of lead, ^{207}Pb or ^{208}Pb. All natural isotopes of uranium and some of their decay progeny emit *alpha* particles; the other members of both decay series emit *beta* particles or *gama* rays. Although *alpha* particles represent high-energy radiation, the long half-lives of uranium isotopes result in relatively low specific activity (ATSDR, 2013).

Table I. *Abundance and basic radioactive properties of natural uranium isotopes.* (Modified from ATSDR, 2013)

Isotope	**Isotopic Composition of Natural Uranium**		**Half-life**	**Specific Activity**
	weight %	activity %	years	Bq g^{-1}
^{234}U	0.0055	48.9	2.45×10^{5}	12.4
^{235}U	0.7200	2.2	7.0×10^{8}	12.4
^{238}U	99.274	48.9	4.47×10^{9}	0.6
Natural uranium				25.4

2.1.3. Cosmic origin of uranium

The origin of uranium leads back to the genesis of chemical elements, more precisely with development of the Universe itself. According to the theories, the Earth's uranium was probably generated in one or more supernovae explosions. The core collapse led to rapid capture of neutrons on seed nuclei at rates greater than disintegration through radioactivity and new super heavy metals were generated. These events must have occurred some 6.5 billion years ago (Hore-Lacy, 2009).

2.1.4. Uranium in the rock

Uranium is usually present in minerals. The primary uranium minerals are generally black or dark brown-coloured. There are only three known primary uranium minerals (uraninite (UO_2), pitchblende (U_3O_8) and davidite ($(Fe, Ce, U)_2(Ti, Fe, V, Cr)_5O_{12}$). Sometimes primary minerals are altered to form the brilliantly coloured and

fluorescent secondary uranium minerals. The most common are coffinite, schoepite or gummite (Bleise et al., 2003).

In 1972 the existence of natural fission reactors was discovered at Oklo in Gabon. This natural phenomenon is very high grade uranium deposit where chain fission reactions occurred approximately 2 billion years ago. The reactors are characterized by abnormal uranium isotopic compositions, containing fission products and end member elements originating from short lived fission products. Nowadays, Oklo is the only known location of natural fission reactor all around the world. Researchers determined 16 separate natural reactors within Oklo (GauthierLafaye et al., 1996).

2.1.5. Uranium in the soil

Natural uranium in soils is mostly mobilized from rocks by the weathering. Typical natural soil background values for uranium differ between 0.79 – 11 mg U kg^{-1} in relation to the parent rock (Kabata-Pendias and Pendias, 2001). Uranium speciation, mobility and leaching (vertical transport to groundwater) is mainly influenced by pH, redox potential, concentration of complexing agents, porosity of soil, soil particle size and sorption properties, as well as the amount of water available. A higher soil cation-exchange-capacity will retain more uranium, while carbonate in the soil increases the mobility of uranium through the formation of complexes (Ebbs et al., 1998). In strongly reducing environments, uranium occurs at U^{4+}. Tetravalent uranium forms phosphate complexes such as $UO_2HPO_4^0$ and $UO_2(HPO_4)_2^{2-}$ and hydroxide complexes such as UO_2OH^+, $(UO_2)_2(OH)_2^{2+}$, $(UO_2)_2(OH)_5^+$ and $(UO_2)_3(OH)_7^-$, which are strongly adsorbed and very immobile in soils (Langmuir, 1978). In oxidizing environments, uranium predominates at U^{6+} and creates complexes with carbonates such as $UO_2CO_3^0$, $UO_2(CO_3)_2^{2-}$ and $UO_2(CO_3)_3^{4-}$ (Langmuir, 1978; Lee et al., 1993).

2.1.6. Uranium in the water

Concentration of uranium in the groundwater is generally higher than in surface water. It is related to geological bedrock. Uranium in the surface water can disperse over large distances. As in soil, the mobility of uranium in waters depends on

many factors such as pH, redox potential, sorbing characteristics of sediments and the suspended solids in the water. Chen et al. (1986) concluded that uranium concentration in the open ocean is about 3.3 mg U m^{-3}. The average concentration in the Pacific Ocean is by 1 % higher than in the Atlantic Ocean. Seko et al. (2003) declare that the total amount of uranium in the World Ocean is 4.5 billion tons, which means the average concentration 3 mg U m^{-3}.

The average uranium concentration in rivers ranges from 0.2 to 0.6 μg U kg^{-1}. These data vary due to the differences in the selected river samples. The lowest uranium concentrations are found in large tropical rivers such as Amazon, Orinoco and Zaire; the highest values are reported in Himalayan Rivers (Palmer and Edmond, 1993). Dissolved uranium concentrations in a given river may also vary spatially and temporally.

Many studies have been undertaken on the concentrations of uranium in drinking water. The World Health Organization (WHO) has advised a maximum contaminant level for uranium in drinking water. The limit is 15 μg U L^{-1} (WHO, 2012), but this limit varies among different countries. The highest limit is in the U.S.A. (30 μg U L^{-1}). Canada and Australia have lower limit (20 μg U L^{-1}). The Czech Republic tightened the limit up in 2007 from 30 μg U L^{-1} to 15 μg U L^{-1}. The National Institute of Public Health in the Czech Republic registers 13 water supplies exceeding this value (Kozisek and Jeligova, 2013).

2.1.7. Uranium in the atmosphere

Uranium is introduced into the atmosphere primarily by weathering of rocks and soils. Another natural phenomenon that may increase the concentration of natural uranium in the air is a volcanic eruption. After the eruption of Mount St. Helens, increased levels of uranium were observed in rainwater in some places in the U.S.A. But nowadays, atmosphere is rich in uranium mainly due to human activities including mining, milling, burning or smoking. Production of phosphate fertilizers, which contain uranium, can also be a source of uranium in the air. Uranium is probably largely present as particulate matters (ATSDR, 2013).

The amount of uranium in the air varies widely. Increased concentrations are recorded in the vicinity of mining and industrial areas. Additionally, the higher uranium concentrations in the air can possibly be associated with the tobacco smoke which contains significant quantities of uranium and other radionuclides such as ^{210}Po. Smoking two packs of cigarettes produces 50 ng of uranium in a form that may be inhaled (WHO, 1998).

2.1.8. Uranium toxicity for humans and animals

Uranium represents both chemical and radiological hazard for human and animals, with a greater risk by acute chemical toxicity rather than radiological one. Uranium is either inhaled, absorbed through the skin or ingested. The health effects related with oral or dermal exposure to uranium appear to be connected with by its chemical properties. The target organs of chemical toxicity are kidneys, while exposure by inhalation may also include a radiological toxicity. The ionizing radiation is associated with carcinogenic effects in lungs and bones. But *alpha* particles released by uranium cannot penetrate the skin (ATSDR, 2013). The WHO established the tolerable daily intake for uranium of 0.6 µg U kg^{-1} body weight (WHO, 1998).

The chemical toxicity of uranium varies according to the route of exposure and chemical forms. As previously mentioned, the organs which are the most affected one are the kidneys. Ingested uranium cause injure of the proximal tubules in nephritis (Weir, 2004). Less soluble uranium species are less toxic and insoluble species are not dangerous for kidneys. These compounds are generally more toxic for lungs, because of the longer retention time in the lung tissue. Generally, the hexavalent uranium is more toxic than tetravalent uranium, because it tends to form relatively soluble complexes (ATSDR, 2013).

About 95 % of the uranium ingested in food or water is eliminated via feces, and the remainder enters the blood and forms complex with carbonate ($UO_2HCO_3^+$). Most of this uranium is distributed via bloodstream to the bone (66 %), liver (16 %), kidneys (8 %) and soft tissue (10 %) followed by excretion in the urine. The retention half-life of uranium in the rat kidney and bones has been estimated to be about 15 and 300 - 5000 days, respectively (Weir, 2004).

Soldiers participating in the Gulf War have claimed to be suffering from a variety of symptoms which are generally termed as Gulf War Syndrome (GWS). The syndrome consists of muscle pain, tiredness, bladder dysfunction, loss of balance or diarrhea. Research findings suggest that syndrome is caused by exposing to a variety of damaging hazards including pesticides, skin insect repellents, medical agents and chemical warfare agents such as depleted uranium (Jamal, 1998).

2.1.9. Uses of uranium

Before the discovery of radioactivity in 1896, the uranium was not considered to be such a dangerous element. Sodium and ammonium diuranates were used to color ceramics and glasses. It produces colors that ranged from lemon yellow to red orange. Uranium has also occasionally been used as a catalyst in certain chemical reactions, in photographic films and in dentistry for obtaining a natural color of dental porcelains (Sairenji *et al.*, 1982). The high density of uranium makes it a potentially suitable material for counterbalance weight and ballast. Industries, where the uranium is used, include the aircraft industry, the oil and gas industry. The uranium is also used in F1 engines for the rapid acceleration or in the manufacture of keels for yachts (WHO, 2001). Chemical phosphate fertilizers often contain high amounts of natural uranium. Fertilizers are processed from phosphate rock that can contain high concentrations of uranium (Wetterlind et al., 2012).

The first nuclear fusion was initiated in 1942 by Italian physicist Enrico Fermi. Since that, uranium potential for use as an energy source had been discovered. In nuclear reactors, uranium serves as source of neutrons. For use as nuclear fuel, natural uranium must be enriched in the isotope ^{235}U. The by-product of this enrichment process is depleted uranium, which is less radioactive than natural uranium. Depleted uranium has numerous applications. It is used in medicine as a radiation shield in radiotherapy, as a counterweight and ballast, or in military as a component of heavy tank armour and armour-piercing munitions. It was used in number of military conflicts. It has been estimated that in the Gulf war approximately 300 tonnes of depleted uranium were used. The huge amount of depleted uranium was also used in

Kosovo conflict. During the air strikes, about 10 tons of armor-piercing munitions were fired (WHO, 2001).

2.1.10. Uranium industry

Uranium resources can be grouped on the basis of geological settings. The International Atomic Energy Agency (IAEA) assigned deposits into 13 main categories based on the geological setting (IAEA, 2009). The unconformity-related deposits have the most economic significance. They include some of the largest and richest deposits that are in Canada and Australia. Other important deposits are situated in Kazachstan, Zair and JAR. It is estimated that about 5.5 million tons of uranium ores exists in world reserves.

The steps necessary to produce uranium include mining, milling, conversion to uranium hexafluoride (UF_6), enrichment, reduction to metal or oxidation to uranium oxide, and fabrication into the desired shape. There are three ways how to mine the uranium ores: open-pit mining, *in situ* leaching, and underground mining. The choice of mining method depends on many factors such as the size, shape, depth, thickness and permeability of the ore deposits and the proximity to groundwater. Open-pit and underground mining have been historically the most commonly used mining methods. However, an increasing proportion of uranium, now 45 %, is produced by *in situ* leaching (ATSDR, 2013).

2.1.11. Uranium mining in the Czech Republic

Mining of uranium ore for glass and ceramic industry began in 19th century in Joachimsthal. Rapid development of surveying and extracting works after the World War II was reflected in the large growth of mining in other localities. Among the biggest deposits on our territory belong Příbram, Rožná, Hamr, Horní Slavkov and Zadní Chodov. From 1946 to 1989, Czechoslovakia had excavated approximately 100,000 tons of uranium. Currently, only one underground mining area - Rožná - exists in the Czech Republic. Whereas the Government of the Czech Republic shut down uranium mining in Stráž pod Ralskem in 1996, uranium is still exploited as a by-product of the remediation process (Calla, 2008). In 2012, the Government rejected the

request to establish an exploration area to search for uranium in Ploužnice in Liberec District. The Government proposes to assess the possibility of uranium mining in Brzkov and Horní Věznice in Jihlava District. In addition, *in situ* leach mining of uranium is considered in the vicinity of Liberec (Calla, 2012).

The uranium mining and processing have negative impacts on the environment by means of abandoned waste accumulation, waste dump or water contamination. Contaminated areas in the Czech Republic spread out more than 0.5 million ha. The most contaminated areas are Stráž pod Ralskem, Rožná and Mydlovary. In the past, contaminated areas were remediated *ex* situ. These techniques are very expensive and have also negative impact on the environment. Nowadays, *in situ* techniques are generally preferred. The one of the promising *in situ* technique is phytoremediation. Results from study in Mydlovary showed that phytoremediation can be applied to the practical situation (Soudek et al. 2007).

Since 1992, remediation is realized by a state company DIAMO. For example, it has been calculated that the remediation process in Stráž pod Ralskem could finish in 2035 and the total costs of this process are expected to be higher than CZK 40 million (Ekert and Muzak, 2010).

2.2. Phytoremediation

Phytoremediation is defined as the use of plants to remove, destroy or sequester contaminants from soil, water and air (Cunningham et al., 1995; Salt et al., 1995). Plants are able to a certain degree to absorb what they need and exclude what they do not. Baker (1981) categorized plants into three groups according to their strategies for growing on metal contaminated soils. Metal excluders reduce metal uptake by the roots. Metal indicators reflect in their tissues the metal levels in the soil. And metal (hyper) accumulators accumulate metal in their tissues to levels far exceeding those present in the soil. Metal hyperaccumulation is generally restricted to species growing at a given locality due to a great variation in physical, chemical and biological factors which exist in the vicinity of contaminated areas (Brooks et al., 1977). Researches of hyperaccumulative phenotype clearly offer the potential for development of phytoremediation techniques (Chaney et al. 1983). Phytoremediation

technology predominantly uses an accumulation ability of plant. Radionuclide decontamination can be more difficult than phytoremediation of the other heavy metals due to the radiation hazards.

Phytoremediation of heavy metals consists of four different technologies where each has a different mechanism of action for remediation and is suitable for different type of pollution. These include:

- **Phytoextraction** – refers to the ability of plants to take up heavy metals and translocate them to the aboveground plant tissues, which are harvested with conventional agricultural methods and smelted for potential metal recycling. The unique ability of hyperaccumulators to accumulate high aboveground levels of metals makes these plants suitable for phytoextraction, however, their generally low biomass and slow growth rate means that the total mass of removed metals will tend to be low. So plants that remove a lower concentration of metals, but have a much greater biomass, are more useful than the hyperaccumulators (Cunningham et al., 1995). A wide range of potential amendments like organic acids or EDTA have been proposed to enhance phytoextraction (Salt et al., 1998).
- **Rhizofiltration** – uses plant roots to precipitate and concentrate metals from polluted waters. Plants are harvested with roots and similarly as in the case of phytoextraction smelted. Either aquatic or terrestrial plants can be used, but terrestrial plants are more suitable due to their greater biomass. Sunflower (*Helianthus annuus* L.), indian mustard (*Brassica juncea* L.) or wetland grasses are the most promising terrestrial plants. In greenhouse experiments sunflower removed more than 95 % of uranium from contaminated water in 24h (Dushenkov et al., 1997). Rhizofiltration has been successfully used to remove radionuclides in the Chernobyl zone, in Ukraine. The constructed wetlands are the best known and utilized kind of rhizofiltration (EPA, 2000).
- **Phytostabilization** – is the use of metal tolerant plants to inhibit the mobility of metals in the soil, thus reducing the risk of further secondary environmental contamination. However, it is important to keep in mind that phytostabilization of radionuclides does not remove the source of radioactivity from the site. The

mobility of metals is reduced by the accumulation of metals by roots, absorption onto roots, or precipitation within the root zone. Preferentially plants with dense root system are chosen due to better stabilization of the soil and prevention erosion. Roots also help to minimize water percolation in soil, and thus reduce metals leaching. Stabilization can be achieved by adding amendments such as lime or organic matter that are able to chelate metals (Cunningham and Berti, 2000). Phytostabilization may be especially useful in controlling tailings from an open pit uranium mines (Dushenkov, 2003).

- **Phytovolatilization** – is a process, in which plants take up metals from the soil and release them as volatile form into the atmosphere through transpiration. Phytovolatilization has been used to remove mercury, arsenic or selenium (Salt et al., 1998).

2.2.1. Transfer factor for uranium

The overall process of uranium uptake by plants from contaminated soil and water was quantified by the International Union of Radioecologists (1989) as the transfer factor (TF), defined as the ratio between the radionuclide content in the plant tissue (µg g^{-1}) or its activity (Bq kg^{-1}) and the radionuclide content or the activity in the dry soil (based on 0-20 cm layer). The TF-values depend on plant species as well as environmental conditions. Vandenhove et al. (2009) compiled data concerning TF values for natural radionuclides and grouped them according to crop group, and also to soil group and organic matter content in the soil. For soils, the highest TF value was derived for the organic soil, whereas the lowest TF value was observed for sandy soils. Leafy vegetables showed the highest TF value, following by fruits, tubers and other vegetables, whereas crops showed relatively low TF value. The observed order of TF value is in agreement with other study (Lauria et al., 2009), where lettuce (*Lactuca sativa* L.) presented the highest TF value, followed by carrot (*Daucus carota* L.) and bean (*Phaseolus vulgaris* L.). Li et al. (2011) have proposed another parameter, the phytoremediation factor (PF). According to authors, this parameter indicates the ability of plant to remove the target element from the contaminated soil as well as plant adaptability to the environment. Common reed (*Phragmites australis* (Cav.) Trin.

Ex Steud.) assigned the highest PF value for uranium, thorium, barium and lead among plant species collected from the uranium mill tailings repository in South China.

2.2.2. Finding suitable plant species for phytoremediation of uranium

Different plant species have been studied as possible target plants for phytoremediation technique. These plants should accumulate high concentrations of the target element and produce a high biomass. Among 11 plant species tested, tepary bean (*Phaseolus acutifolius* A. Gray) and red beet (*Beta vulgaris* L.) showed the greatest uranium accumulation (3.2 mg U kg^{-1} DW in the presence of 5 µM uranium in cultivation solution) (Ebbs et al., 1998). Soudek et al. (2011a) tested 20 different plant species grown in hydroponic solution with addition of uranium (0.1 mM and 0.5 mM U L^{-1}). The amount of accumulated uranium was influenced by uranium concentration in solution. Maize (*Zea mays* L.) has been identified as the most efficient accumulator of uranium. Relatively high uranium content in plant tissues was also found in barley (*Hordeum vulgare* L.). This study showed significant variations between cultivars of the same species. *Cannabis sativa* cv. Benico was able to accumulate more uranium in its tissues compared to the low uranium content in other two tested cultivars cv. Juso11 and cv. Silesia. Shahandeh and Hossner (2002) found the highest uranium accumulation in shoots of sunflower (*Helianthus annuus* L.) and indian mustard (*Brassica juncea* (L.) (24.6 and 21.8 mg U kg^{-1} DW, respectively) grown in the soil treated with 100 mg U kg^{-1}. Sunflower plants were successfully used in the pilot-scale rhizofiltration system (Dushenkov et al., 1997). The hairy root culture of indian mustard (*Brassica juncea* L.) has been shown to be an efficient candidate for uranium removal from aqueous solutions. This plant culture took up 20-23 % of uranium from the solutions containing up to 5000 µM U L^{-1}, while the hairy root culture of white goosefoot (*Chenopodium amaranticolor* Coste & Reyn.) showed retarded growth at higher concentrations of uranium and took up only 13 % of uranium from solution at the same concentration treatment (Eapen et al., 2003). Macrophytes, especially common reed (*Phragmites australis* (Cav.) Trin. Ex Steud.), have been recommended for phytoremediation of uranium contaminated soils and waters (Soudek et al., 2007; Cerne et al., 2011; Li et al., 2011; Caldwell et al., 2012). It has been observed that

common reed grown on the uranium tailing pile accumulated 8.6 mBq g^{-1} DW and 2.4 mBq g^{-1} DW uranium in leaves and stems, respectively (Cerne et al., 2011). Aquatic mosses were also recognized as good candidates for phytoremediation of waters contaminated with uranium. They have great ability for rapid accumulation mainly due to the large surface area and the absence of cuticle. Mosses grown in water contaminated with uranium (on average 630 mg U kg^{-1}) contained as high as 12 500 mg U kg^{-1}, what represents approximately 1 % of DW (Caldwell et al., 2012). Furthermore, fern, especially netted chain fern (*Woodwardia areolata* (L.) T. Moore), may have also potential for phytoremediation of wetland soils contaminated by uranium (Knox et al., 2008). Aquatic plants, especially duckweed *Lemna* sp., have been reported to remove uranium from wastewater efficiently (Mkandawire and Dudel, 2005; Charles et al., 2006). Gibbous duckweed (*Lemna gibba* L.) accumulated uranium from wastewater particularly in the first two days (Sasmaz and Obek, 2009). This plant species was also successfully used for phytoremediaton of arsenic contamination in the tailing water of abandoned uranium mine sites. The study revealed that the TF values for uranium were significantly lower than the values for arsenic. Thus, arsenic may pose more health risk than uranium (Mkandawire and Dudel, 2005). Recent findings indicated that tobacco (*Nicotiana tabacum* L.) is promising plant for phytoextraction of uranium, especially varieties Virginia and Burley, which were classified as an uranium hyperaccumulators (Stojanovic et al., 2012).

2.2.3. Agricultural crop contamination by uranium

There have been reports on uranium bioavailability by agricultural (Stojanovic et al., 2009) or medicinal plants (Sasmaz and Yaman, 2008; Desideri et al., 2010; Morsy et al., 2010; Oufni et al., 2011). High uranium concentrations are found near the uranium mining sites as well as in the solid mining waste and sludge from mine water treatment. Anke et al. (2009) investigated the harmful effect of uranium exposure on vegetation, waters, vegetable and animal foodstuffs and beverages due to uranium mining and processing. In comparison with the control site, plants cultivated near uranium waste dump were found to store till eightfold uranium contents. Leafy vegetables and herbs accumulated more uranium, whereas fruits, grains and tubers

stored less uranium as was also documented by (Vandenhove et al., 2009). Relatively low uranium content was observed in tubers of potatoes (*Solanum tuberosum* L.) grown near the old uranium mine in Spain. The accumulation of all measured radionuclides was enhanced by irrigation by mine water. Furthermore, 90 % of the accumulated uranium was stored in the peel of tubers (Carvalho et al., 2009). This result is in agreement with finding of Saric et al. (1997) for various bulk vegetables, which also presented the highest uranium content in the peel. Based on these results, the risk of human exposure to uranium through the consumption of peeled bulk vegetables grown in vicinity of areas contaminated by uranium is relatively low (Neves et al., 2012). However, the health risk for human can pose the usage of the gum tragacanth milkvetch *(Astragalus gumnifer)* for medical purposes. Sasmaz and Yaman (2008) observed high content of uranium in shoot of this medicinal plant grown around the abandoned mining sites in Turkey. Similarly, other studies showed the great ability of medicinal plants to accumulate uranium, but it depends on plant species and environmental conditions (Desideri et al., 2010; Oufni et al., 2011). Interestingly, it was found that the ordinary consumption of fruits from desert date (*Balanites aegyptiaca* (L.) Delile) grown on naturally radioactive soil lead to decrease of the blood sugar and the total cholesterol in tested population. The greatest decline was reached by ingestion of fruits with the highest content of uranium (2.35 mg U kg^{-1}) (Morsy et al., 2010). It is known that the phosphate fertilizers contain radionuclides, which naturally occur in phosphate ores and hence may alter the overall radioactivity in soil and crop (Falck and Wymer, 2006). In consequence, the use of phosphate fertilizers in conventional farming management may increase the human exposure to radiation via food chain. Unexpectedly, it was observed no significant differences in content of uranium in vegetables from either conventional or organic farming management (Lauria et al., 2009).

