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Full Length Article

Tetracycline Accumulation in Pea Seedlings and its Effects on Proteome and Enzyme Activities

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Abstract

Among antibiotics, tetracyclines are the most commonly used and detected in the environment. In this study, the amount of tetracycline taken up from soil by pea seedlings was analyzed, identified its main site of accumulation in plants and determined also changes in the protein profile of pea. The study demonstrates that pea seedlings take up tetracycline from soil and transport the drug via roots to over-ground parts and then accumulate it in the youngest parts, such as upper stem and leaves. After the taken up of drug, the activity of guaiacol peroxidase is modified and changes in the profile of proteins, as determined by two-dimensional gel electrophoresis occur. The majority of proteins (~40%) visualized possessed molecular weight between 25 and 37 kDa. Only 8% of the proteins had molecular weight lower than 20 kDa, and 2% greater than 75 kDa. The number of spots in the control samples was 194, which is less by 49 than at the concentration of 150 mg kg^{-1} of soil. Isoflavone reductase was present only in seedlings growing with tetracycline. Tetracycline uptake from soil modify mainly the changes in biochemical processes connected with protein.

Keywords: Tetracycline uptake; Pea seedlings; Enzyme activity; Proteins profile

Introduction

Tetracyclines are widely-used antibiotics in the treatment of humans and veterinary medicine due to a wide spectrum of antibacterial activity and low costs (Liu *et al*., 2013). The mechanism of tetracycline toxicity involves the inhibition of bacterial protein biosynthesis thanks to binding with a 30S ribosome subunit in the bacterial cell. The inhibition of granulocytic activity and a drop in antibody production cause an anti-inflammatory effect of tetracyclines (Kuzin *et al*., 2001; Connell *et al*., 2003).

The use of antibiotics on an enormous scale, their irrational administration with feed for prophylactic purposes and the direct addition of antibiotics to ponds are a source of their presence in soil and surface and ground waters, sewage and even in drinking water (Daghrir and Drogui, 2013). Fertilization of fields with animal excrement is also a reason behind the transmission of antibiotics to the environment (Kwon *et al*., 2011). In addition, in many countries in the world such as Canada, USA and Korea, antibiotics are used as growth stimulators (Daghrir and Drogui, 2013). Communal and hospital sewage and wastes from drug-producing factories are also reason for antibiotics being found in the environment (Bagda *et al*., 2013).

In human and veterinary medicine, there are hundreds of different antibacterial drugs (Kümmerer and Henninger, 2003). The average global use of antibiotics ranges from 100 to 200 thousand tons per year, of which over 39% are used in veterinary medicine (Luo *et al*., 2010; Cheng *et al*., 2014).

Tetracycline is commonly administered in the treatment of human diseases such as lung diseases, infections caused by *Helicobacter pylori* or for acne, in which the treatment duration extends to as long as five months. A single dose of tetracycline administered orally averages 20 mg kg^{-1} BW, while a daily amount of this antibiotic per an adult human reaches 2 g depending on the type of infection (Walker-Renard *et al*., 1994; Stenström *et al*., 2008; Alikhan *et al*., 2010). The whole dose of ingested antibiotic is not metabolized and a part of it is excreted from the body (Shi *et al*., 2012). The conducted studies indicate that 30% of the ingested dose of tetracycline is excreted with urine in an unchanged form and 20 – 60% is eliminated with feces (Agwuh and MacGowan, 2006; Huang *et al*., 2013). Therefore, one person treated with tetracycline excretes daily as much as 500 mg of the drug in an unchanged form.

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Tetracycline retains antibacterial properties in the environment for a certain period of time (Chander *et al*., 2005). It has been found that within 5 months, tetracycline in a liquid fertilizer undergoes degradation by 50% (Winckler and Grafe, 2000). Tetracyclines show solid and durable adsorption to soil particles while being simultaneously resistant to biodegradation (Huang *et al*., 2013). It has been demonstrated that tetracyclines, similar to fluoroquinolones may persist in soil from several months to as long as few years (Hamscher *et al*., 2005; Rosendahl *et al*., 2012). The proven efficacy of tetracycline in water and soil may result in this antibiotic entering the food chain (Zhao *et al*., 2010; Wei *et al*., 2011). It has also been demonstrated that plants are capable of uptaking drugs from soil (Adomas *et al*., 2013). This ability constitutes a potential risk to consumers ingesting different parts of plants.

This study analyzed the amount of tetracycline uptaken from soil by pea seedlings and identified the organs in which this antibiotic is accumulated. The impact of tetracycline present in a plant on its growth, water potential, content of chlorophyll, activity of guaiacol peroxidase and the profile of proteins in the tissues of pea were also determined.

Materials and Methods

Germination and Growth

Pea seeds (*Pisum sativum* L., cv. Cysterski) were germinated in rolls of blotting germination paper (Anchor Paper, USA) at room temperature (24/20°C day/night) for 7 days. Three seedlings per replicate were then transplanted in 150 ml pots filled with soil. Cultivation was conducted for another 7 days at 23/19°C day/night with a 16 h of light provided with 3 klx sodium-vapor lamps. Plants were fertilized twice with a diluted Hoagland's medium (Sigma-Aldrich). On day 7, aqueous tetracycline solutions were added to the pots (with 14-day-old plants) in order to produce concentrations of 3, 15, 30, 50 and 150 mg kg^{-1} of soil. Seedlings irrigated with distilled water served as control sample.