2.2.4. Biomonitoring

Since last years, researchers focused on the ability of various plants to act as biomonitors for environmental contamination. Results from the monitoring could be potentially utilized for research connected with the phytoremediation. Epiphytic

lichens have been shown to be ones of the most efficient biomonitors. The results from active biomonitoring with tube lichen (*Hypogymnia physodes* (L.) Nyl.) reflected the contamination by radionuclides around the uranium mine in Slovenia (Jeran et al., 1995). The distribution of the element in the annual growth of trees, dendroanalysis, has been proposed as a technique of biomonitoring. Edmands et al. (2001) utilized black oak (*Quercus velutina* lamb.) as a bioindicator of uranium contaminated groundwater. The isotopic fractionation has determined the occurrence of uranium in tree rings dating back to 1937. The high content of uranium (>3 ppb) was indicated in sapwood and drop rapidly to low concentrations in heartwood (0.3-0.4 ppb). On the contrary, using of another analytical method of dendroanalysis did not identified significantly measurable amount of uranium in tree rings. The probable explanation may be the low contamination of groundwater around a former uranium foundry in Ohio (Mitchell et al., 2008). Another biomonitoring study of various plant species showed that plants are linear accumulators of uranium (Caldwell et al., 2012). This study also confirms correlation of uranium in plant tissues with other heavy metals or micronutrients, and suggests that active uptake mechanism may influence uranium accumulation. Sasmaz and Yaman (2008) showed that shoots of gum tragacanth milkwetch (*Astragalus gumnifer* Labill.) and roots of *Verbascum cheiranthifolium* Boiss. and *Euphorbia maccroclada* Boiss. can be used as biomonitors of uranium contamination.

2.3. Uranium accumulation in plants

2.3.1. Uranium uptake by plants

Uranium accumulation and distribution in plants has been reported by several authors. Generally, plants primarily store uranium in belowground tissues (Jain and Aery, 1997; Duschenkov et al., 1997; Soudek et al., 2011a; Lhotsky, 2011), but the mechanism of uranium uptake by plants has not been identified so far. The influence of soil pH and chemical speciation of uranium on its bioavailability was observed. Ebbs et al. (1998) showed that the free uranyl ion (UO_2^{2+}), which predominates in solution at pH 5.0 – 5.5, was the uranium species most taken up and translocated to the shoots by peas (*Pisum sativum* L.). In comparison, uptake of uranium by roots was highest at pH

6 and 8. The reduction in uranium uptake by peas in the presence of phosphate in hydroponic solution was also documented. This finding has already been described in *Arabidopsis thaliana* (L.) Heynh. (Misson et al., 2009), sunflower (*Helianthus annuus* L.) (Tome et al., 2009), duckweed (*Lemna gibba* L.) (Mkandawire et al., 2007), oilseed rape (*Brassica napus* L.) and wheat (*Triticum aestivum* L.) (Laurette et al., 2012b). These authors suggest that phosphate enhanced uranium precipitation mainly on root epidermis but inhibits its accumulation in all plant tissues. Soudek et al. (2011a) supported this finding by identification of enhanced uranium accumulation by various hydroponically cultivated plant species under phosphate deficiency. In contrast, the presence of phosphate in cultivation medium did not reduce uranium uptake by bean (*Phaseolus vulgaris* L.) (Laroche et al., 2005) and indian mustard (*Brassica juncea* (L.) Czern.) (Tome et al., 2009). These plants may have different abilities to retain the uranium-rich precipitates on their roots. Surprisingly, the presence of phosphate increased uranium uptake of *in vitro* cultivated root cultures of horseradish (*Armoracia rusticana* Gaerth. Mey. et Scherb.) (Soudek et al., 2011b). The recorded high uranium content in roots may be caused by the medium residues on root surface. Vandenhove et al. (2007) showed that plants, namely ryegrass (*Lolium perenne* L.), are able to uptake $UO_2PO_4^-$, but not UO_2PO_4, and translocate it to the shoots.

The addition of citrate or carbonate to the solution enhanced translocation of uranium from the roots to the shoots in plants (Laurette et al., 2012a; Laurette et al., 2012b). Other studies also indicated enhanced uranium accumulation in plants caused by the addition of citrate (Ebbs et al., 1998; Tome et al., 2009; Mihalik et al., 2011), or by the addition of other chelating agents such as ethylenediaminetetraacetate (EDTA) (Ebbs et al., 1998; Tome et al., 2009), [S,S]-ethylenediamine disuccinic acid (EDDS) (a structural isomer of EDTA) (Duquene et al., 2009) or tartaric acid (Soudek et al., 2011a). For example, the addition of EDTA at 5 mM U L^{-1} resulted in a 1000-fold increase of uranium content in shoot of indian mustard (*Brassica juncea* (L.) Czern.) (Huang et al., 1998). According to some authors, uranium-rich citrate complexes are taken up by plants (Huang et al., 1998), while for other authors these complexes dissociate at the vicinity of plant root and uranium is taken up as UO_2^{2+} (Ebbs et al., 1998; Laurette et al., 2012a; Laurette et al., 2012b). The possible reason for the

increase uranium accumulation is the ability of chelating agents to form complexes with uranium which could be easily taken up by plants (Soudek et al., 2011a).

Anke et al. (2009) focused on the influence of geological origin of the soil with the uranium content on the vegetation. Granite soils produce significantly uranium-richest vegetation. The flora on limestone soils contains about 25 % less uranium; and about 50 % less uranium has flora on gneiss soils and various type of sandstones. Shahandeh and Hossner (2002) observed the highest uranium accumulation in sunflower (*Helianthus annuus* L.) and indian mustard (*Brassica juncea* (L.) Czern.) grown on calcareous soils, and lowest uranium accumulation in clayey acid soils with relatively high Fe, Mn and organic fractions. Adsorption of uranium on clay minerals was probably responsible for its low uptake by plants, whereas carbonate complexes in calcareous soils enhanced uptake of uranium by plants. Particularly, the uranium-rich carbonate complexes were mainly stored on the surface or inside the roots. In fact, uranium complexation with carbonate in cultivation medium drastically increased translocation of uranium to the shoots of studied plants (Huang et al., 1998). Duquene et al. (2006) also observed higher uranium accumulation in plants from alkaline soils rather than from acid soils, which they attributed to the presence of highly soluble uranyl carbonate complexes. Lauria et al. (2009) supported this finding by identification of correlation between exchangeable Ca and Mg ions in soil and uranium content in plants. They observed the increase in uranium accumulation in plants with the increase of soil pH, caused by soil liming with dolomite ($CaCO_3 \times MgCO_3$), and formation of uranium-rich carbonate complexes.

2.3.2. Distribution of uranium within plant body

Uranium is mainly accumulated in roots. Localization of uranium in plant tissues has been already documented in early studies. Easton and Hanchey (1972) identified in oat leaves the crystallized uranium in the cell wall and in vicinity of the plasmalema of vascular cells, but not in the protoplast. It was confirmed that the Casparian strip and suberinized lamella are effective barriers to the uranium radial translocation in plant roots (Robards and Robb, 1972). Recently, Laurette et al. (2012ab) used advanced ultrastructural methods to evaluate the impact of uranium mobility in plants. The

uranium was accumulated mainly in root epidermis, precipitated in the intercellular spaces, cell walls and membranes of endocytotic vesicle. In the parenchyma cortex, uranium was localized in cell vacuoles and cell walls (**Figure 1**). In leaves, uranium was mainly localized in cell walls and membranes of vascular cells, some uranium was also observed in leaf epidermis and mesophyll cells.

In lichens, there has been documented that uranium is accumulated in the exciple and epithecium, and uranium is adsorbed to melanin-like pigments (McLean et al., 1998).

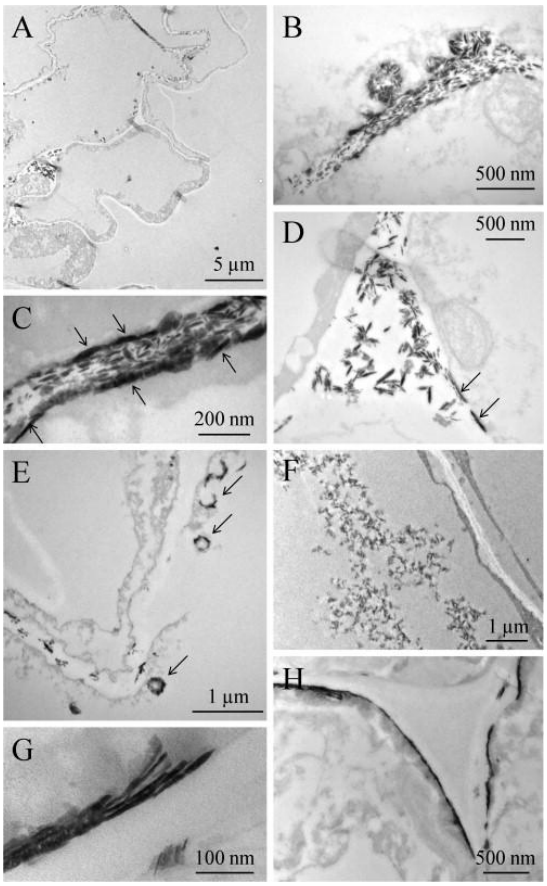


Figure 1. *Uranium distribution in plant root analyzed by TEM.*
Observation of sunflower (A-F) and oilseed rape (G and H) roots exposed to 100 µM U L^{-1} for 72 h (Adopted from Laurette et al., 2012b).

2.3.3. Effect of mycorrhiza on uranium accumulation

Plant associated microorganisms including mycorrhizal fungi may have significant effects on uranium accumulation in plants (Rufyikiri et al., 2002; Rufyikiri et al., 2003). It can affect uranium speciation and thus uranium bioavailability by modifying the pH, extracellular binding, transformation and formation of complexes (Duschenkov, 2003).Rufyikiri et al. (2003) indicated that the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* N.C. Shenck & G.S. Sm. was able to take up, adsorb and translocate uranium to roots of carrot (*Daucus carrota* L.) cultivated *in vitro*. The uptake of uranium in fungal biomass was highly influenced by the pH, while the translocation was correlated with the number of fungal hyphae. Chen et al. (2005) observed that uranium translocation to shoots was greatly reduced by mycorrhizal colonization due to uranium sequestration in fungal structures. Furthermore, uranium accumulation in plants was influenced by the addition of

phosphorus to the growth solution. Chen et al. (2008) investigated the role of AM fungus *Glomus intraradices* N.C. Shenck & G.S. Sm. on uranium accumulation in two plant species, i.e. barrel medic (*Medicago truncatula* Gaertn.) and ryegrass (*Lolium perenne* L.). This study showed that ryegrass produced more extensive root system and greater biomass than medic plant, while medic plants showed greater ability to accumulate uranium in their tissues. Mycorrhizal colonization reduced both uranium uptake and accumulation in shoots, whereas enhanced uranium accumulation in roots, even in the case of ryegrass with low colonization levels. Authors concluded that ryegrass inoculated by AM fungus is an efficient plant species for phytostabilization of uranium.

2.3.4. Uranium toxicity for plants

There is contradictory information on the phytotoxicity of uranium to plants. Some studies report that uranium enhances the plant growth at very low concentrations. Gulati et al. (1980) observed that the wheat yield increased significantly to a certain level with increase of uranium concentration. Another study observed the increase of growth of wheat in the lowest uranium treatment (1 μg U g^{-1}) (Aery and Jain, 1998). Meyer et al. (1998) confirmed the evidence of hormesis (i.e. the existence of generally favorable biological responses to low exposures to toxins and other stressors) in plants exposed to uranium. They suggested that a potential mechanism for the hormesis may be enhanced uptake of phosphorus due to the interaction of uranium with phosphate to form complexes. Sheppard et al. (1992) reviewed the data on uranium toxicity to plants. They identified no effect for uranium in soil of 100 mg U kg^{-1}. There is evidence that the toxic effect of uranium is the result of chemical toxicity rather than the radiation-related toxicity. This idea is based on minimal radiation measured in soil and air around experimental plants (Sheppard et al., 1983).

2.4. Effect of uranium on plant metabolism

It was already shown that heavy metals induced multiple changes in plant metabolism (summarized in thesis Lábusová, 2010). However, only limited information is available on uranium toxicity in plants at a molecular level. Cellular membranes and enzymes are first targets of metal toxicity. Their damage causes other secondary changes in metabolism, such as dislocation in uptake of minerals, inhibition of photosynthesis and changes in metabolism of carbohydrates. Schutzendubel and Polle (2002) distinguish three main mechanisms of molecular metal toxicity:

1. Production of reactive oxygen species (ROS) by autoxidation and Fenton reaction.
2. Blocking of essential functional groups in biomolecules.
3. Displacement of essential metal ions from biomolecules.

2.4.1. Effect of uranium on nutrient contents

Chemical toxicity of uranium is predominantly caused by the high affinity of uranyl cation (UO_2^{2+}) for oxygen binding centers. According to the classification of metal ions by (Nieboer and Richardson, 1980), the uranium belongs among class A metal ions, which seek out oxygen binding sites (caboxylate, carbonyl, alcohol, phosphate or phosphodiester). It resembles Ca^{2+} and Mg^{2+} but UO_2^{2+} forms complexes of higher stability than other two ions. Hence, UO_2^{2+} seeks out same binding sites as Ca^{2+} and Mg^{2+}. This competition for binding sites can result in nutrients depletion. Boileau et al. (1985) observed a 80% decrease in Ca^{2+} content in reindeer lichen (*Cladonia rangiferina* (L.) Weber. Ex F.H. Wigg.) grown in cultivation medium containing different species of uranium. It is assumed that extracellular Ca^{2+} was probably removed due to the competition between UO_2^{2+} and Ca^{2+} for cell wall exchange sites. But this phenomenon is not supposed to be important for naturally grown plants due to lower uranium concentration in soil solution (Straczek et al., 2009). Therefore, these authors used for experiments the hairy root culture of carrot which was grown in the gel solution. That experimental design allows same conditions in the gel solution as in the soil but in standardized and reproducible orders. In contrast, Ca^{2+} concentration in the root culture increased with uranium concentration,

probably because of precipitation in the apoplasm of dying roots. Uranium exposure causes decrease in concentrations of other nutrients in plant tissues, e.g. Mn, K, P, S (Vanhoudt et al., 2008). Viehweger and Geipel (2008) observed that the absence of iron in the cultivation solution lead to lower iron uptake whereas enhanced uranium accumulation in roots and shoots of *Arabidopsis halleri* L. plants respond to iron deficiency by production of root exudates. These exudates reduce the biologic inaccessible Fe^{3+} to Fe^{2+}, which can be consequently taken up by plants. Therefore, a reduction of Fe^{3+} to Fe^{2+} at the root surface can be probably accompanied by a similar reduction of U^{6+} to U^{4+}, which is more bioavailable for plants.

UO_2^{2+} has also a strong affinity to phosphate and carboxyl groups. Gunther et al. (2003) showed that uranium co-localized with phosphate in lupine (*Lupinus angustifolius* L.) tissues. Their observation was confirmed by further studies (Vanhoudt et al., 2008; Misson et al., 2009; Laurette et al., 2012b). All studies concur in the opinion that uranium precipitates with P and Ca^{2+} on the root epidermis. Phosphate-rich granules were found also inside the root where they are localized on cell walls and membranes of parenchymal or vascular cell. Some of them were not fixed on membranes, but were rather free in the intercellular spaces (Laurette et al., 2012b). Intracellular phosphate-rich granules were also observed in vacuoles or at the high uranium concentration treatment (50 µM U L^{-1}) in the nucleus, where may cause DNA damages (Misson et al., 2009).

As mentioned previously, the uranium strongly interacts with phosphate, both in the soil and inside the plant. Phosphate supply in the soil or cultivation medium can significantly decrease uranium bioavailability and toxicity in plants (Ebbs et al., 1998; Rufyikiri et al., 2006). It is mainly due to the formation of mostly insoluble precipitates of $(UO_2)_3(PO_4)_2 \times 6H_2O$ in the substrate. In this condition, only 1.5 % of uranium is in the soluble UO_2HPO_4 form, which may be retained by roots (Laurette et al., 2012b). Contrary, uranium was shown to disturb phosphate homeostasis in plants. The presence of uranium in substrate caused the induction of the high affinity phosphate transporter gene *PHT1;4* expression and the increase in phosphate remobilization from leaves to roots. In roots, this phosphate is used to immobilize uranium by forming phosphate-rich granules (Misson et al., 2009).

2.4.2. Genotoxicity of uranium

Panda et al. (2001) found that exposure of onion bulb (*Allium cepa* L.) to uranium ions results in increased micronuclei formation, but frequencies of cells with micronucleus or with chromosome aberrations were inconsistent in root meristem cells. This implicate that uranium was neither clastogenic nor aneugenic. However, the number of chromatide exchange significantly increased already at the lowest uranium concentration (25 μM U L^{-1}). This was interpreted through interacting with DNA and/or interfering with DNA replication-repair processes. The severe DNA damages were also observed in root cells of dwarf bean (*Phaseolus vulgaris* L.) at the highest uranium concentration (1000 μM U L^{-1}) (Vandenhove et al., 2006). At the lower uranium concentration, the comet assay did not indicate increased DNA migration. This means that no strand breaks were occurred or that efficient DNA repair mechanisms were activated. In shoot cells, no DNA damage was observed.

2.4.3. Effect of uranium on photosynthesis

Boileau et al. (1985) documented uranium toxicity on photosynthesis. Under uranium exposure, a reduction in the total ^{14}C-fixation rates and decreased photosynthetic activity was observed in reindeer lichen (*Cladonia rangiferina* (L.) Weber. Ex F.H. Wigg.). Authors ascribed this effect to the interference of UO_2^{2+} with bicarbonate transfer into the lichen cell and blocking of the carbohydrate transport from algal partner. Decrease in the total chlorophyll content under uranium exposure was observed in water hyacinth (*Eichhornia crassipes* (Mart.) Solms) (Hafez and Ramadan, 2002), sunflower (*Hellianthus annuus* L.) (Jagetiya and Purohit, 2006) and *Arabidopsis halleri* L. (Viehweger and Geipel, 2010). Similar phenomenon was also observed in couch grass (*Triticum repens* (L.) Gould) and greater plantain (*Plantago major* L.), where larger decline in chlorophyll contents was observed in case of uranium than thorium treatment (Shtangeeva et al., 2006). In sunflower, the gradual and significant decreases in chlorophyll *a*, *b* and total chlorophyll contents were observed in plants grown on uranium tailings with maximum decrease at the 75% tailing concentration (tailing mixed with garden soil) (Jagetiya and Purohit, 2006). On the contrary, Hafez and Ramadan (2002) observed increased content of chlorophyll *a*

while chlorophyll *b* content decreased in water hyacinth (*Eichhornia crassipes* (Mart.) Solms). Uranium caused slighter alterations in chlorophyll contents than other studied metal ions contained in liquid wastes (Cd^{2+}, Pb^{2+}, Ni^{2+}, Th^{4+} and Fe^{3+}). Decrease in chlorophyll biosynthesis may be explained by replacement of the central Mg^{2+} by uranium in chlorophyll molecules (Jain and Aery, 1997) as it was previously described for other heavy metals (Kupper et al., 1996). Additionally, the drop of chlorophyll contents could be caused by a peroxidative breakdown (Viehweger and Geipel, 2010).

It was reported repeatedly that the photosystem I is not inhibited by heavy metals (or slightly), whereas the photosystem II is inhibited completely (Clijsters and Vanassche, 1985; Appenroth et al., 2003). Decrease in fluorescence in response to copper exposure showed that only chlorophyll molecules associated with photosystem II, which is responsible for most of the fluorescence, were accessible to heavy metal ions at low concentrations. At higher concentrations combined with shade, magnesium-substitution took place in all chlorophyll molecules (Kupper et al., 1996). Unfortunately, the impact of uranium on the individual photosystem has not been studied yet.

2.4.4. Effect of uranium on carbohydrate metabolism

Beside the crucial role of carbohydrates as substrates in carbon and energy metabolism and in polymer biosynthesis, they are also involved in stress responses and signaling pathways. Sugar sensing and signaling mechanisms are the main factors controlling growth and development of plants. Generally, a low concentration of carbohydrates in the plant tissues increases photosynthesis, mobilization and export of supplies; however a high concentration promotes the growth and storing of nutrients (Koch, 1996). So, carbohydrates play an important role in protecting plants against varying environmental conditions. A common function of carbohydrates in plant responses to abiotic stress is the accumulation as a compatible solutes that are non toxic for cell at high concentrations (Wang et al., 2000). Frequently observed compatible solutes are carbohydrates (e.g. sugars and sugar alcohols), amino acids and other amino compounds (prolin, glycinbetain). Apart from their role in osmotic protection, they have also osmoprotective functions. Due to their hydrophilic

structure, they could replace water at the surface of biomolecules, thus preserving their biological functions (Hasegawa et al., 2000).

The heavy metal stress leads to alterations in carbohydrate metabolism, but there are a limited number of studies on this aspect. Furthermore, plant responses to heavy metal stress differ among particular plant species, and also vary depending on particular heavy metal. The effect of uranium on carbohydrate metabolism is even less studied. (Hafez and Ramadan, 2002) detected in leaves of water hyacinth (*Eichhornia crassipes* (Mart.) Solms) the reduction in content of total soluble carbohydrates in response to uranium exposure. Authors attributed the reduction to alterations in photosynthetic apparatus. Equivalent results were observed by Morsy (2008) but only for some tested plants (e.g. desert date (*Balanites aegyptiaca* (L.) Delile, *Capparis galeata* Fresen. and henbane (*Hyoscyamus muticus* L.)). Other plants had antagonistic responses to the uranium exposure (e.g. colocynthis (*Citrullus colocynthis* (L.) Schrad) and caper bush (*Capparis spinosa* L.)).

2.4.4.1. Carbohydrate metabolism under heavy metal exposure

There are three main enzymes involved in sucrose metabolism. Sucrose phosphate synthase (SPS) catalyzes the synthesis of sucrose. SPS play a major role in sucrose biosynthesis (Huber and Huber, 1996). Sucrose synthase (SS) and invertase (IT) are involved in the sucrose breakdown. SS is a cytosolic and reversible enzyme that catalyzes sucrose degradation/synthesis and supplies ADP-glucose and UDP-glucose for synthesis of cell wall polysaccharides and starch (Baroja-Fernandez et al., 2003). In contrast to SS, IT catalyzes irreversible hydrolysis of sucrose to glucose and fructose. Plants carry three types of IT based on their pH optima and solubility. Soluble acid IT is located in vacuoles, whereas an extracellular acid IT is bounded to the cell wall. The soluble alkaline IT is located in cytoplasm (Sturm, 1999). Generally, heavy metal exposure generates decrease in the activity of sucrose synthesizing enzymes, i.e. SPS in plant tissues (Verma and Dubey, 2001; Mishra and Dubey, 2008; Choundhury et al., 2010). These findings concord with the observed decrease in the sucrose content under heavy metal exposure (Costa and Spitz, 1997; Guangqiu et al., 2007). Increased activities of SS and IT are found to be parallel with increased conversion of non-

reducing sugars (sucrose) to reducing sugars (hexoses) under heavy metal stress (Verma and Dubey, 2001). Increase in reducing sugars contents may provide an adaptive mechanism to protect the membranes and biomolecules (Choudhury et al. 2010). However, some plants increase the content of sucrose in their tissues or accumulate sucrose in certain organs during heavy metal stress. As an example might be taken cucumber (*Cucumis sativus* L.) that accumulated sucrose in leaves under copper exposure. It can be attributed to the reduction in phloem up-loading and translocation of assimilates to other plant organs. Moreover, the observed decline in magnesium content in leaves could contributed to the reduction in net assimilation rate and to the accumulation of assimilates in leaves (Alaoui-Sosse et al., 2004). Decrease SS and IT activity with a slight increase of sucrose content was reported in nodules of white lupin (*Lupinus albus* L.) as a response to heavy metal exposure. Inhibition of SS limits the availability of carbon to the bacteroid respiration and thus might be responsible for the decline in N_2 fixation (Sanchez-Pardo et al., 2013).

Activity of alkaline IT is not inhibited by heavy metals, whereas activity of both acid ITs is altered by heavy metal ions. Differences between the acid and alkaline IT may relate to their molecular structure (Roitsch et al., 2004). Moreover, acid ITs are involved in the regulation of sink strength and hexoses production following abiotic and biotic stress that require fast induction of sink metabolism for the ability to activate a cascade of defense responses and to mediate physiological adaptations (Sturm, 1999; Roitsch et al., 2003). The important role of acid ITs in tolerance of plants to heavy metal stress was confirmed by (Xiong et al., 2008).

Raffinose family oligosaccharides (RFOs) have been proposed to fulfill important role in plant stress responses. RFOs are accumulated during heavy metal stress. Recent results suggest that RFOs can act in the osmoprotection (Sun et al., 2010), and also in the antioxidative defense (Van den Ende and Valluru, 2009). The important role of fructans in heavy metal stress conditions was also established. Their role is either to quench reactive oxygen species (ROS) produced during stress or indirectly stimulate other antioxidant. Changes in contents of fructans correlate with levels of antioxidants like glutathione or ascorbate. Fructans react with ROS, to form oxidized fructan free radicals (OFFRs), these OFFRs may be rapidly reduced again to

fructans by classical antioxidants (Van den Ende and Valluru, 2009). In addition to their roles as a source of hexoses, fructans were proved to stabilize membranes during abiotic stress by inserting polysaccharides into the lipid headgroup site of membranes (Livingston et al., 2009).

2.4.5. Effect of uranium on N-containing metabolites

It is clear that nitrogen metabolism has significant role in response of plants to heavy metals. Accumulation of some N-metabolites displays a variety of functions such as metal chelation, antioxidant defence, protection of macromolecules, and also signaling. Review of (Sharma and Dietz, 2006) fairly summarized available information on accumulation of N-metabolites, mainly proline, under heavy metal stress (**Figure 2**).

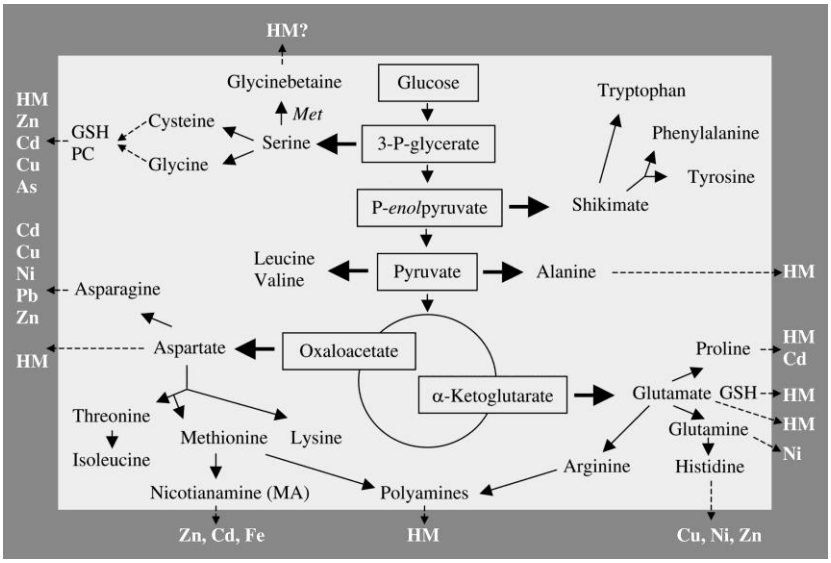


Figure 2. *Schematic depiction of N-containing metabolites involved in plant responses to heavy metal stress* (Adopted from Sharma and Dietz, 2006).

Lhotsky (2011) ascertained alterations in contents of amino acids, mainly glutamine, asparagine, serine and proline, in hydroponically cultivated tobacco (*Nicotiana tabbacum* L.) at high concentration of uranium (500 µmol U L^{-1}). Alterations in amino acids contents took place mainly in leaves. In roots, only proline content increased significantly under uranium exposure. The biggest divergences from control plants showed plants grown in the medium with addition of organic acids, mainly citric and tartaric acids. Negligible alterations in amino acids contents were found in plants grown in the medium without phosphate, although they showed the highest uranium

accumulation. The author supposed that increase in amino acids contents in treatments with organic acids was due to the joint influence of uranium and these acids. A matter of great interest is that contents of most amino acids decreased after 7 days of uranium exposure, whereas after 14 days their levels increased in comparison with values at the beginning of the experiment. Experiments with water thyme (*Hydrilla verticillata* (L.f.) Royle) also showed increased contents of proline and total phenolics in plant tissues under uranium exposure (Srivastava et al., 2010). Proline is known for its beneficial functions under heavy metal exposure, i.e. metal binding, antioxidative defense, and signaling (Matysik et al., 2002).

Another scientist in his dissertation (Morsy, 2008) studied uranium effect on amino acids representation in different plant species. His experiments were carried out using the plants grown in natural field conditions like colocynthis (*Citrullus colocynthis* (L.) Schrad), caper bush (*Capparis spinosa* L.), henbane (*Hyoscyamus muticus* L.) and *Capparis galeata* Fresen. The presence of uranium in soil had different impact on amino acids contents from one amino acid to another. Levels of glycine, alanine, serine, histidine, tyrosine, lysine, and threonine were mostly elevated in leaves of tested plants. However, levels of leucine and methione increased in roots under uranium stress. These alterations in amino acids levels are not explained in this study.

Additionally, complexation of U^{6+} with the amino acids threonine (Gunther et al., 2006), and glycine or cysteine (Gunther et al., 2007) was observed in aqueous solution. Glycine, cysteine and glutamic acid are fundamental elements of phytochelatins, which play an important role in detoxification of heavy metals. Another study showed an increase in concentrations of flavonoids in cell suspensions of canola (*Brassica napus* L.) treated by uranium. Due to a strong complex formation between flavonoid quercetin and heavy metal, they supposed that flavonoids are important part of defence mechanism against uranium (Viehweger and Geipel, 2008).

2.4.6. Antioxidative defence system

Uranium causes oxidative stress in plant tissues as other heavy metals, which results in an increased production of reactive oxygen species (ROS). The chief toxicity of ROS resides in initiation of cascade reactions that can result in the cell death by lipid peroxidation, enzyme inhibition, protein oxidation, and DNA or RNA damages. ROS are also produced in plants under normal circumstances. They are not only toxic byproducts of aerobic metabolism, but also serve as signaling molecules and control whole plant metabolism. The formation of ROS under normal conditions must be tightly regulated. Mittler et al. (2004) affirmed that at least 152 genes are involved in controlling the level of ROS in *Arabidopsis thaliana* (L.) Heynh. The major sources of ROS in normal plant cells are organelles with a highly oxidizing metabolic activity such as chloroplast and mitochondria. ROS are also formed on other membranes such as peroxisome, glyoxisome, tonoplast or plasma membrane. To regulate the amount of ROS in cells, plants use an antioxidative defence system comprising numerous enzymes and compounds of low molecular weight (Noctor and Foyer, 1998).

Responses of the individual plant parts to oxidative stress triggered by uranium are different. Results from study on *Arabidopsis thaliana* (L.) Heynh. provide an evidence that RBOHD (isoenzyme of NADPH-oxidase) and LOX1 (cytosolic isoenzyme of lipoxygenase) are involved in early oxidative burst in the roots during uranium stress, which could influence the root-to-shoot signaling. Latest evidence exists for the influence of *LOX1* expression on jasmonate biosynthesis (Keunen et al., 2013). Furthermore, it is suggested that jasmonates are key signaling molecules during heavy metal stress (Maksymiec and Krupa, 2002). Vanhoudt et al. (2011ab) showed that the oxidative stress related responses was only present at the highest tested uranium concentration of 100 µM U L^{-1}. The activity of superoxide dismutase (SOD) as an important enzymatic ROS scavenger immediately increased in roots which was accompanied by a simultaneous increase in *FSD1* (plastidic iron SOD) transcript level whereas a down regulation of *CSD1* (cytoplasmic copper/zinc SOD) and *CSD2* (plastidic copper/zinc SOD) expression was found. A decrease in expression of *CSD1* and *CSD2* could be mediated by sucrose via miR398 expression that its down regulation was found in response to copper stress (Dugas and Bartel, 2008). In the roots, catalase

(CAT) was observed also as a rapid enzymatic ROS scavenger during uranium stress, particularly *CAT1* (peroxisomal catalase), was responsible for a fast trigger in CAT activity. An increased in peroxidase (PX) activity was observed at a later stage of uranium stress (Vanhoudt et al., 2011ab). Significantly increase activities under uranium stress were observed for two peroxidase isoenzymes - guaiacol peroxidase (GPX) and syringaldazine peroxidase (SPX) (Vandenhove et al., 2006; Vanhoudt et al., 2008). Equivalent results were observed in several studies with different heavy metals and plant species (Cuypers et al., 2002; Smeets et al., 2008). Peroxidases are known to have key roles in synthesis of lignin. It was shown that SPX is mainly located extracellularly and therefore it is implicated in lignification processes (Cuypers et al., 2002). GPX is also associated with cell wall lignification (Mazhoudi et al., 1997), and consequently with a decrease of plant growth. The activity of ascorbate peroxidase (APX) increased in roots under uranium exposure and seemed to play an important role in the regeneration of oxidized ascorbate (Vanhoudt et al., 2011ab). In roots, the regeneration of ascorbate by glutathione was hampered under uranium exposure. This may be explained in the drop of glutathione reductase (GR) activity observed for roots of beans (*Phaseolus vulgaris* L.) exposed to uranium (Vandenhove et al., 2006).

In leaves, the oxidative stress is probably generated via root-to-shoot signaling while an increase of membrane damage was also indicated. In roots, the important role in lipid peroxidation was attributed to *LOX1* under uranium stress whereas *LOX2* (plastidic lipoxygenase) seemed to be more important in responses of leaves to uranium stress (Vanhoudt et al., 2011ab). Smeets et al. (2008) also reported that *LOX1* transcript levels were affected only in roots under cadmium stress while no significant effect was observed in leaves. The up-regulation of *LOX2* expression in uranium-treated leaves could be attributed to the chloroplast as the important leaf sensing site during uranium stress. The ascorbate redox balance seemed to be the most important modulator (more than glutathione redox balance) of early uranium stress responses of leaves. The ascorbate pool significantly increased under uranium exposure characterized by an augmentation towards its reduced form (Vanhoudt et al., 2008). Moreover, ascorbate has another important role in regeneration of α-tocopherol, which was found to be the potential ROS scavenger in chloroplasts during heavy metal

stress (Sun et al., 2010). Uranium also induced accumulation of the total glutathione content but it seemed to be more sensitive than ascorbate under uranium stress (Vanhoudt et al., 2011ab).

Srivastava et al. (2010) studied biochemical responses to uranium exposure in water thyme (*Hydrilla verticillata* (L.f.) Royle) as a representative of aquatic plants. Their results indicated that antioxidative enzymes played a major role in early ROS scavenging while on longer durations, thiolic metabolites such as glutathione took over their function in antioxidative defence mechanism. An additional glutathione defence mechanism towards uranium toxicity includes the ability of reduction U^{+6} to U^{+4} by the complex formation between glutathione and uranium ion (Viehweger et al., 2010).

2.4.7. Simultaneous effect of uranium with other pollutants

In areas contaminated by uranium, other contaminants are usually present. Plant responses to these multiple contaminants also need to be studied. Scientists in the Belgian Nuclear Research Centre focused on responses of *Arabidopsis thaliana* (L.) Heynh. seedlings exposed to uranium in combination with cadmium. Their results showed the harmful effect of both uranium and cadmium on seedlings. Cadmium highly increased the accumulation of uranium in roots. Moreover, cadmium was taken up less by roots but showed higher translocation to shoots. Also micronutrient contents, especially those of manganese and iron, were strongly affected in roots by the addition of cadmium as a second stressor, while leaf macronutrient contents were mostly influenced by uranium. This can be illustrated by the observed increase in calcium and magnesium contents under uranium exposure and mixture conditions (Vanhoudt et al., 2010). Relatively low production of H_2O_2 in leaves of *Arabidopsis thaliana* (L.) Heynh. was observed under uranium exposure while cadmium and mixture conditions induced significant H_2O_2 production after 24h and 168h, respectively. In uranium treatment, the increased lipoxygenase activity was probably able to keep ROS production under control (Horemans et al., 2011). Mkandawire and Dudel (2005) showed that arsenic could transfer more easily from contaminated waters to high trophic levels than uranium. It was presented in duckweed (*Lemna gibba* L.) grown in tailing waters of abandoned uranium mine sites. Chinese brake fern

(*Pteris vittata* L.) has been identified as an arsenic hyperaccumulator (Cao et al., 2004), it can accumulate arsenic up to 22 630 mg As kg^{-1} DW. Therefore, Chen et al. (2006) used this fern as a possible species for phytoremediation. They demonstrated that fern could greatly accumulate uranium as well as arsenic. The inoculation of fern by AM fungus *Glomus intraradices* N.C. Shenck & G.S. Sm. increased uranium accumulation. It is possible that increased content of uranium in fern was a result of uranium adsorption by mycorrhizal hyphae.

Previous studies have shown that effect of multiple heavy metal stress can be different markedly from the effects induced by the stressor individually. Ecosystems are also obviously exposed to combinations of anthropogenic and natural stressors such as ionizing radiation. Therefore, combined effects of uranium and Ionizing radiation should not be neglected when evaluating the impact of uranium on the environment. Study focused on effects of *Arabidopsis thaliana* (L.) Heynh. plants exposed to uranium and *gamma* radiation showed that harmful effects were mostly generated by uranium presence (Vanhoudt et al., 2010). The lack of deepen assessments of combined stressor exposures and resulting possible interactions urgently needs to be resolved.

3. Materials and methods

3. 1. Experiments with the tobacco plants

3.1.1. Plant material and cultivation conditions

For experiments, the tobacco (*Nicotina tabacum* L.) cv. La Burley 21 was used. This cultivar was chosen according to the results from previous experiments performed at the IEB. The tobacco seeds were sown in moist perlite and cultivated for two months. The seedlings were watered with Hoagland medium every 3 days (Hoagland and Arnon, 1938). Plants ready for experiments were placed for two weeks into Araponics boxes (Araponics SA, Belgium) supplemented with 2 L of Hoagland hydroponic medium to acclimate to the hydroponic conditions (**Figure 3**). The hydroponic medium was prepared by mixing of the storage solutions and diluting by distilled water with pH adjusted to 7.6 using 0.1M NaOH. The composition of medium is shown in the **Table II**. The Plants were cultivated in the culture chamber under light conditions (72 µmol m^{-2} s^{-1}; 16/8 h photoperiod) at 20°C and 60% humidity. The last experimental replication was conducted in the greenhouse during October (10/14 h photoperiod) at 20-25°C.

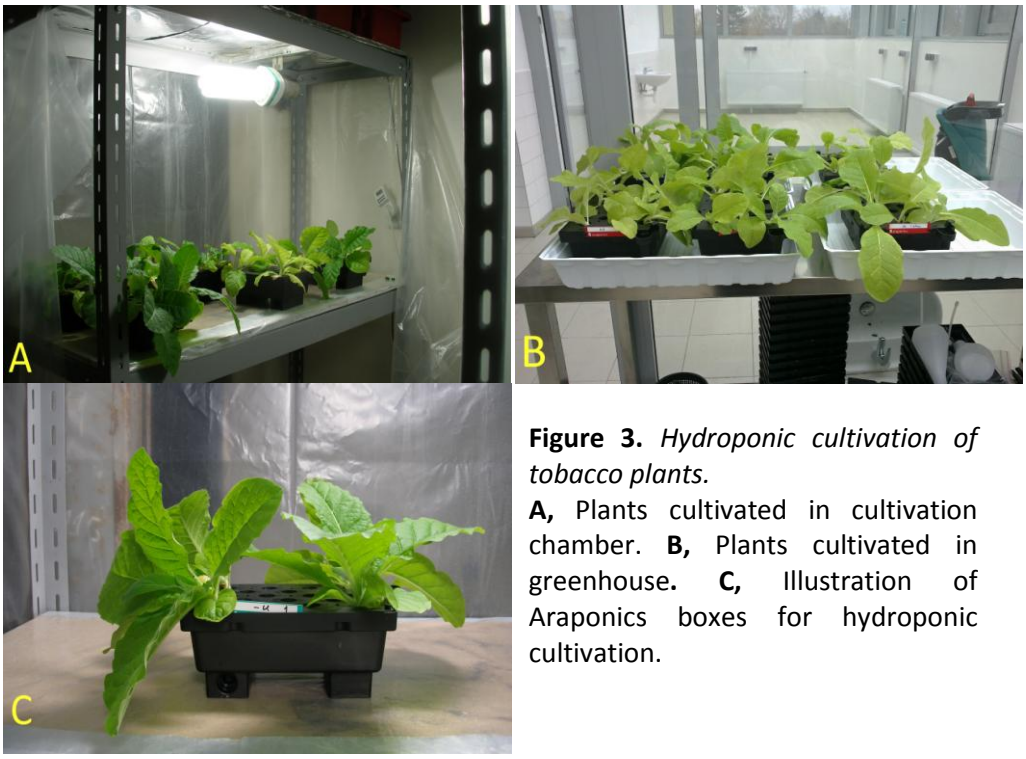


Figure 3. *Hydroponic cultivation of tobacco plants.*
A, Plants cultivated in cultivation chamber. **B,** Plants cultivated in greenhouse**. C,** Illustration of Araponics boxes for hydroponic cultivation.

Storage solution	1	2
A Macroelements	$MgSO_4 \times 7H_2O$	369.7
	K_2SO_4	348.51
	$CaCl_2 \times 2H_2O$	588.07
B Phosphates	$NaHPO_4 \times 2H_2O$	291.73
	$Na_2HPO_4 \times 12H_2O$	46.56
C Microelements	H_3BO_4	8.58
	$MnSO_4 \times 4H_2O$	4.64
	$ZnSO_4 \times 7H_2O$	0.66
	$CuSO_4 \times 5H_2O$	0.82
	$Na_2MoO_4 \times 2H_2O$	0.06
D Iron	$FeSO_4 \times 7H_2O$	17.92
Nitrogen	$NaNO_3$	339.98
	NH_4Cl	213.97
	NH_4NO_3	160.09

Table II. *Composition of Hoagland medium utilized for hydroponic cultivation of tobacco plants*.
1, Components of storage solutions. **2,** Concentration of individual components in 1 L of hydroponic medium (mg L^{-1}).

3.1.2. Experimental designs

The tobacco plants were cultivated in Araponics boxes supplemented with 2 L of Hoagland hydroponic medium containing uranyl nitrate ($UO_2(NO_3)_2$) in the final concentration of 0.5mM. The composition of medium was modified by various amendments. The following variants were used:

- Hoagland medium (HM).
- Hoagland medium without phosphate (0P).
- Hoagland medium modified to contain 0.1mM citric acid (CA).
- Hoagland medium modified to contain 0.1mM tartaric acid (TA).
- Hoagland medium with the daily spraying of plants with 0.75mM solution of putrescine (PA).

Seven replications for each treatment were used. The samples for biochemical analysis were harvested after 14 days of the cultivation. Roots were washed three times with distilled water to remove surface uranium precipitates. Then the root length and biomass (scanned with WinRhizo), leaf surface area and shoot length was evaluated (these results will be demonstrated in Otradovcova's thesis). The samples for biochemical analysis were collected from root, stem, young leaf (3-4th) and old leaf (6-8th). In the last experiment, the samples were taken only from the aboveground and belowground parts. The samples were dried with the filter paper, inserted into

beforehand weighted eppendorf tubes, frozen in liquid nitrogen immediately and stored at -80 °C until the following analysis.

3.1.3. Carbohydrate content determination

The deeply frozen sample was freeze-dried (Lyovac GT 2, Finn-Aqua) for 12 hours. Subsequently, the dry weight (DW) was determined and the sample was boiled in 80% methanol (0.5 mL) at 75 °C for 10 min. The solvent was then evaporated for about 3 hours in the centrifugal evaporator (Speedvac Plus SC110 A, Savant). The residue was dissolved in MiliQ ultrapure water in dependence on the sample DW (**Table III**) in an ultrasonic bath (Julabo USR 5414 D) for 15 min and centrifuged (Eppendorf Centrifuge 5414 D) for 10 min at 14,000×g. The supernatant was filtered through a membrane filter (Millex Milipore, porosity 0.45 µm) into 0.5ml eppendorf tube. The samples were kept at -20 °C until the subsequent analysis. The content of carbohydrates was determined using the high-performance liquid chromatography (HPLC) with the refractometric detection. A volume of 10 µL of the sample was used for the HPLC analysis. The parameters of the HPLC: temperature – 80 ° C; flow rate – 0.5 mL min^{-1}; pre-column – Hema-Bio 1000 SB+Q (Watrex, CZ); column – Hi-Plex Ca^{2+} (Polymer Laboratories Ins.) or Pb^{2+} (Watrex, CZ); eluent – Synergy MiliQ ultrapure water 18.2 (Merck Milipore, U.S.A.); pump – isocratic pump HPI-300 (SISw, CZ); detector – refractometer (Shodex R1-71); PC software – Clarity for Windows (DataApex, CZ); standards from Sigma-Aldrich.

Table III. *Volume of MiliQ ultrapure water for carbohydrate extraction.*

DW (mg)	Volume of water (mL)
5 -10	0.3
10 -20	0.5
20 -30	1
<30	1.5

3.1.4. Assays of antioxidative enzymes

3.1.4.1. Extraction

The frozen plant sample was ground with a mortar and pestle in the presence of liquid nitrogen. It was weighted out about 1.8 g of the homogenized plant tissue and then 18 mL (ten times amount of the sample mass) of cold 0.1M Tris (tris(hydroxymethyl)-aminomethane)/HCl buffer (pH 7.8) containing 5 mM EDTA (ethylenediaminetetraacetate), 1%PVP (polyvinylpyrrolidone), 5 mM DTE (dithioerythriol) and 1% Nonidet P40 (4-nonylphenyl-polyethylene glycol) was added. The mixture was cooled in an ice bath on magnetic stirrer for 30 min; then centrifuged at 25,000×g and 4 °C for 30 min (Ultracentrifuge L7, Beckman; rotor Ti 50.2). The volume of the supernatant was measured after the filtration through a nylon mesh (Miracloth Filter, Calbiochem) and poured into a new beaker. The supernatant was subjected to a two-step ammonium sulphate protein precipitation.

3.1.4.2. Protein precipitation by ammonium sulphate

Ammonium sulphate ($(NH_4)_2SO_4$) was gradually added to the beaker containing the sample supernatant according to the following formula:

The amount of $(NH_4)_2SO_4$ = 0.2254 × volume of supernatant.

After 30 min of the mixing in an ice bath, the solution was centrifuged at 25,000×g and 4 °C for 30 min. The supernatant was again filtrated and the measured volume was poured into a new beaker and put on an ice bath. The volume of $(NH_4)_2SO_4$ was again gradually added to the solution according to the following formula:

The amount of $(NH_4)_2SO_4$ = 0.258 × volume of supernatant.

Subsequently, the solution was mixed in an ice bath for 30 min, and then centrifuged at 25,000×g and 4 °C for 30 min. Thereafter, the supernatant was decanted and the resulting 40-80% ammonium sulphate fraction (pellet) was used for the desalination.

3.1.4.3. Protein desalination

The PD-10 column (Sephadex G25-M, Ge Healthcare) was five times rinsed with 25mM Tris/HCl buffer (pH 7.8). The pellet was dissolved in 2.5 mL of Tris/HCl buffer, and then the solution was inserted into the column. The sample was eluted by 3.5 mL

of Tris/HCl buffer and the eluted fraction was collected to the 30mL falcon tube. The eluted protein solution was divided into 500µl aliquots, frozen in liquid nitrogen and stored at -80 °C until the subsequent analysis. After the elution, the PD-10 column was washed with 10mL of 0.1M HCl, and then with 20mL of distilled water. The columns were stored in the fridge until the next use.

3.1.4.4. Protein assay

For determination of the total sample protein content the Bradford protein assay was used (Bradford, 1976). The method is based on the proportional binding of Coomassie Brilliant Blue G-250 dye to the protein. The standard protocol was performed as a 250 µL microplate assay with bovine serum albumin (BSA) standard. The linear part of a curve was ranging from 125 to 1,000 µg mL^{-1}. A volume of the each standard (5 µL) and the protein solution was pipeted into the separate microplate wells. A volume of the dye reagent (250 µL) was added with the multichannel pipet. The sample was incubated for 10 min at the room temperature. The absorbance was measured at 595 nm using UV-visible spectrophotometer (Tecan Infinite N200). A standard curve was created by the plotting values versus their concentration in µg mL^{-1}. The protein content of sample was calculated.

3.1.4.5. Enzyme activity assays

The specific activity of enzyme was measured in the protein solution at 25 °C using spectrophotometer (Tecan Infinite N200). The activity of peroxidase (PX; EC 1.11.1.7) was determined using standard substrates guaiacol and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) according to Drotar et al. (1985). Hydrogen peroxide (H_2O_2) and ascorbate were utilized as substrates for catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APX; EC 1.11.1.11), respectively (according to Verma and Dubey (2003) and Vanacker et al. (1998), respectively). The glutathione S-transferase (GST; EC 2.5.1.18) activity was determined using standard substrates 1-chloro-2,4-dinitrobenzene (CDNB) and p-nitrobenzoyl chloride (pNBC), the procedure of Habig et al. (1974) was utilized. The individual assays are listed in the **Table IV**.

Table IV. *Assays for individual enzymes*.
1, Concentration of reagent in storage solution (mM). **2,** Volume of reagent storage solution for buffer (mL). **3,** Pippeted volume of buffer to microplate well (µL). **4,** Pippeted volume of protein solution to microplate well (µL).

Enzyme	Substrate	Reagent	1	2	3	4
PX	Guaiacol	Tris/HCl (pH=6)	50	27	190	10
		Guaiacol	3.4	0.6		
		H_2O_2	9	0.6		
	ABTS	KH_2PO_4 (pH=7)	50	27	190	10
		ABTS	100	0.54		
		H_2O_2	4.5	0.57		
CAT	H_2O_2	KH_2PO_4 (pH=7)	100	30	140	10
		H_2O_2	200	12		
APX	Ascorbate	KH_2PO_4 (pH=7)	55.56	36	180	20
		Ascorbate	60	0.01		
		H_2O_2	3%	0.041		
GST	CDNB	Tris/HCl (pH=6.4)	0.1	23.7	150	40
		GSH	0.369 g/ 2 mL ethanol	0.5		
		CDNB	0.0122 g/ 2 mL ethanol	1		
	pNBC	pNBC	0.0051 g/ 2 mL ethanol	1	150	40

3.1.4.6. Specific enzyme activity calculation

Table V shows the data for calculation of the specific enzyme activity. The final specific activity is related to the protein amount in the sample and thus is expressed as µmol min^{-1} mg^{-1} protein.

Table V. *Data for calculation of specific enzyme activity.*
1, Extinction coefficient (mM^{-1} cm^{-1}). **2,** Optical path length (cm). **3,** Wavelength (nm).

Enzyme	Substrate	1	2	3
PX	Guaiacol	26.6	0.594	420
	ABTS	35	0.594	414
CAT	H_2O_2	0.036	0.463	240
APX	Ascorbate	2.8	0.594	290
GST	CDNB	9.6	0.568	340
	pNBC	1.8	0.568	340

The specific enzyme activity was calculated according to the following equation:

$$Specific\ activity = \left(\left(\frac{\left(\frac{\Delta A}{\Delta t_{sample}} - \frac{\Delta A}{\Delta t_{blank}} \right) . V_{all}}{\varepsilon . d . V_{enzyme}} \right) . 1{,}000 \right) / C_{Bradford}$$

$\frac{\Delta A}{\Delta t_{sample}}$	$Change\ in\ absorbance\ over\ time\ of\ sample$
$\frac{\Delta A}{\Delta t_{blank}}$	$Change\ in\ absorbance\ over\ time\ of\ blank$
V_{all}	$Volume\ of\ cuvette$
V_{enzyme}	$Volume\ of\ buffer$
ε	$Extinction\ coefficient$
d	$Optical\ path\ length$
$1{,}000$	$Conversion\ factor\ for\ \mu g\ to\ mg$
$C_{Bradford}$	$Protein\ content\ in\ sample$

3.2. Experiments with the horseradish roots

3.2.1. Plant material and cultivation conditions

The hairy root culture of horseradish (*Armoracia rusticana* Gaerth. Mey. et Scherb.) was acquired from the IEB. The roots were transferred on the basal liquid hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (**Figure 4**). The medium was prepared by the mixing of storage solutions and diluting by distillated water (**Table VI**). The pH was adjusted to 7.75. The roots were cultivated in 250mL Erlenmeyer flask supplemented with 80 mL MS medium shaking on the orbital shaker. The roots were kept in the dark and at 26 °C. The roots for experiments were treated with $UO_2(NO_3)_2$ in the final concentration of 200µM. The samples for biochemical analyses were harvested after 7, 14 and 21 days. The roots were washed three times in distilled water, wiped off and frozen in liquid nitrogen and stored at -80

°C until the subsequent analysis. The plant material was also harvested for structural analysis that will be presented in thesis of Otradovcova. Eight different biological replicate root samples were used for the analysis.

Table VI. *Composition of MS medium utilized for hairy root culture of horseradish.*
1, Kind of storage solution. **2,** Composition of storage solution. **3,** Concentration of individual components in storage solution. **4,** Volume of storage solution added to 1 L of cultivation medium.

1	2	3	4
A Macroelements	NH_4NO_3	16.5	50 mL
	KNO_3	19	
	$CaCl_2$	3.31	
	$MgSO_4.7H_2O$	3.7	
	KH_2PO_4	1.7	
B Microelements	KI	0.083	5 mL
	H_3BO_3	0.62	
	$MnSO_4.H_2O$	1.69	
	$ZnSO_4.7H_2O$	0.860	
	$Na_2MoO_4.5H_2O$	0.025	
	$CuSO_4.5H_2O$	0.0025	
	$CoCl_2$	0.0013	
C Chelates	$FeSO_4.7H_2O$	2.78	5 mL
	$Na_2EDTA.2H_2O$	3.73	
D Vitamins	Inositol	10	5 mL
		0.05	
	Pyridoxine	0.05	
	Thiamine	0.05	
	Glycine	0.2	
Sucrose			30 g

Figure 4. *Illustration of cultivation hairy root culture of horseradish.*

3.2.2. Carbohydrate content determination

The procedure was the same as for tobacco (see the chapter 3.1.3.).

3.2.3. Assays of activities of carbohydrate metabolism enzymes

Sucrose synthase (SS; EC 2.4.1.13) and two isoenzymes of invertase (IT; EC 3.2.1.26) were assayed according to Kuo et al. (1997) and Appeldoorn et al. (1997). Plant sample weighting 400 mg was homogenized with a chilled mortar and pestle in 1-1.2 mL of 50mM Hepes (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) buffer (pH 7.4) containing 5mM $MgCl_2$, 1mM EDTA, 1mM EGTA (ethylene glycol-bis(aminoethyl ether)-*N,N´*-tetraacetic acid), 10% glycerol, 0.1% BSA, 5mM DTT (dithiothreitol) and 2%

insoluble PVP (polyvinylpyrrolidone). The homogenate was centrifuged at 15,000×g and 4°C for 5 min. The supernatant was used for the determination of sacharose synthase and soluble invertase activity. The pellet was washed two times in Hepes buffer. The resulting pellet was extracted with 1mL MES (2-(*N*-morpholino)ethanesulfonic acid) buffer containing 1M NaCl overnight at 4°C. The extract was centrifuged (Eppendorf Centrifuge 5414 D) for 15 min at 15,000×g and 4°C. The supernatant was used for the determination of insoluble invertase activity. The samples were stored at -80°C until the following analyses.

3.2.3.1. Sucrose synthase assay

The sucrose synthase assay was a two step procedure. The first step consisted of the sample extract (20 µL) incubation with 140 µL of Hepes/sucrose buffer (pH 7.0) containing 28.6mM Hepes and 286mM sucrose; and 40 µL of 20mM uridine diphosphate (UDP) solution at 30 °C for 30 min. The reaction was stopped by heating at 100 °C for 4 min and then the sample was cooled on ice for 4 min and put at -20 °C until the second step.

The UV/VIS spectrophotometer (Unicam Helios α) was used for determination of UDP-glucose content in the sample. The mixture for the second step contained 875 µL of glycine buffer (pH 8.9) consisting of 227mM glycine and 5.7mM $MgCl_2$; and 40 µL of 50mm nicotinamide adenine dinucleotide (NAD^+) solution and 80 µL of the sample from the first step. The sample was incubated for 5 min at room temperature and then 6.7 µL of UDP-glucose dehydrogenase (UDPGD) was added. The absorbance was measured at 340 nm until the reaction stopped (15min).

3.2.3.2. Invertase assay

The invertase assay was also a two step procedure. In the first step, the sample extract (50 µL) was incubated at 30 °C for 45 min with 200 µL of phosphate/citrate buffer containing 32.6mM sucrose, under pH 5.2 or 7.5 for soluble invertase or insoluble invertase, respectively. The reaction was stopped by heating at 100 °C for 4 min and then the sample was cooled on ice for 4 min and put at -20 °C until the second step.

The glucose content in the sample was determined with Glucose (HK) Assay Kit (Sigma Aldrich). A volume of 0.5 ml glucose assay reagent was mixed with 0.5 µL of the sample from the first step in the cuvette. The absorbance was measured at 340 nm on UV/VIS spectrophotometer (Unicam Helios α) until the reaction stopped (20 min).

3.2.3.2. Specific enzyme activity calculation

The specific activity of enzyme was calculated according to the following equations. The final specific activity is related to the protein amount in the sample and thus is expressed as µmol min^{-1} g^{-1} FW.

$$Concentration_{G/UDPG} = \left(\frac{V_{all}.M_{G/UDPG}}{\varepsilon_{NADH}.d.V_{sample}.1{,}000}\right).\Delta A_{340}$$

ΔA_{340} $Measured\ absorbance$

V_{all} $Volume\ of\ sample\ in\ assay\ 2$

$M_{G/UDPG}$ $Glucose/UDP-glucose\ molecular\ weight$

ε_{NADH} $NADPH\ extinction\ coefficient$

d $Optic\ path\ length$

V_{sample} $Volume\ of\ extract\ from\ assay\ 1$

1,000 $Conversion\ factor\ for\ \mu g\ to\ mg$

$$Specific\ activity = \frac{v.C_{G/UDPG}.V_{buffer}}{m.t}$$

v $Volume\ of\ sample\ from\ assay\ 1$

V_{buffer} $Volume\ of\ extraction\ buffer$

m $Fresh\ weight\ of\ sample$

t $Reaction\ time$

3.3. Statistical analysis

The basic arithmetic operations were performed in Microsoft Excel. Statistical analyses of antioxidative enzymes activities were carried out using the Statistica (StatSoft, Inc., Tulsa, Oclahoma, U.S.A.). Analysis of variance was performed using one-way ANOVA analysis and taking $p < 0.05$ as a significant to determine the effect of medium supplements to the activity of antioxidative enzymes. Statistical analysis of enzymes of carbohydrate metabolism was performed in the R 2.9.1. statistical software package (R Development Core Team 2009). Differences between the measurements were statistically tested with one-way ANOVA analysis followed by Tukey-Kramer test ($p < 0.05$). Statistically insignificant values on the level of probability $p < 0.05$ are indicated by the same symbol above columns in figures. The error bars indicate the standard deviation of analyzed values

4 Results

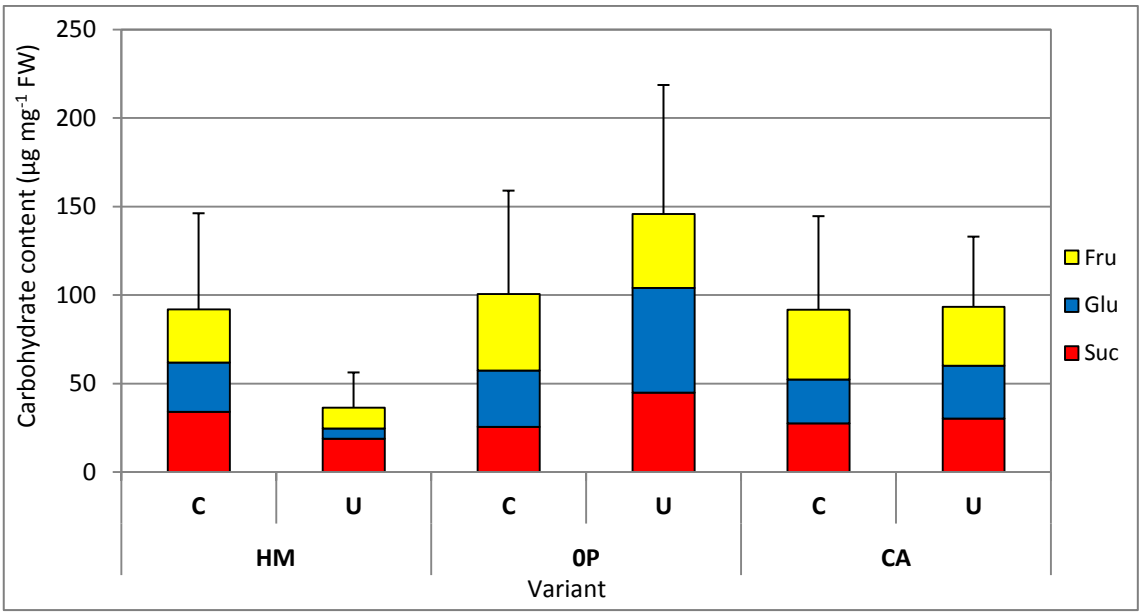
4.1. Effect of uranium on biochemical processes in the tobacco plants

The effects of uranium on metabolism of the tobacco plants (*Nicotiana tabacum* L.) cv. La Burley 21 were investigated. The plants were exposed to Hoagland hydroponic medium containing 500 µM U L^{-1} for 14 days. In the first experiment, all five variants of cultivation conditions (see the chapter 3.1.2.) were used (Hoagland medium (HM), Hoagland medium without phosphate (0P), Hoagland medium containing citric acid in the concentration of 0.1 mM (CA), Hoagland medium containing tartaric acid in the concentration of 0.1 mM (TA), and Hoagland medium with the daily spraying of plants with 0.75mM solution of putrescine (PA)). The results from this preliminary experiment were used only for the formulation of appropriate variants for the following experiments. In other three experiments only three variants were used, i.e. HM, 0P and CA. An analysis of carbohydrate distribution within the plant body was performed in every experimental replication. The activities of antioxidative enzymes were measured in the last experiment merely. The plant samples for the analysis of enzyme activities were also harvested before uranium treatment (the bars in graphs are marked as 0 and have a lighter shade). Uranium treatments had negative effects on plant biomass markedly, except for HM variant in the last experiment. In this case, plants treated with uranium ions had higher biomass than untreated plants (**Figure 5**).

Figure 5. *Appearance of plants in last experiment.* **A**, HM variant. **B**, 0P variant. **C**, CA variant (C – control, U – 500 µM U).

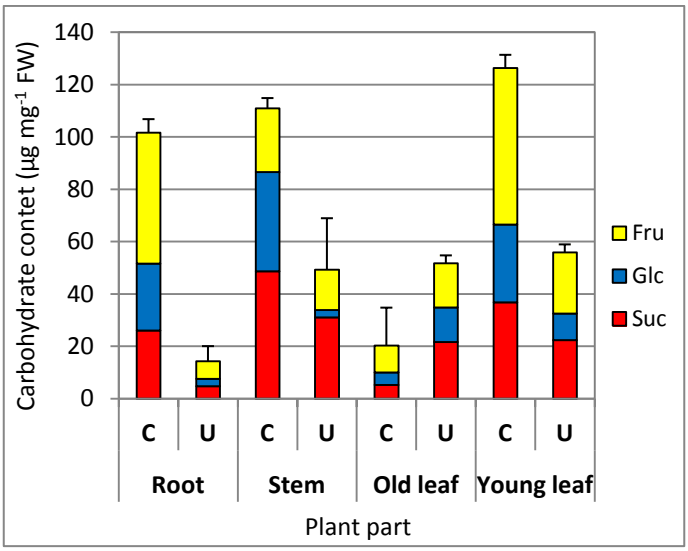
4.1.1. Effect of uranium on distribution and spectrum of carbohydrates

The results of carbohydrate content determination are demonstrated from two experiments. In the first experiment, carbohydrate contents were measured in the roots, shoots, old leaves and young leaves. The individual cultivation conditions had distinctive effects on carbohydrate contents. **Graph 1** shows the changes in the total carbohydrate content in the whole plant exposed to uranium ions depending on the composition of hydroponic medium. In case of HM variant, the total carbohydrate content decreased in uranium-treated plants, while in 0P variant the highest accumulation of carbohydrates was determined (130 µg mg^{-1} FW).

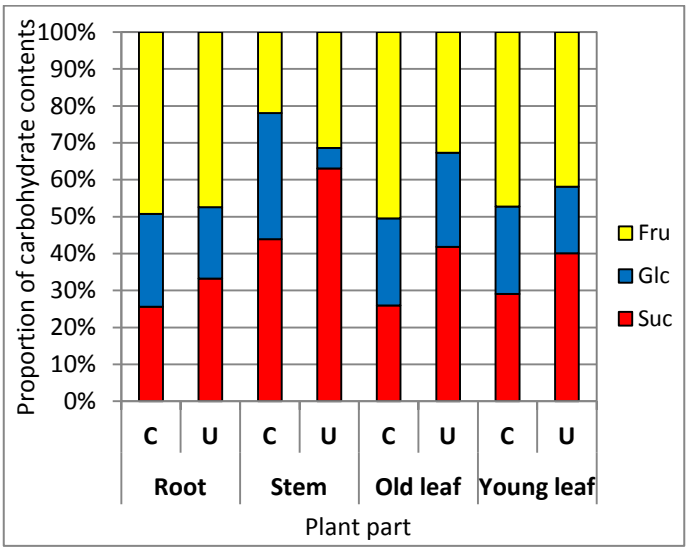


Graph 1. *Carbohydrate contents in tobacco cultivated under 500 µM $UO_2(NO_3)_2$ in dependence on composition of cultivation medium*. (1. Experiment)
Hoagland medium (**HM**), Hoagland medium without phosphate (**0P**), Hoagland medium with 0.1mM citric acid (**CA**), control plants (**C**), uranium-treated plants (**U**). Bars represent standard deviations of total carbohydrate content.

Negligible changes in carbohydrate content were detected in uranium-treated plants of CA variant. **Graph 2** demonstrates uranium impact on carbohydrate contents in the individual parts of the plant grown in the standard Hoagland medium. The content of carbohydrates decreased in all plant parts steadily, except for the old leaves which were observed to accumulate carbohydrates. **Graph 3** demonstrates the changes in carbohydrate proportion of uranium-treated plants grown in standard Hoagland medium. The amount of sucrose was generally higher in all plant parts exposed to stress conditions. An apparent decrease in the amount of glucose was found in the stems of uranium-treated plants.

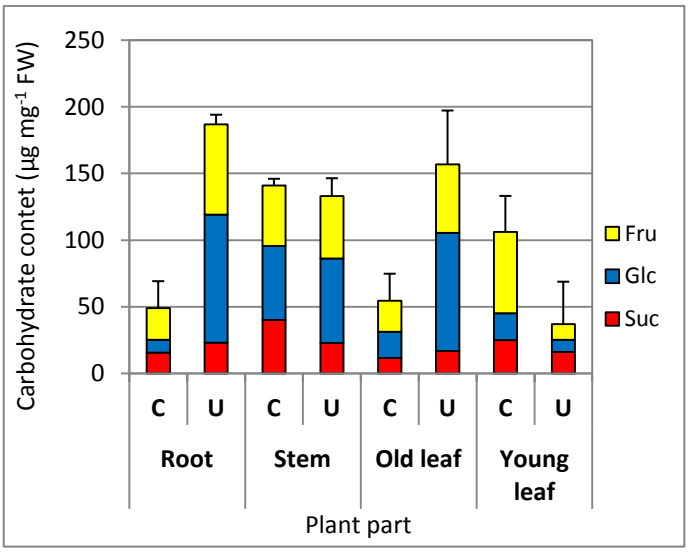


Graph 2. *Carbohydrate contents in different parts of tobacco cultivated in Hoagland medium under 500 µM $UO_2(NO_3)_2$.*
1. experiment
Bars represent standard deviations of total carbohydrate content.

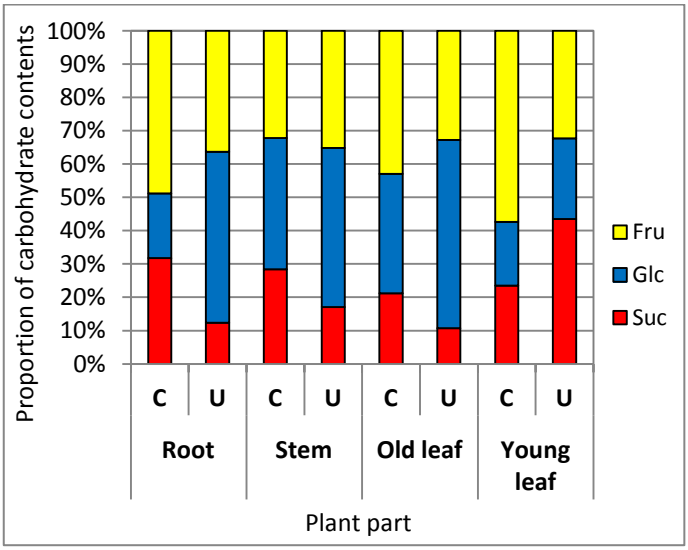


Graph 3. *Carbohydrate spectrum in different parts of tobacco cultivated in Hoagland medium under 500 µM $UO_2(NO_3)_2$.*
1. experiment

In case of 0P variant (**Graph 4**), the total carbohydrate content increased in the whole uranium-treated plant revealing their remarkable accumulation in the roots and old leaves (186 and 156 µg mg^{-1} FW, respectively), whereas the young leaves showed a decrease in carbohydrate contents with drop to 35% in uranium-treated plants compared to the control plants. No changes were observed in stems of uranium-treated plants. Moreover, the changes in carbohydrate proportion of the young leaves resulting from uranium treatment were also different compared to the other parts of the plant body that generally accumulated hexoses under stress conditions. Carbohydrate accumulation shifted towards higher sucrose in the young leaves (**Graph 5**).

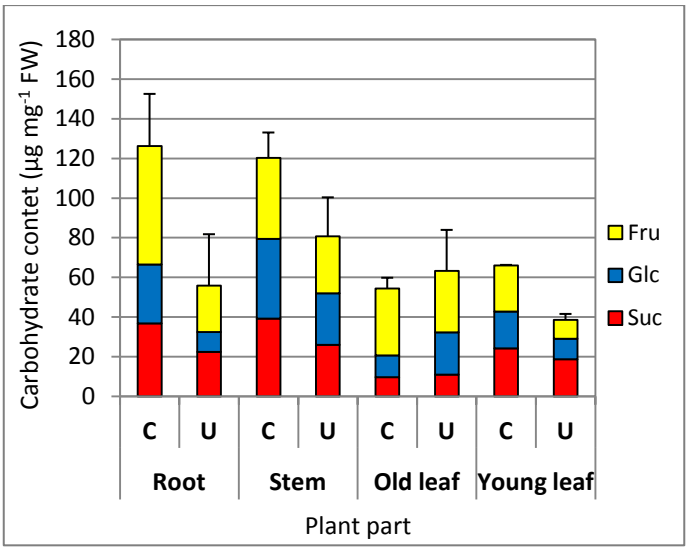


Graph 4. *Carbohydrate contents in different parts of tobacco cultivated in Hoagland medium without phosphate under 500 µM $UO_2(NO_3)_2$.*
1. experiment
Bars represent standard deviations of total carbohydrate content.

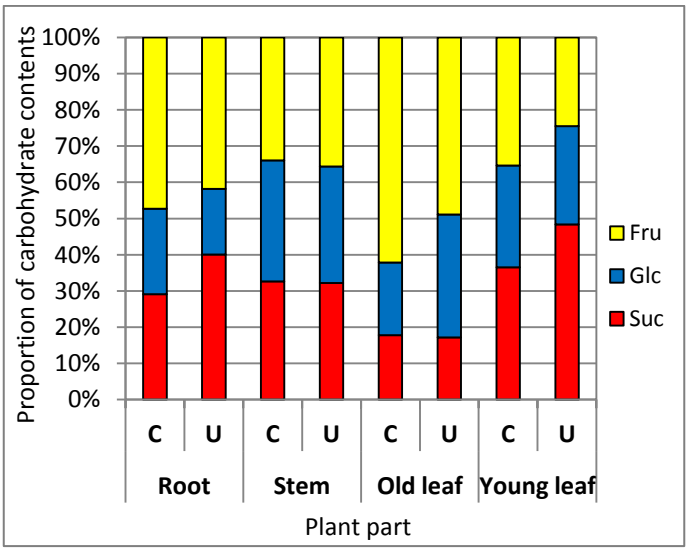


Graph 5. *Carbohydrate spectrum in different parts of tobacco cultivated in Hoagland medium without phosphate under 500 µM $UO_2(NO_3)_2$.*
1. experiment

In case of CA variant, the total carbohydrate content remained relatively stable in the whole plant, but the changes in carbohydrate contents in the individual parts of the plant body was observed. Excepting the old leaves, carbohydrate contents declined in all plant parts (**Graph 6**). Uranium exposure caused an increase of sucrose content in the roots and young leaves, whereas its content was unchanged in the stems and old leaves. Furthermore, uranium exposures had no effect on the amount of other two carbohydrates in the stems. In the old leaves, the amount of glucose increased at the expense of fructose amount (**Graph 7**).

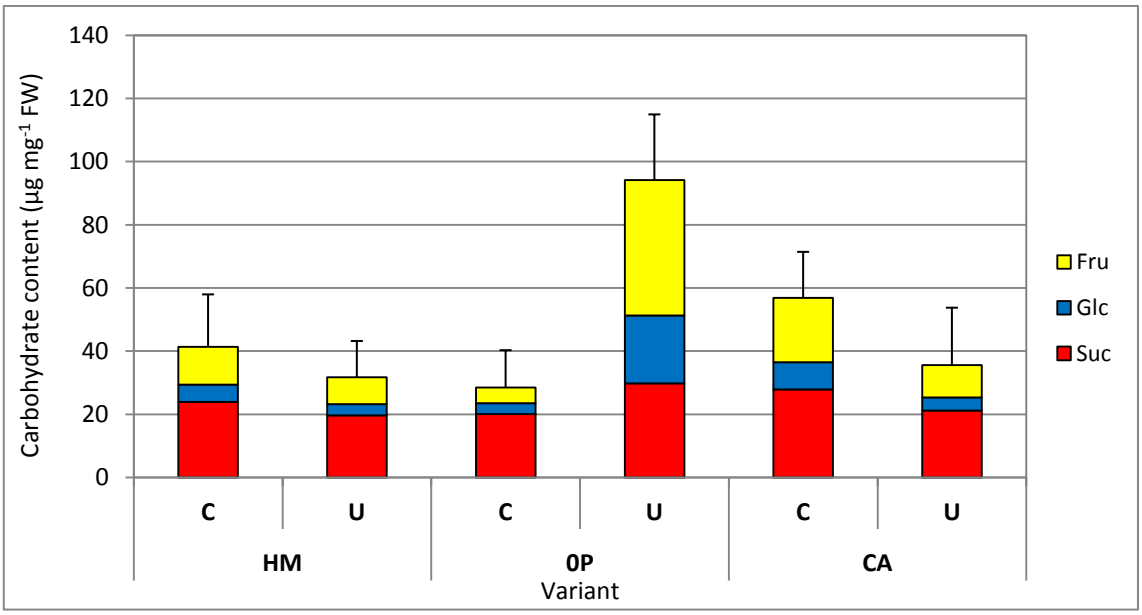


Graph 6. *Carbohydrate contents in different parts of tobacco cultivated in Hoagland medium with 0.1mM citric acid under 500 µM $UO_2(NO_3)_2$.*
1. experiment
Bars represent standard deviations of total carbohydrate content.



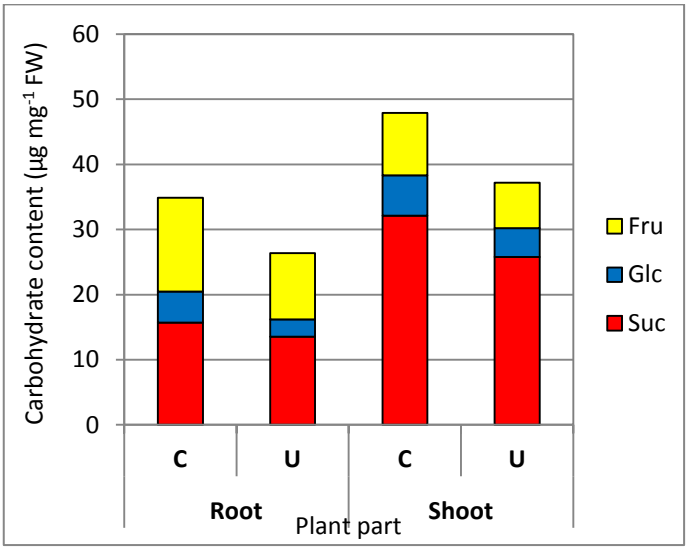
Graph 7. *Carbohydrate spectrum in different parts of tobacco cultivated in Hoagland medium with 0.1mM citric acid under 500 µM $UO_2(NO_3)_2$.*
1. experiment

In comparison with the first experiment, the total carbohydrate content was determined only in the individual plant parts, i. e. roots and shoots, in the second experiment. The results showed that the total carbohydrate content was also affected by uranium treatment in the whole plant depending on the medium composition. With the respect of the total carbohydrate content, the observations were similar in the whole plants as for the first experiment, except for the plants grown in CA variant. Threefold increase in the content of carbohydrates was observed in the whole plant of 0P variant treated with uranium ions (94 μg mg^{-1} FW) (**Graph 8**).

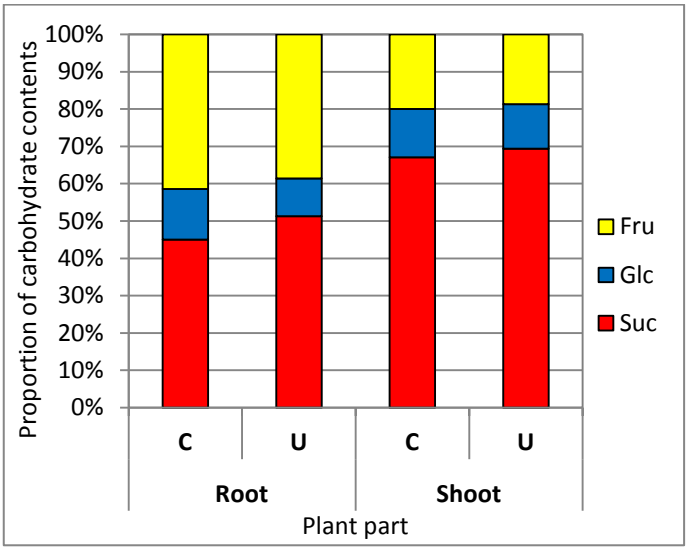


Graph 8. *Carbohydrate contents in tobacco cultivated under 500 μM $UO_2(NO_3)_2$ in dependence on composition of cultivation medium*. (2. Experiment)
Hoagland medium (**HM**), Hoagland medium without phosphate (**0P**), Hoagland medium with 0.1mM citric acid (**CA**), control plants (**C**), uranium-treated plants (**U**). Bars represent standard deviations of total carbohydrate content.

Graph 9 shows the effect of uranium on carbohydrate contents in the individual parts of plant grown in the standard Hoagland medium. Uranium treatment caused a decrease of carbohydrate contents the both roots and shoots. The proportion of carbohydrates remained stable in all plant parts in dependence on uranium exposure (**Graph 10**).

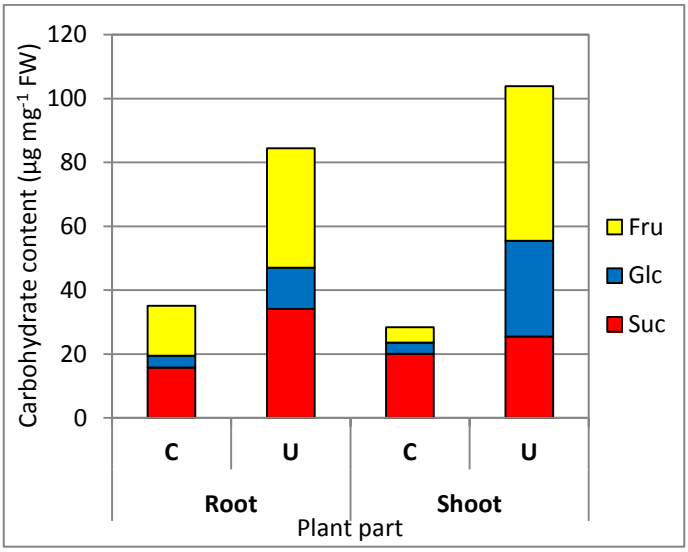


Graph 9. *Carbohydrate contents in different parts of tobacco cultivated in Hoagland medium under 500 µM $UO_2(NO_3)_2$.*
2. experiment
Bars represent standard deviations of total carbohydrate content

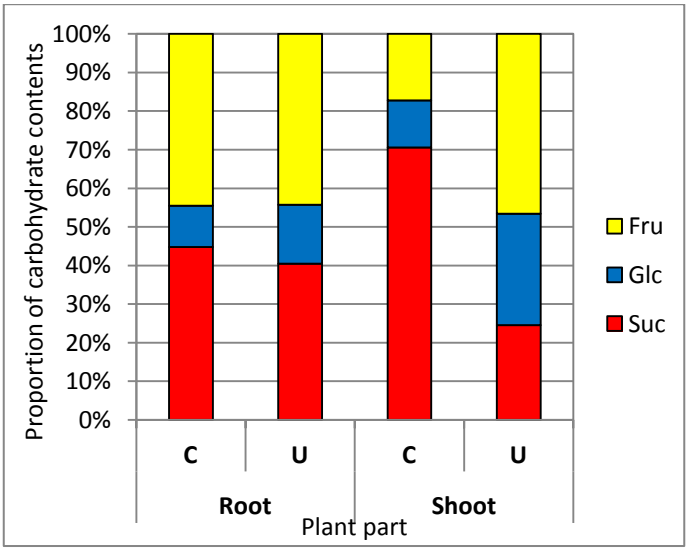


Graph 10. *Carbohydrate spectrum in different parts of tobacco cultivated in Hoagland medium under 500 µM $UO_2(NO_3)_2$.*
2. experiment

In case of 0P variant, uranium exposure influenced markedly the total carbohydrate content in all parts of the plant body. The plants accumulated carbohydrates under stress conditions. The highest amount of carbohydrates was found in uranium-treated shoots (104 µg mg^{-1} FW) that means up two fold increase compared to the untreated shoots (**Graph 11**). The amount of hexoses increased in all parts of plants exposed to uranium ions, with their substantial accumulation in the shoots (**Graph 12**).

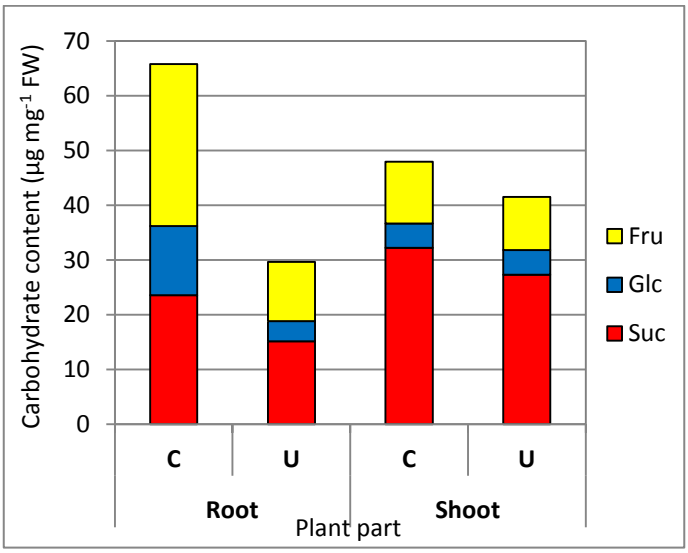


Graph 11. *Carbohydrate contents in different parts of tobacco cultivated in Hoagland medium without phosphate under 500 µM $UO_2(NO_3)_2$.*
2. experiment
Bars represent standard deviations of total carbohydrate content.

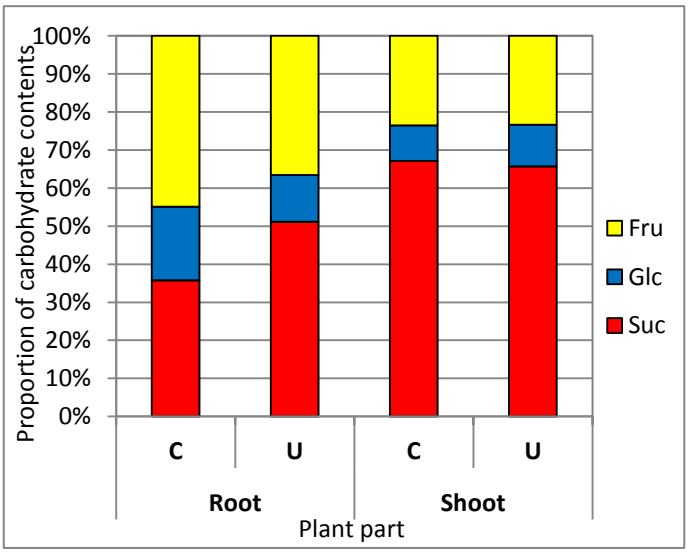


Graph 12. *Carbohydrate spectrum in different parts of tobacco cultivated in Hoagland medium without phosphate under 500 µM $UO_2(NO_3)_2$.*
2. experiment

In case of CA variant, the total carbohydrate content decreased in the roots exposed to uranium ions, whereas the shoots were found to accumulate carbohydrate under stress conditions (**Graph 13**).The spectrum of carbohydrates was attributed toward the increase of sucrose in uranium-treated roots. No changes were observed in carbohydrate spectrum of uranium-treated shoots (**Graph 14**).



Graph 13. Carbohydrate contents in different parts of tobacco cultivated in Hoagland medium with 0.1mM citric acid under 500 µM $UO_2(NO_3)_2$.
2. experiment
Bars represent standard deviations of total carbohydrate content



Graph 14. *Carbohydrate spectrum in different parts of tobacco cultivated in Hoagland medium with 0.1mM citric acid under 500 µM $UO_2(NO_3)_2$.*
2. experiment

4.1.2. Effect of uranium on activities of selected antioxidative enzymes

This experiment aimed to investigate the impact of uranium exposure on the antioxidative defence system of the tobacco plants. The changes in the activities of selected antioxidative enzymes, i.e. peroxidase (PX), catalase (CAT), ascorbate peroxidase (APX) and glutathione S-transferase (GST) in the tobacco roots and shoots were assayed by incubating the plant extracts in a mixture containing the appropriate cofactor. The protein concentration of the sample was calculated according to the standard curve from Bradford protein assay (**Figure 6**).

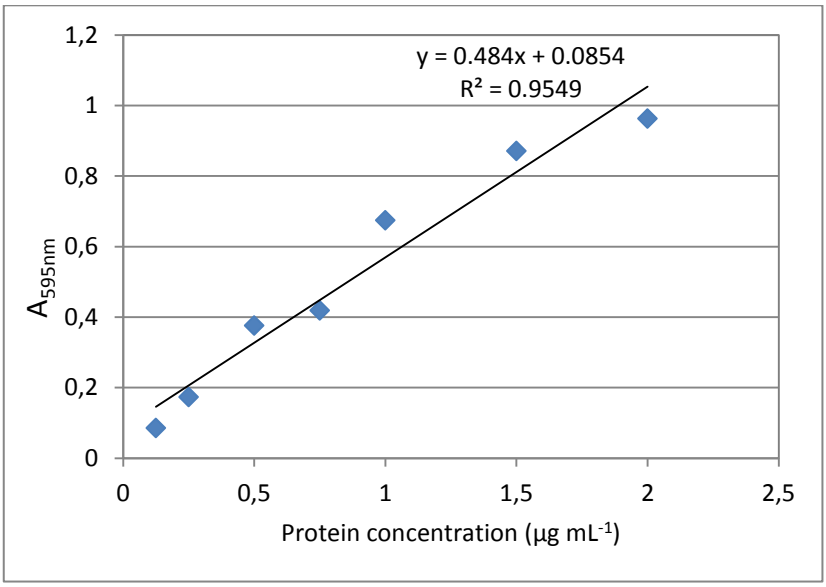


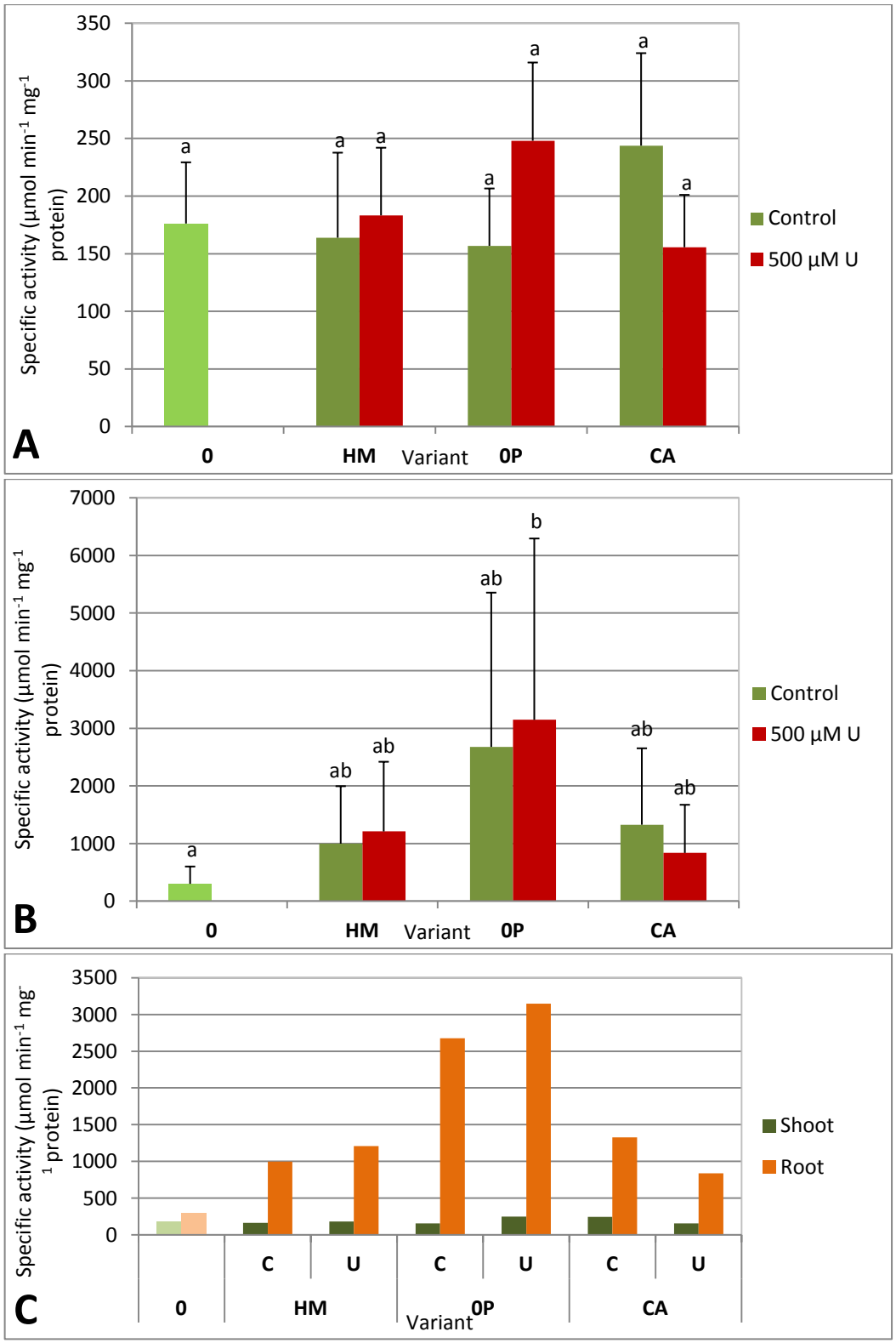
Figure 6. *Standard curve obtained from Bradford protein assay by measuring absorbance at 595 nm for standards.* Equation for the standard curve is: y = 0.484× + 0.0854 (R^2 = 0.9549).

4.1.2.2. Peroxidases

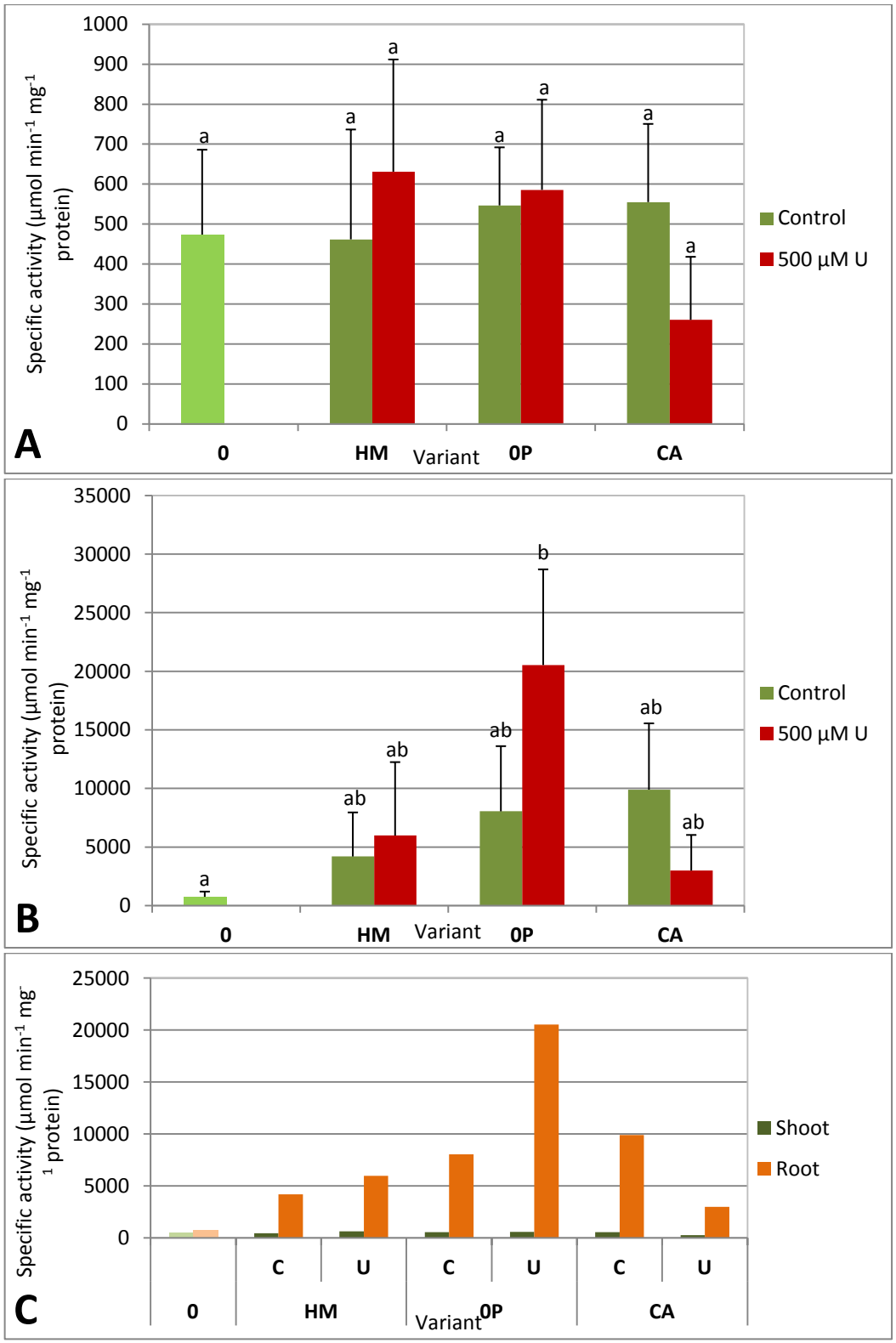
Due to the existence of a high number of PX isoenzymes (Penel et al., 1992, adopted from Mika and Luthje, 2003), the characterization of the individual isoenzymes is difficult. Therefore, in my experiments, guaiacol was used as a substrate which is metabolized by a wider array of PXs, and thus it may serve as a marker for general PX activity. For more detailed analysis, ABTS was used as another substrate for the determination of PX activity.

The specific activity of guaiacol peroxidase (GPX) in the tobacco shoots depending on medium variants is showed in the **Graph 15A**. No significant differences were observed. Uranium exposure caused an increase of GPX activity in the plants of HM and 0P variants. Surprisingly, the addition of citric acid (CA) enhanced the activity of GPX in the control plants, whereas the plants treated with uranium had the lowest GPX activity (157 µmol min^{-1} mg^{-1} protein). In the roots, the highest GPX activity was observed in the plants of 0P variant treated with uranium ions (3148 µmol min^{-1} mg^{-1} protein). The roots of the control plants from the same variant had also relatively high GPX activity compared to the other medium varints (**Graph 15B**). In the roots, GPX activity was appreciably higher compared to the shoots (**Graph 15C**).

Graph 16A shows the specific activity of peroxidase assayed with substrate ABTS (ABTS-PX) in the tobacco shoots depending on the medium composition. For HM and 0P variants, ABTS-PX activity increased in uranium-treated shoots. In comparison with GPX, the greatest increase was observed in the shoots of HM variant (631 µmol min^{-1} mg^{-1} protein). In case of CA variant, ABTS-PX activity was higher in the control plants, while plants treated with uranium ions had the lowest ABTS-PX activity (260 µmol min^{-1} mg^{-1} protein). However, no significant differences in ABTS-PX activity were observed in the tobacco shoots. In the tobacco roots, ABTS-PX had the same trend as an observation of the root GPX activity. The significantly highest ABTS-PX activity was observed in uranium treated roots of 0P variant, the activity was as much as twofold higher compared to the control plants in the same variant (**Graph 16B**). **Graph 16C** shows that ABTS-PX activity was greatly higher in the roots than shoots.



Graph 15. *Activity of **GPX** in shoots (**A**) and roots (**B**) of tobacco cultivated under 500 µM $UO_2(NO_3)_2$ in dependence on different composition of medium*.
Hoagland medium (**HM**), Hoagland medium without phosphate (**0P**), Hoagland medium with 0.1 mM citric acid (**CA**). GPX activity in plants before uranium treatment is marked as 0 (lighter shade bars). Bars represent standard deviations and different letters indicate significant differences between variants $p < 0.05$. Schematic graph **C** demonstrates differences of GPX activity between shoots and roots.



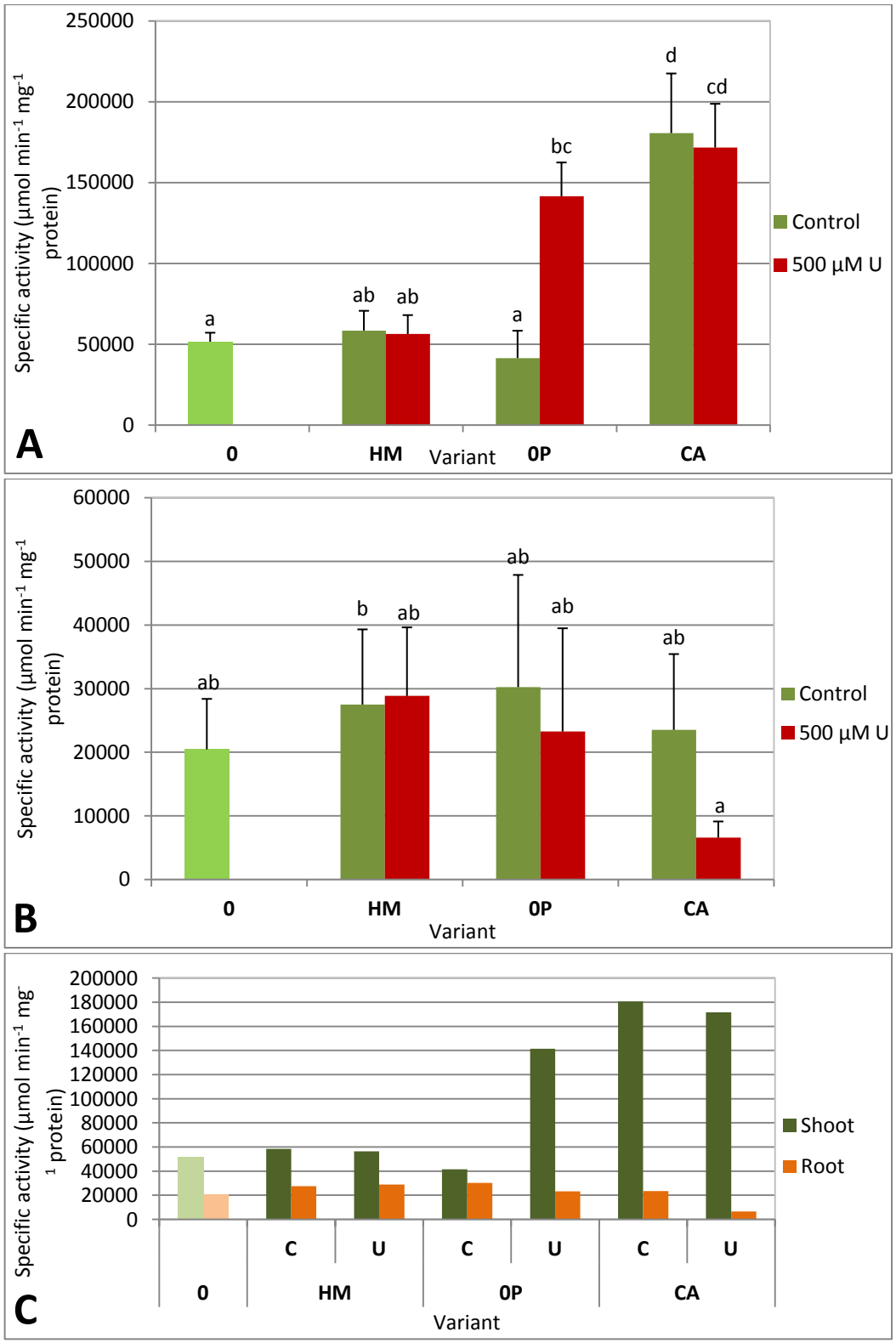
Graph 16. *Activity of **ABTS-PX** in shoots (**A**) and roots (**B**) of tobacco cultivated under 500 µM $UO_2(NO_3)_2$ in dependence on different composition of medium.*
Hoagland medium (**HM**), Hoagland medium without phosphate (**0P**), Hoagland medium with 0.1 mM citric acid (**CA**). ABTS-PX activity in plants before uranium treatment is marked as 0 (lighter shade bars). Bars represent standard deviations and different letters indicate significant differences between variants $p < 0.05$. Schematic graph **C** demonstrates differences of ABTS-PX activity between shoots and roots.

4.1.2.3. Catalase

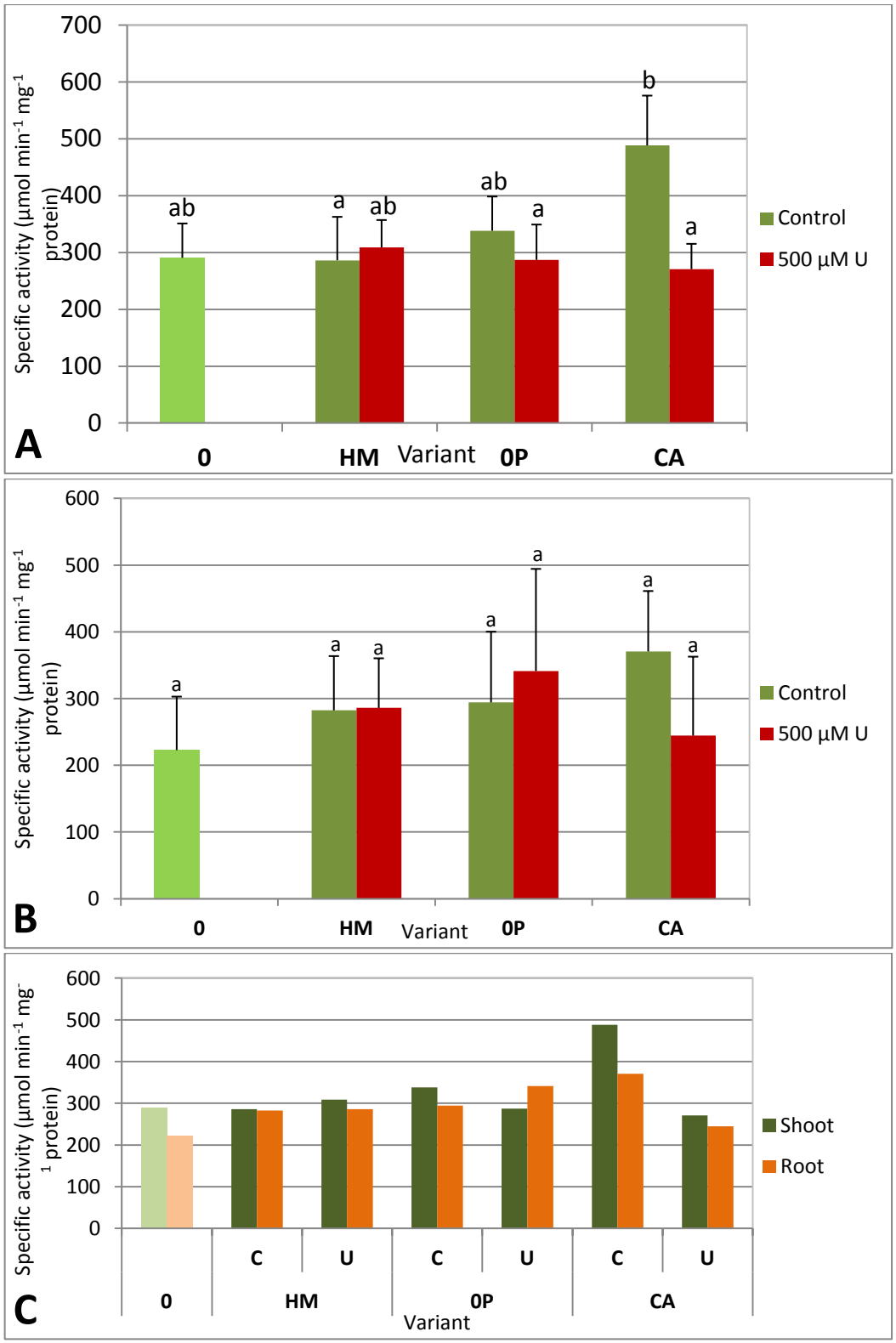
The activity of catalase (CAT) was relatively stable under uranium exposure in the shoots of HM variant. The significant increase of CAT activity was observed in the tobacco shoots of 0P variant treated by uranium, although, the greatest CAT activity (1,777 µmol min^{-1} mg^{-1} protein) was found in the shoots of the control plants from CA variant. In this variant, CAT activity declined under uranium exposure, whereas the values were still higher markedly compared to the plants 0 variant (**Graph 17A**). In the roots, CAT activity slightly increased in uranium-treated plants of HM variant, while for 0P and CA variants the decline in CAT was observed under uranium exposure. In case of CA variant, CAT activity decreased significantly (6,564 µmol min^{-1} mg^{-1} protein) (**Graph 17B**). Compared with the activities of PXs, CAT activity was higher in the tobacco shoots than roots (**Graph 17C**).

4.1.2.4. Ascorbate peroxidase

A slightly increased activity of ascorbate peroxidase (APX) in tobacco shoots treated with uranium ions was observed in HM variant. In case of 0P and CA variants, the decline in APX activities was detected in the shoots of uranium-treated tobacco plants compared to the control plants of the similar variant. The highest APX activity was found for uranium untreated shoots of the plants from CA variant (488 µmol min^{-1} mg^{-1} protein), when the plants were exposed to uranium ions, APX activity significantly decreased in the treated shoots (**Graph 18A**). In the roots, APX activity also increased in untreated roots of plants from CA variant but no significant differences in APX activity was detected in the roots (**Graph 18B**). For all medium variants, except for uranium-treated plants from 0P variant, APX activity was slightly greater in the shoots than roots of tobacco plants (**Graph 18C**).



Graph 17. *Activity of **CAT** in the shoots (**A**) and roots (**B**) of tobacco cultivated under 500 µM $UO_2(NO_3)_2$ in dependence on different composition of medium.*
Hoagland medium (**HM**), Hoagland medium without phosphate (**0P**), Hoagland medium with 0.1 mM citric acid (**CA**). CAT activity in plants before uranium treatment is marked as 0 (lighter shade bars). Bars represent standard deviations and different letters indicate significant differences between variants $p < 0.05$. Schematic graph **C** demonstrates differences of CAT activity between the shoots and roots.

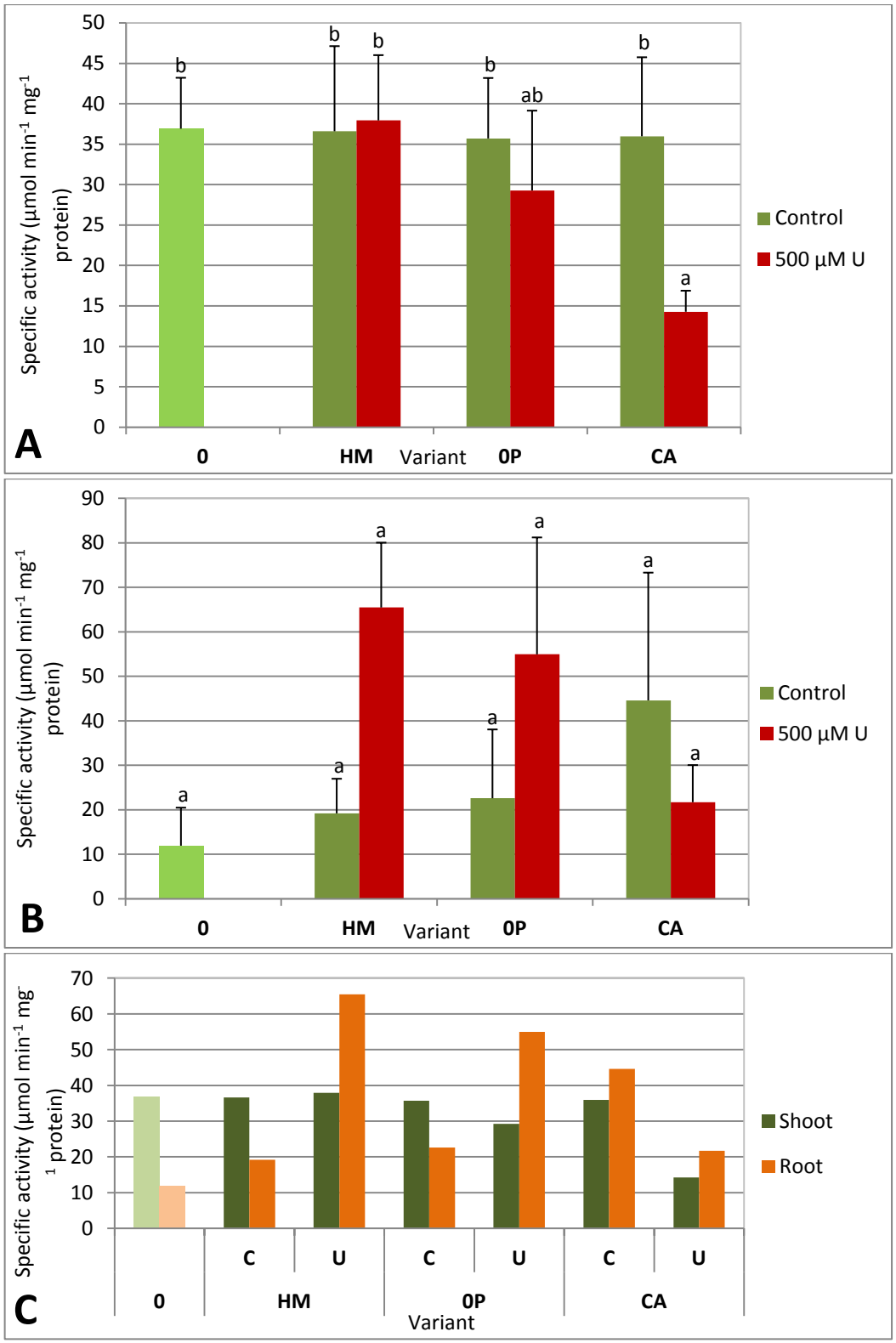


Graph 18. *Activity of **APX** in shoots (**A**) and roots (**B**) of tobacco cultivated under 500 µM $UO_2(NO_3)_2$ in dependence on different composition of medium.*
Hoagland medium (**HM**), Hoagland medium without phosphate (**0P**), Hoagland medium with 0.1 mM citric acid (**CA**). APX activity in plants before uranium treatment is marked as 0 (lighter shade bars). Bars represent standard deviations and different letters indicate significant differences between variants $p < 0.05$. Schematic graph **C** demonstrates the differences of APX activity between shoots and roots.

4.1.2.5. Glutathione S-transferases

Tobacco (*Nicotiana tabaccum* L.) contains multiple GST isoenzymes that differ in their substrate specifity and inducibility by various heavy metals (Lyubenova et al., 2009). In my experiments, I used CDNB and pNBC as substrates for the determination of GST activity. pNBC was found not to induce specific GST activity. Therefore, the results are only presented for CDNB substrate.

In case of HM variant, negligible changes of GST activity in the shoots of the plants were observed. In case of 0P and CA variants, GST activity decreased in shoots of the plants treated with uranium ions. GST activity was as much as twofold lower in the shoots exposed to uranium ions than in untreated shoots of the plants from CA variant (**Graph 19A**). In the roots, GST activity increased in the tobacco under uranium exposure in case of HM and 0P variants. In case of CA variant, GST activity increased in uranium untreated roots. In the roots, no significant changes have been found in GST activity (**Graph 19B**). GST activity showed a different trend for shoots and roots related to medium variants. In HM and 0P variants, uranium treatments showed upper GST activity in the roots; while uranium untreated plants had higher GST activity in the shoots. The addition of citric acid caused the increase of GST activity in the roots of tobacco plants (**Graph 19C**).



Graph 19. *Activity of **GST** in the shoots (**A**) and roots (**B**) of tobacco cultivated under 500 µM $UO_2(NO_3)_2$ in dependence on different composition of medium.*
Hoagland medium (**HM**), Hoagland medium without phosphate (**0P**), Hoagland medium with 0.1 mM citric acid (**CA**). GST activity in plants before uranium treatment is marked as 0 (lighter shade bars). Bars represent standard deviations and different letters indicate significant differences between variants $p < 0.05$. Schematic graph **C** demonstrates the differences of GST activity between shoots and roots.

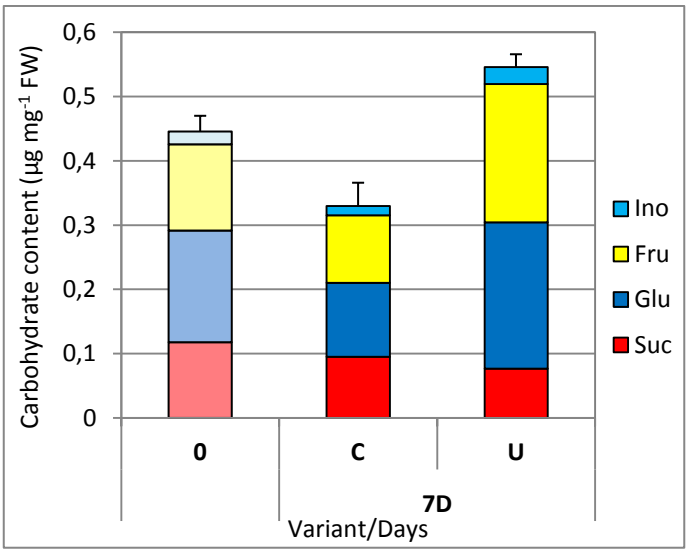
4.2. Effect of uranium on carbohydrate metabolism of the hairy root culture of horseradish

The aim of this part of the thesis was to examine uranium effects on carbohydrate metabolism in the hairy root culture of horseradish (*Armoracia rusticana* Gaerth. Mey. et Scherb.) cultivated *in vitro*. The roots were exposed for 7 and 14 days to 200 μM U L^{-1} in MS medium. Carbohydrate content determination was performed in three experiments. The activities of enzymes of carbohydrate metabolism were measured in the last experiment merely. The root samples for analysis of carbohydrate content determination were also harvested at the beginning of the experiments (the bars in graphs are marked as 0 and have a lighter shade). In the second experiments, carbohydrate content determination was performed in the roots that were exposed 14 days to uranium ions and then 7 days in uranium free MS medium.

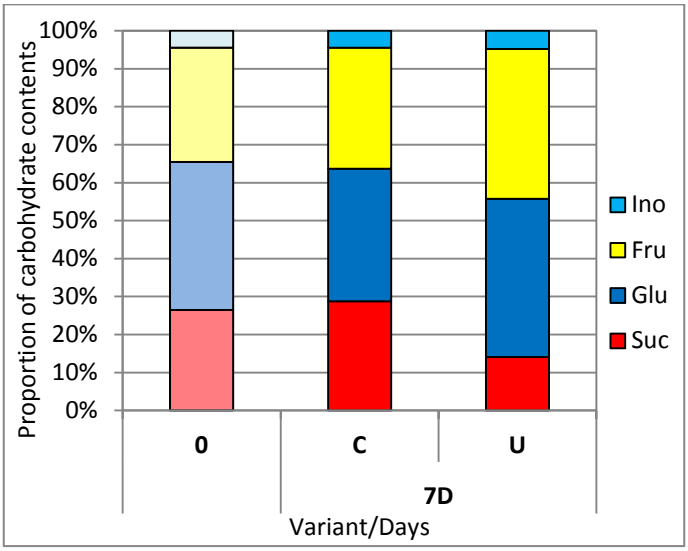
4.2.1. Effect of uranium on content and spectrum of carbohydrates

The results from carbohydrate analysis are demonstrated from all three experiments. Sucrose, glucose, fructose and inositol were determined as the main proportional representative carbohydrates in the roots. In the first experiment, the root samples were harvested at 0 and 7 days of the cultivation. The total carbohydrate content was higher at 0 days than at 7 days (**Graph 20**) whereas the spectrum of carbohydrates was equal comparatively (**Graph 21**). The uranium exposition caused the increase in the total carbohydrate content (**Graph 20**). The amount of all individual carbohydrates, except sucrose content, increased in the roots exposed to uranium ions (**Graph 21**).

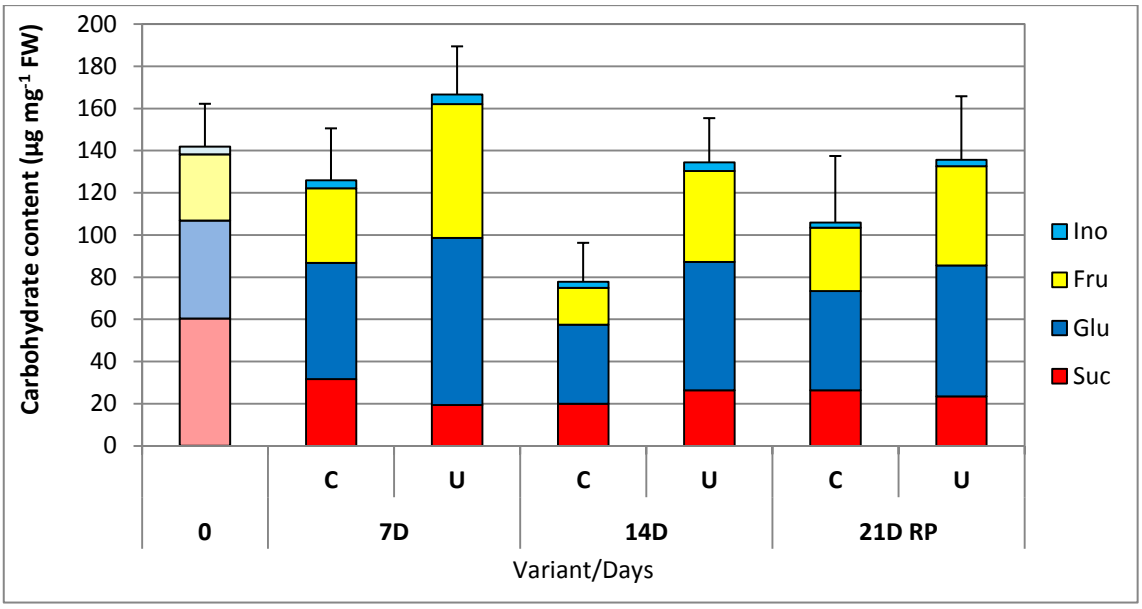
In the second experiment, the root samples were harvested at 0, 7, 14 and 21 days of cultivation. The results also showed an increase in the total carbohydrate content in uranium-treated roots. The highest accumulation of carbohydrates was detected in the roots after 7 days of uranium exposure (167 μg mg^{-1} FW). The amount of carbohydrates increased even in the recovery phase (after 21 days) (**Graph 22**). The great changes were observed within the spectrum of carbohydrates. The proportion of sucrose in the total carbohydrate spectrum decreased in the roots exposed to uranium ions. The roots produced mainly glucose as a major carbohydrate (**Graph 23**).



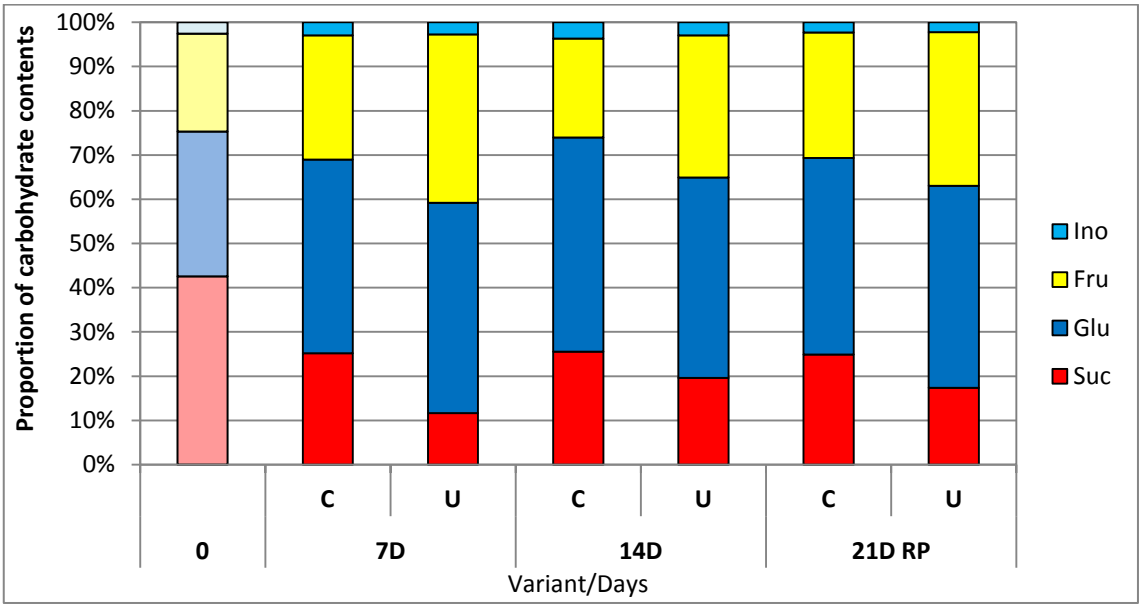
Graph 20. *Carbohydrate contents in hairy roots cultivated in MS medium under 200 µM $UO_2(NO_3)_2$ for 7 days (**7D**).*
1. experiment
Carbohydrate contents observed in hairy roots before uranium treatment is marked as **0** (lighter shade bars). Bars represent standard deviations of total carbohydrate content.



Graph 21. *Carbohydrate spectrum in hairy root of horseradish cultivated in MS medium under 200µM $UO_2(NO_3)_2$ for 7 days (**7D**).*
1. experiment
Carbohydrate spectrum observed in hairy roots before uranium treatment is marked as **0** (lighter shade bars).

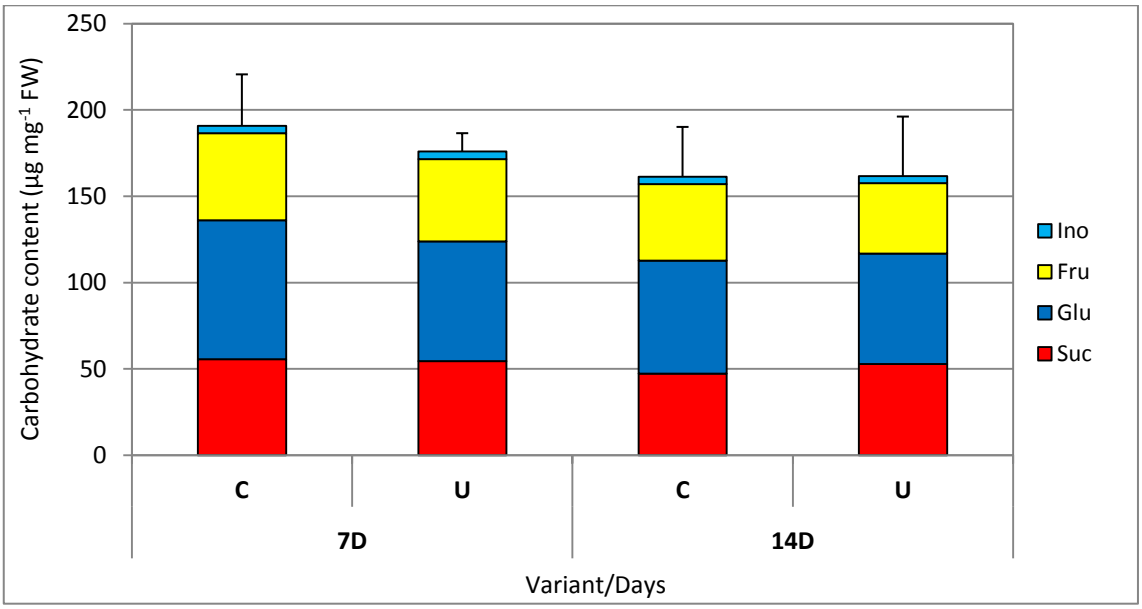


Graph 22. *Carbohydrate contents in hairy roots cultivated in MS medium under 200 µM $UO_2(NO_3)_2$ for 7 (**7D**) and 14 (**14D**) days.Carbohydrate contents determined in recovery phase are marked as **21D RP.*** (2. experiment)
Carbohydrate contents observed in hairy roots before uranium treatment is marked as **0** (lighter shade bars). Bars represent standard deviations of total carbohydrate content.

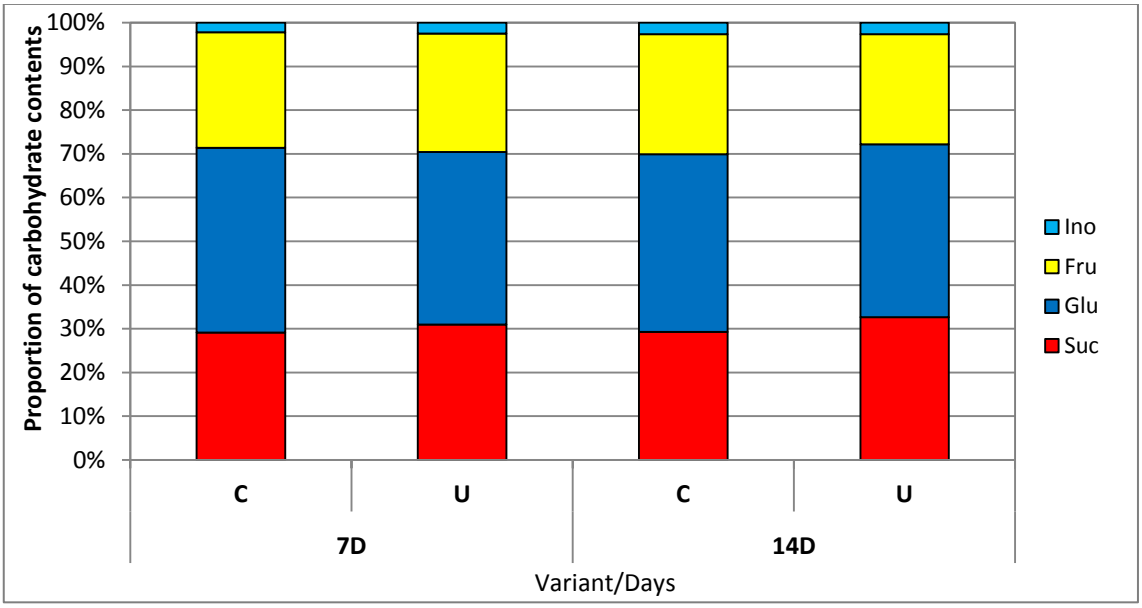


Graph 23. *Carbohydrate spectrum in hairy root of horseradish cultivated in MS medium under 200µM $UO_2(NO_3)_2$ for 7 (**7D**) and 14 (**14D**) days. Carbohydrate spectrum determinated in recovery phase is marked as **21D RP***. (2. experiment)
Carbohydrate spectrum observed in the hairy roots before uranium treatment is marked as **0** (bars have a lighter shade).

Different results were observed in the last experiment. The content of carbohydrates slightly declined in the roots exposed to uranium ions for 7 days. After 14 days, carbohydrate composition in uranium-treated roots remained stable as in the control roots (**Graph 24**). No changes were observed for the spectrum of carbohydrates in the roots (**Graph 25**).

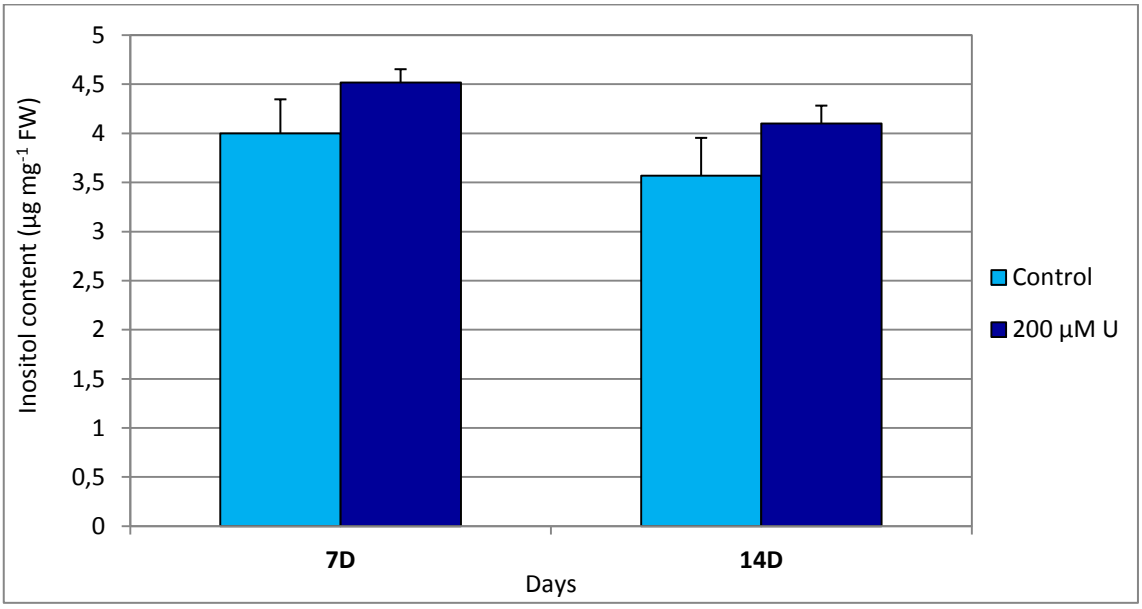


Graph 24. *Carbohydrate contents in hairy roots cultivated in MS medium under 200 µM $UO_2(NO_3)_2$ for 7 (**7D**) and 14 (**14D**) days*. (3. experiment)
Bars represent standard deviations of total carbohydrate content.



Graph 25. *Carbohydrate spectrum in hairy roots cultivated in MS medium under 200 µM $UO_2(NO_3)_2$ for 7 (**7D**) and 14 (**14D**) days.* (3. experiment)

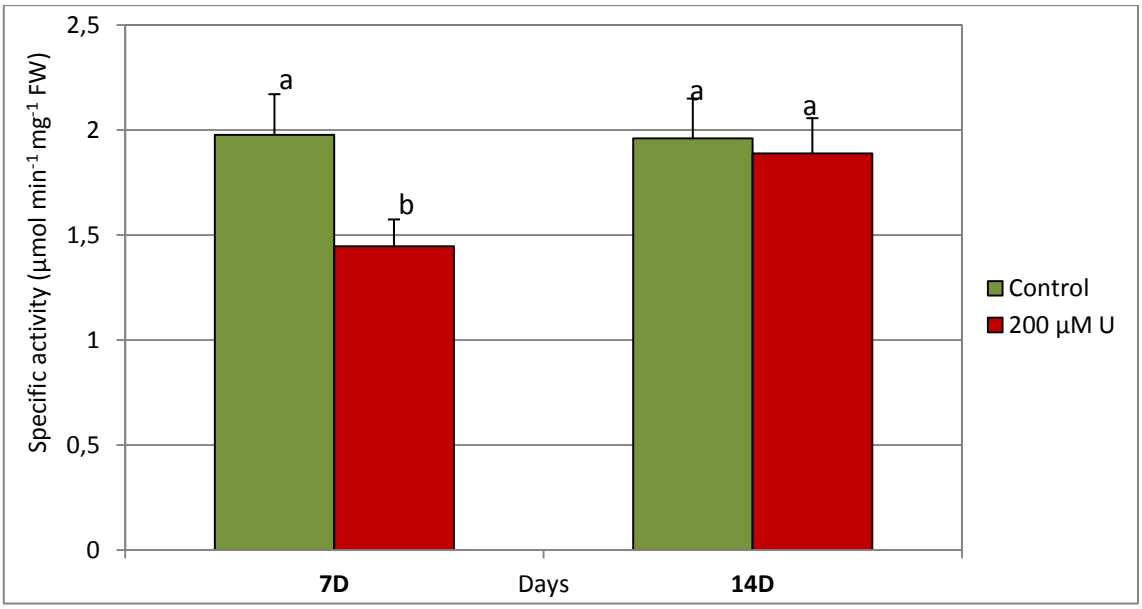
Graph 26 also shows the collective results from the second and third experiments and demonstrates an increase of inositol amount in the roots treated with uranium ions.



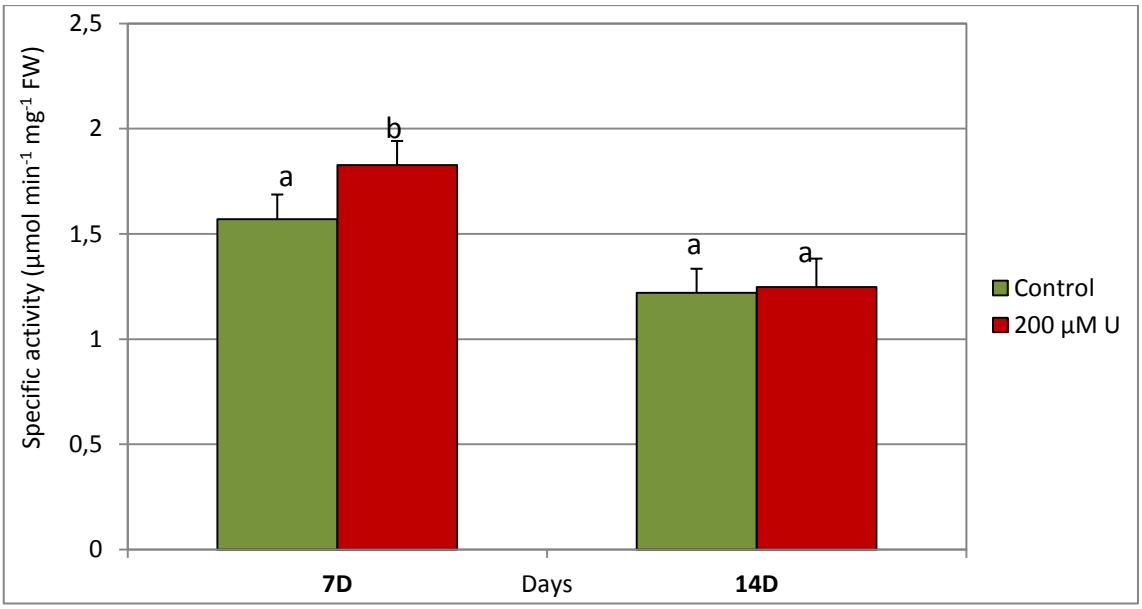
Graph 26. *Inositol content in hairy roots cultivated in MS medium under 200 µM $UO_2(NO_3)_2$ for 7 (**7D**) and 14 (**14D**) days.*
Results are determined from 2. and 3. experiment. Bars represent standard deviations of total carbohydrate content.

4.2.2 Effect of uranium on activities of enzymes involved in sucrose breakdown

After 7 and 14 days of uranium treatment, the activities of enzymes of carbohydrate metabolism were determined in the hairy root culture of horseradish. An analysis was performed for the most important enzymes involved in sucrose metabolism, i.e. sucrose synthase and two isoenzymes of invertase. The activity of insoluble invertase has not been detected; therefore the results are demonstrated for sucrose synthase and soluble invertase. As shown in **Graph 27**, the activity of sucrose synthase was significantly reduced in the roots grown for 7 days MS medium with the addition of uranium ions. Negligible changes were observed in sucrose synthase activity after 14 days of uranium treatment, but they were not statistically significant. Soluble invertase activity was found to be increased in the roots by about 15% after 7 days of uranium exposure. The soluble invertase activity was observed to be lower in both, control and uranium treated variant, in the roots after 14 days of the cultivation (**Graph 28**).



Graph 27. *Activity of **sucrose synthase** in hairy roots cultivated in MS medium under 200 µM $UO_2(NO_3)_2$ for 7 (**7D**) and 14 (**14D**) days*. (3. experiment)
Bars represent standard deviations of enzyme activity and different letters indicate significant differences on level probability $p < 0.05$.



Graph 28. *Activity of **soluble invertase** in hairy roots cultivated in MS medium under 200 µM $UO_2(NO_3)_2$ for 7 (**7D**) and 14 (**14D**) days*. (3. experiment)
Bars represent standard deviations of enzyme activity and different letters indicate significant differences on level probability $p < 0.05$.

5 Discussion

5.1. Methodical approaches

The effect of uranium on plant metabolism was investigated in intact tobacco plants (*Nicotiana tabacum* L.) and horseradish hairy root cultures (*Armoracia rusticana* Gaerth. Mey. et Scherb.). These model plant cultures were used for the determination of changes in carbohydrate metabolism and antioxidative defence reactions under uranium exposure in the laboratory conditions. Based on previous results from the IEB, I used uranium concentration of 500 µM and 200 µM for experiments with the tobacco and horseradish, respectively.

5.2. Effect of uranium on biochemical processes in the tobacco plants

A reduction in the plant biomass was observed under uranium stress, but differences in response were observed in connection with the individual medium composition. One exception was observed for the plants grown in Hoagland medium in the second experiment. In this case, uranium had positive effect on plant biomass accumulation. It can be explained by the process of hormesis that was found in grass (little bluestern (*Schizachyrium scoparium* (Michx.) Nash)) exposed to uranium in the concentrations ranging from 5 to 500 µM U L^{-1} (Meyer et al., 1998). Together with the master student Otradovcova, we harvested the tobacco plants and evaluated them for the root length (scanned with WinRhizo), leaf surface area and shoot length. Otradovcova will discuss these structural changes elicited by uranium exposure in her master thesis.

5.2.1. Effect of uranium on distribution and spectrum of carbohydrates

The changes in carbohydrate spectrum and distribution in the relation to uranium exposure were determined in the tobacco (*Nicotiana tabacum* L.) cv. La Burley 21. The spectrum of carbohydrates in the tobacco was established consisting mainly of glucose, fructose and sucrose. Investigated different effect of medium amendments on carbohydrate metabolism under uranium exposure was established (**Graph 1, 8**). The content of total carbohydrates decreased markedly in response to

uranium exposition in the tobacco grown in standard Hoagland medium, whereas negligible changes in the total carbohydrate contents were observed in plants grown in medium with the addition of citric acid. Increased accumulation of carbohydrates was reported in plants grown in medium with the absence of phosphate exposed to uranium ions. In case of standard medium, decrease in the total carbohydrate contents may be explained by the reduction of ^{14}C-fixation rates in uranium-treated plants which was observed by Boileau et al. (1985). These data are also in agreement with the findings on cadmium-treated plants (Greger and Lindberg, 1986; Costa and Spitz, 1997; Podazza et al., 2006). With the respect to the individual plant parts, a decrease in the total carbohydrate content was observed in all parts, except the old leaves (**Graph 2**). In the second experiment, the data showed a decline in carbohydrate contents in the whole plant, i.e. roots and shoots, under uranium exposure (**Graph 9**), reflecting the sampling method.. Results obtained by Lhotsky (2011) showed that uranium accumulation within the leaves of the tobacco plants was in the oldest leaves predominantly, but uranium content in all leaves was lower than in the roots and shoots. Carbohydrate accumulation in the old leaves can be attributed to the plant response toward uranium ions that are predominantly accumulated to high concentration in these leaves. In the first experiment, the proportion of sucrose in the total carbohydrate spectrum increased in uranium-treated plants compared to the control plants of the same medium variant (**Graph 3**). The second experiment showed that the changes in the carbohydrate spectrum were not dramatic compared to the untreated plants. However, the higher proportion of sucrose in total carbohydrates was found in the aboveground parts of the plant body compared to the belowground parts (**Graph 10**). The both experiments established that the proportional representation of fructose in the whole carbohydrate spectrum was higher than for glucose. Sucrose, the most frequently translocated carbohydrate of higher plants, is known to play an important role in stress responses as osmoprotectant (Hoekstra and Golovina, 1999), cryoprotectant (Reyes-Diaz et al., 2008), signaling molecule (Tabaei-Aghdei et al., 2003) or rapid source of energy to support growth at unfavourable conditions (Strand et al., 2003). My results show that, in the tobacco plants, sucrose is preserved as an important carbohydrate species of plant response to uranium ions.

The absence of phosphate in medium led to an increase of the total carbohydrate accumulation in the whole plant exposed to uranium ions (**Graph 4, 11**). Carbohydrate content was threefold higher in the roots of uranium-treated plants than in the control plants of the same medium variant. Appreciable increase was also observed in the old leaves. However, the unchanged carbohydrate levels were detected in stems, though that carbohydrate amount declined in young leaves of uranium-treated plants (**Graph 4**). Lhotsky (2011) showed that, compared with other medium variants, the absence of phosphate led to considerably higher accumulation of uranium in all parts of the tobacco plants with the highest content in the roots. Similar behaviour has been demonstrated by Ebbs et al. (1998) or Mkandawire et al. (2007). It was assumed that the increase in uranium accumulation was due to the better bioavailability of dissolved uranium ions in the hydroponic medium that in the presence of phosphate creates insoluble complex with uranium. Moreover, plants respond to phosphate deficiency by various biochemical and morphological adaptations including an increased secretion of organic acids and acid phosphatase, higher root-to-shoot ratio and remobilization of phosphate to roots (Raghothama, 1999). The enhanced phosphate remobilization from the leaves to the roots was also observed under uranium exposure in phosphate-deficient as well as phosphate-sufficient conditions (Misson et al., 2009). So, the absence of phosphate in the hydroponic medium could intensify the remobilization of phosphate within the plant body during exposition to uranium ions. This phosphate was shown to immobilize uranium ions in the roots by forming the mentioned complexes. Phosphate co-localization with uranium on the root epidermis was also observed by Laurette et al. (2012ab). Preferential distribution of the photosynthetic carbon to the roots and an increase in the root-to-shoot ratio has been well documented for plants limited by phosphate (Cakmak et al., 1994). Therefore, the higher carbohydrate levels observed in the roots of uranium-treated plants (**Graph 4**) may be explained by carbon remobilization during the phosphate deficiency. Nevertheless, the higher carbohydrate accumulation was detected in the old leaves and shoots of uranium-treated plants in the first and second experiments, respectively (**Graph 4, 11**). Therefore, the increase in the total carbohydrate levels was probably caused mainly by the presence of uranium

ions. Whether the root-to-shoot ratio was changed in tobacco plants under uranium exposure, will be discussed in the master thesis of Otradovcova. Moreover, it was identified that genes involved in phosphate metabolism are regulated by sucrose, as well as by hexoses (Liu et al., 2005). These authors concluded that sugars are crucial for signal transduction in phosphate-limited conditions and this regulation is conserved across the plant species. Taken together, the observed increase in carbohydrate levels in uranium-treated tobacco plants reveals a putative interplay between stresses caused by the phosphate deficiency and the toxicity of uranium ions. The proportion of hexoses, especially glucose, in the total carbohydrate spectrum increased to a great extent in the whole plant body treated with uranium ions, except the young leaves (**Graph 5, 12**). An accumulation of hexoses under heavy metal stress has also been observed (Devi et al., 2007; Verma and Dubey, 2001). Although, hexoses represent reducing sugars, thus they cannot serve as compatible solutes, they were identified to play important role in the cell osmotic adjustment during heavy metal stress (Choundhury et al., 2010). Their accumulation has also been observed in wheat (*Triticum* spp.) exposed to the other abiotic stresses such as drought (Rascio et al., 1994) or salinity (Kerepesi et al., 1998). Salinity caused an increase of the total carbohydrate content of apple (*Malus domestica* Borkh.) in favour to hexoses, while raffinose was accumulated particularly under low temperature (Krsek, 2009). It has been concluded that individual plant species may exploit the high diversity of carbohydrate spectrum in the specific response to various stresses (Rejskova et al., 2007). In view of the fact that phosphate-limited plants enhance sucrose accumulation in their tissues (Li et al., 2008), thus a decrease in sucrose level in uranium-treated plants was not probably caused by phosphate-limited conditions.

Different effects on carbohydrate metabolism in plants cultivated in Hoagland medium with the addition of citric acid were observed for individual experiments. Negligible changes in the total carbohydrate contents were observed in the first experiments (**Graph 1**) whereas carbohydrate contents decreased to 60 % in uranium-treated plants of the second experiments (**Graph 8**). The positive effect of citric acid on uranium accumulation in the tobacco plants was discussed in literature survey. Lhotsky (2011) found that uranium accumulation in the tobacco plants was relatively high

probably due to the usage of the free acid form of citric acid. This form was observed to be more effective for uranium accumulation comparing to potassium citrate (Huang et al., 1998). An increase in uranium uptake in the presence of citric acid has been also found by other scientists. According to some authors, uranium-rich citrate complexes are taken up by plants (Huang et al., 1998) while for other authors these complexes dissociate at the vicinity of plant root and uranium is taken up as a free uranyl ions (UO_2^{2+}) (Ebbs et al., 1998; Laurette et al., 2012ab). The addition of citric acid to the cultivation medium also enhances uranium translocation to the (Mihalik et al., 2011; Laurette et al., 2012ab). Soudek et al. (2011a) found that the addition of tartaric acid enhanced uranium uptake and translocation within the plant body more than citric acid supplementation. It is known that the exudation of natural low molecular weight organic acids (NLMWOA) by the roots is enhanced during exposure to various stresses (Delhaize et al., 1993). These compounds including citric or malic acid play a significant role in heavy metal solubility and nutrient mobility (Mench and Martin, 1991; Jones et al., 1996). Carbohydrates are involved in the metabolism of organic acids through the phosphoenolpyruvate carboxylase (PEPC). So, the addition of citric acid may enhance the exudation of NLMWOA by uranium-treated roots because of the stress conditions; therefore we would reckon a decrease in carbohydrate contents due to their elevated usage in the synthesis of NLMWOA. Unfortunately, I observed the decline in carbohydrate levels only in the first experiment. Taken these observations into the account, the hypothesis that the higher usage of carbohydrates for NLMWOA synthesis during uranium stress has not been verified. The results showed the only common trend in the both experiments, relatively sharp reduction in the total carbohydrate content of uranium-treated roots (**Graph 6, 13**). With the respect of changes in carbohydrate spectrum during uranium exposure, I can conclude that sucrose could play an important role in the root response towards high concentration of uranium ions due to the observed increase in sucrose proportion in the total carbohydrate contents. The increase was observed in both experiments (**Graph 7, 14**).

In conclusion, the results show that the deficiency of phosphate in hydroponic medium generated the greatest alterations in carbohydrate metabolism of tobacco plants under uranium stress. These changes in carbohydrate distribution and

proportion may be caused both by uranium ions and the absence of phosphate due to the important role of phosphate in carbohydrate metabolism. It may be assumed that accumulated carbohydrates are involved in plant responses toward toxic uranium ions and starvation of important nutrient – phosphate. One more conclusion from the experiments is that the tobacco plants may exploit the high diversity of carbohydrate spectrum in the specific response to stress caused by the presence of uranium ions and changes in the medium composition.

5.2.2. Effect of uranium on activities of selected antioxidative enzymes

To investigate the possible role of oxidative stress connected with uranium exposure, the antioxidative defense mechanism was examined in the tobacco plants. Induction of the oxidative stress after exposure to various heavy metals has been extensively studied in many plant species (Chaoui et al., 1997; Schutzendubel and Polle, 2002). Vanhoudt et al. (2008) identified that oxidative stress related responses are triggered after exposure to uranium in the concentration 100 µM U L^{-1}. The tobacco plants, used in my experiment, were exposed to uranium concentration 500 µM U L^{-1} for 14 days and activity of selected antioxidative enzymes, i.e. peroxidase (PX), catalase (CAT), ascorbate peroxidase (APX) and glutathione S-transferase (GST) were investigated. Potential effect of various medium compositions was also tested and will be discuss one by one.

The present results indicated negligible change or low increase in the activities of all tested antioxidative enzymes in the tobacco plants grown in Hoagland hydroponic medium supplemented with uranium ions. Vanhoudt et al. (2011ab) observed an increase in PX activity at a later stage of uranium stress specifically after 7 days of uranium exposure. This is affirmed by my observations of a slight increase in GPX activity (PX assayed with guaiacol as a substrate) but more enhanced PX activity was determined by ABTS as a substrate (ABTS-PX) in tobacco shoots treated by uranium ions (**Graph 15A, 16A**). The root PX activity was higher compared to shoot GPX and ABTS-PX activity in both stressed and control plants (**Graph 15C, 16C**). In contrast to the higher PX activity in the roots, the activity of catalase (CAT) was higher in the tobacco shoots of both treated and control tobacco plants. No differences in

CAT and ascorbate peroxidase (APX) activity were observed in the tobacco plants exposed to uranium ions (**Graph 17, 18**), indicating that these enzymes were not involved in plant defence responses against uranium-induced oxidative stress. These findings is in agreement with results of Martins et al. (2011) that showed no alterations in APX and also CAT activity in tobacco (*Nicotiana tabacum* L.) under cadmium stress.

The absence of phosphate in the hydroponic medium had influence on the activities of antioxidative enzymes in the tobacco plants. The relationships between phosphate starvation and enhanced antioxidative defense mechanism were observed in the roots (Juszczuk et al., 2001), but not in the leaves (Cakmak et al., 1994) of bean (P*haseolus vulgaris* L.). The present results showed that the activity of GPX and ABTS-PX increased in the roots of the both uranium-treated and control plants grown in the medium without phosphate. Enzyme activity grown to be stronger in the whole plant after the addition of uranium ions (**Graph 15, 16**). Significant increases of CAT activity were observed in uranium-treated tobacco shoots compared to the roots that exhibit slightly decreased CAT activity under uranium exposure (**Graph 17**). However, the analysis of CAT transcripts showed an induction of its expression as a consequence of uranium exposure, but no change in CAT activity in the roots and shoots of *Arabidopsis thaliana* L. (Vanhoudt et al., 2011ab). It may be suggested that uranium could promote posttranslational modifications of CAT protein as was suggested by Romero-Puertas et al. (2007). Another assumption proposes that an increase in CAT activity in tobacco shoots and PXs activities in the whole plant could be enhanced both by uranium exposure and phosphate limitation. This can be affirmed by findings of Juzsczuk et al. (2001) who found the higher PX and CAT activity in phosphate-limited plants, whereas activity of APX and SOD were not affected by phosphate starvation. My results also showed insignificant changes in APX activity of uranium-treated tobacco plants (**Graph 18**). Nevertheless, Vanhoudt et al. (2011a) found that APX is an important ROS-scavenger in plants under uranium stress and seems to have a crucial role in regeneration of oxidized ascorbate. Concerning GST activity, the significant decrease in tobacco shoots was observed under uranium exposure, whereas insignificant increase in GST activity was detected in uranium-treated roots. Furthemore, the root-to-shoot ratio of GST activity changed toward higher activity in uranium-treated roots (**Graph**

19). The increase in root GST activity may be affirmed by the enhanced GST activity in the hairy roots culture observed by Soudek et al. (2011b).

The present results showed that the addition of citric acid to the hydroponic medium caused considerable increases in activities of all tested antioxidative enzymes in the tobacco plants that have not been exposed to uranium ions. With the addition of uranium ions, enzyme activities decreased (**Graph 15-19**). The supplementation of the soil with citric acid for phytoextraction purposes is recommended as an effective enhancer of heavy metal accumulation in plants, but Huang et al. (1998) point out that the amount of added citric acid to the soil has to be controlled. My results confirmed that citric in the concentration of 0.1 mM was toxic for the tobacco plants and enhanced activities of antioxidative enzymes. The decreases in enzyme activities under uranium exposure may be explained by the forming of uranium-citrate precipitates that not trigger oxidative burst as much as free citrate.

In conclusion, the changes in the medium composition had various effects on the plant antioxidative defense responses induced by uranium exposure. Generally, the tobacco plants grown in Hoagland medium showed the least alterations in activities of antioxidative enzymes. However, the presence of free citric acid in medium enhanced enzyme activities more than was observed under combined conditions with uranium. The results from experiment with phosphate absence in the medium showed generally increase of enzyme activities in tobacco plants exposed to uranium ions.

5.3. Effect of uranium on carbohydrate metabolism of the hairy root culture of horseradish

The alterations in the carbohydrate metabolism in the relation to uranium exposure were examined in the hairy root culture of horseradish (*Armoracia rusticana* Gaerth. Mey. et Scherb.) cultivated *in vitro*. The changes in carbohydrate levels, spectrum and activity of enzymes involved in sucrose breakdown were determined. Structural changes of uranium-treated roots will be also discussed in Otradovcova's master thesis.

5.3.1. Effect of uranium on content and spectrum of carbohydrates

The spectrum of carbohydrates in the hairy root of horseradish has not been examined yet. The present results showed that the roots contain mainly sucrose, glucose, fructose and inositol, with the majority proportion of hexoses, i.e. glucose and fructose. Uranium treatments caused marked perturbations in the total carbohydrate content but the observed changes were different in the individual experiments. The results from the first and second experiments showed an increase of the total carbohydrate content in the roots mainly after 7 days of uranium exposure (**Graph 20, 22**). The level of carbohydrates also increased after 14 days of uranium exposure but their total amount was lower than at the 7 day of the treatment (**Graph 22**). I also examined patterns of recovery phase, following the roots exposure to uranium ions for 14 days. Even 7 days after transferring the roots into an uncontaminated medium the treated roots still showed an enhanced accumulation of carbohydrates. The differential recovery of common duckweed (*Lemna minor* L.) to various heavy metals was observed by Drost et al. (2007). They assumed that good recovery of stressed plants depend on the distribution of the metal within the plant body and its mobility during plant growth. According to this hypothesis, uranium ions could simply be tightly retained in the root cells and made the roots recovery impossible. Therefore the persistent high carbohydrate contents observed in uranium-treated roots during recovery phase may were caused due to the still high uranium concentration in the plant cells. The results from the last experiment showed slight decline of the total carbohydrate content in the roots treated with uranium ions for 7 days. After 14 days,

no alterations in carbohydrate levels were observed among the treated and control roots (**Graph 24**).The present results indicated that the amount of glucose, fructose and inositol increased in plants treated with uranium ions (**Graph 21, 23, 25**). An increase in the amount of fructose was higher compared to glucose content. This observation may be explained by enhanced consumption of glucose for synthesis of inositol that its content increased twofold in roots exposed to uranium-ions (**Graph 26**). Inositol is an essential cyclic polyols for all organisms that generates diversified derivates such as phosphatidylinositols or methyled derivate pinitol. The synthesis of inositol is a highly conserved two-step biochemical pathway consisting from conversion of glucose-6-phosphate to *myo*-inositol-3-phosphate followed by dephosphorylation with the participation of *myo*-inositol monophosphate (MIPase) to form free inositol (Loewus et al., 2000). Several studies reported the accumulation of polyols during water, salt or temperature stress. Their primary functions reside in the cell osmotic adjustment but they have other protective effects on macromolecules in stressed plants. Some of these metabolites could act as efficient scavengers of ROS (Vernon and Bohnert, 1992; Smirnoff, 1993; Agarie et al., 2009). The accumulation of inositol was also reported in cadmium-treated poplar (*Populus tremula* L.) (Kieffer et al., 2008). Recently, this finding was confirmed by the observed increase of MIPase transcript levels in the roots of *Miscanthus sinensis* Andersson treated with chromium ions (Sharmin et al., 2012).

In summary, it was shown that, in the hairy roots, uranium induced accumulation of carbohydrates. The role of inositol appears to be important in plant responses to uranium stress. These finding also assumed that uranium can be tightly retained in the cell compartments and thus its toxicity remain even after the uranium exposure. Different results from the last experiment can be explained by the different plant origin due to the long time period between the second and third experiment.

5.1.2. Effect of uranium on activities of enzymes involved in sucrose breakdown

The activities of enzymes involved in sucrose breakdown, i. e. sucrose synthase (SS) and two isoenzymes of invertase (soluble and insoluble), were determined in hairy roots from the third experiment. The activity of insoluble invertase has not been detected or the activity was very low, whereas the activity of soluble invertase increased in the hairy roots mainly after 7 days of uranium exposure. Following 7 days of uranium exposure, the invertase activity remained relatively stable. The assumption that insoluble invertase has not been detected may be associated with subcellular localisations of different invertases. Because of the vacuolar and cytoplasmic localization of soluble invertases, the reason for observed differencies may be explained by the important roles of plasma membrane and tonoplast as efficient barriers against uranium ions. In contrast, the activity of insoluble invertase that is bounded to the cell wall may be reduced by excess of uranium ions in the apoplastic space. The first possibility means direct inhibition of invertase activity by binding uranium ions on sulphudryl groups. The second possibility to explain invertase inhibition is an induced enzyme breakdown by ROS generated by uranium stress. Similar results were also observed in plants exposed to copper stress (Xiong et al., 2008; Huang et al., 2011). These authors concluded that acid invertases including the both soluble and insoluble have an important role in the tolerance of metallicolous plants to heavy metal stress. Podazza et al. (2006) also observed significant reduction in cell wall invertase compared to vacuolar invertase activity that was not changed in Rangpur lime citrus (*Citrus limonia* L. Osbeck). They explained this difference in the activities of invertases through an alteration in the sucrose/H^+ symport due to a reduction of H^+ATPase plasma membrane activity. The presented results indicate the decrease of sucrose synthase activity in the hairy roots treated with uranium ions. After 14 days of uranium exposure, the activity of sucrose synthase remained relatively constant.

Take together, the analysis of enzymes of carbohydrate metabolism affirmed the results of carbohydrate content determination from the third experiment with the hairy roots because of the similar plant material origin. The observed reverse behaviour of SS and IT activities under uranium exposure may result in relatively stable

(or slight decline) levels of carbohydrates in uranium-treated hairy roots that were observed in the experiments. In addition, the changed activities of enzymes of carbohydrate metabolism observed in uranium-treated roots could be explained by a direct effect of uranium ions on the protein level, or by an indirect metal ion effect. However, further experiments are required to achieve deeper knowledge in carbohydrate metabolism under uranium stress.

6. Conclusions

The first objective of this master thesis was to evaluate the responses of hydroponically cultivated tobacco (*Nicotiana tabacum* L.) cv. La Burley 21 to stress caused by uranium exposure. The results indicate:

- Uranium exposure causes alterations of carbohydrate metabolism in the tobacco plants. The changes are in the distribution of individual carbohydrates within plant body as well as in carbohydrate spectrum, but these alterations are different in dependence on the modification of cultivation conditions. The absence of phosphate ions in cultivation medium generate the greatest alterations in carbohydrate metabolism manifested by enhanced carbohydrate accumulation and increased proportion of hexoses in the total carbohydrates.
- Oxidative stress induced by uranium exposure enhances antioxidative enzyme activities in the tobacco plants. The citric acid alone in cultivation medium elicits the higher activities of all tested antioxidative enzymes compared to combined conditions with uranium ions.

The second objective of this master thesis was to evaluate the responses of the hairy root culture of horseradish (*Armoracia rusticana* Gaerth. Mey. et Scherb.) cultivated *in vitro* to stress caused by uranium exposure. The findings show:

- The hairy roots contain mainly sucrose, glucose, fructose and inositol.
- Uranium exposure causes the preferential accumulation of hexoses in the roots.
- Uranium induced inositol accumulation is observed.

In summary, the plant responses to uranium stress differ among particular plant species, type of culture as well as cultivation conditions. For phytoremediation purposes it should be emphasized that extensively recommended usage of citric acid for enhanced uranium accumulation has to be done in a carefully controlled manner.

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