Determination of Seedling Growth, Water Potential and Chlorophyll Content

In 17-days-old seedlings, osmotic pressure and chlorophyll content in pea leaves was performed on the third day after adding tetracycline solutions to the pots. Osmotic pressure was measured with a Scholander bomb (MODEL 300) and the content of chlorophyll in pea leaves was determined with a Minolta SPAD-502 chlorophyll meter.

Activity of Guaiacol Peroxidase

The activity of guaiacol peroxidase was measured on the third day after adding tetracycline solutions to the pots. Pea seedlings (leaves and stems; 500 mg) were homogenized in a porcelain mortar at 4ºC in 5 mL of isolation buffer [0.1 M Tris-HCl (Sigma), 8.75% polyvinylpyrrolidone (Sigma), 0.1M KCl (PPH Stanlab), 0.28% Triton X-100 (Sigma)]. The extract was centrifuged for 30 min at 2,800 *x g* at 4ºC. After which, the amount of isolated protein was determined with the method by Lowry *et al.* (1951).

Guaiacol peroxidase activity was determined spectrophotometrically (CECLI, CE2021 2000 series). Fifty (50) µL of the plant extract and 25 µL of 0.06% H_2O_2 (CHEMPUR) were added to 2 mL of a reaction mixture (0.1 M KH_2PO_4 (CHEMPUR), 100 µL 1% guaiacol (Sigma). The growth rate of absorbance was measured at λ=470 nm wavelength at room temperature. One unit (U) corresponded to oxidation of 1 µM H_2O_2 during one minute.

Tetracycline Content in Plants

The content of tetracycline in plants was determined on day 1, 2 and 3 after adding the tested tetracycline solutions to the pots. The above-ground part of each 17-days-old seedling was divided into three parts (a 40 mm bottom of stem, a 40 mm upper stem, and leaves). The weighing portions of 500 g of the selected plant parts were homogenized in a porcelain mortar with 2 mL of methanol and centrifuged for 30 min at 9,000 *x g*. The obtained supernatant was transferred onto nylon filters (mesh diameter 0.22) (Sigma). Plant sap was squeezed from fresh plant material with mortar and pestle. For all extractions, SPE cartridges Chromabond®Easy, 3 mL/200 mg, Macherey-Nagel, Dtiren, Germany were used. SPE cartridges were rinsed with methanol and after loading with plant saps were eluted with 250 µL methanol. Tetracycline content in seedlings was analyzed by HPLC according to Pailler *et al.* (2009) with small modifications. Briefly, the chromatographic system consisted of a Water Aliance 2695 HPLC system (Waters Corp.) with a binary high-pressure gradient pump, an automatic injector and a column oven. The chromatographic column was an Atlantis T3 column (150×3.0 mm, 3 µm) (Waters Corp.) at 40º C. The MS–MS analyzer consisted of Quattro micro® API MS (Waters Corp.) using electrospray in the positive mode (ESI+). N_2 was used as nebuliser, drying, curtain and collision gas. A chromatographic gradient was applied for the separation of the analytes depending on the ionization mode employed, with a total chromatographic run of 18 min. Gradient elution was carried out with aqueous 0.1% formic acid: 0.1% formic acid in acetonitrile at flow rate of 0.45 mL/min. Validation of the method included the assessment of selectivity, linearity (1 to 11 µg/mL), limits of detection (8 ng/mL) and quantification (26 ng/mL). Chromatographic system and data collection were controlled with a MassLynx 4.1. chromatographic software interfaced to a personal computer.

Two-dimensional Gel Electrophoresis

Two-dimensional electrophoresis of proteins was performed

with seedlings (stem and leaves) growing on a substrate with the highest dose of tetracycline (150 mg of the drug/kg of soil). The seedlings (2 g) were homogenized in a porcelain mortar in an extraction buffer [500 mM TRIS with pH=8 (Sigma), 50 mM EDTA (Sigma), 700 mM of sucrose (Sigma), 100 mM KCl (PPH Stanlab), 0.07% 2-mercaptoethanol (Sigma)]. A 2.5 mL dose of phenol was added to the extract and the mixture was vortexed for 10 min. The extract was centrifuged at 4°C for 10 min at 5,500 *x g*. After centrifuging, a phenol phase was collected and 1.8 mL of the extraction buffer was then added. Following 3-minute mixing, the samples were centrifuged for 20 min at 3,200 x g. The phenol phase was transferred to a new tube and mixed with a four-fold volume of 100 mM ammonium sulphate (Sigma) dissolved in cold methanol (Chempur). The mixture was incubated for 12 h at -20°C. After 12 h, precipitated proteins were centrifuged at 4°C/3,200 *x g*/15 min and the obtained sediment was washed two times in cold acetone (Chempur) centrifuging it each time at 4°C/5,500 *x g* for 5 min. The sediment was dried at room temperature.

The dried sediment was dissolved in 9 M urea (POCH), 20 g/dm^3 CHAPS (Sigma), 0.3% DTT (Sigma) and incubated for one hour at room temperature. Next, the samples were ultrasonified in an ice bath for 15 sec and then centrifuged at 14,000 *x g*/5 min. The procedure was repeated three times. The prepared solution of proteins was used in further analyses.

IPG, 7 cm, pH 4–7 strips (Ready-Strip, Bio-Rad) were actively rehydrated for 12 h in a rehydration buffer (Bio-Rad) with 300 µg of isolated protein. Isoelectrofocusing was performed under the following conditions: 250 V/15 min, 4000 V/2 h 30 min, and then with rapidly increasing voltage up to 20,000 V.

After isoelectric focusing, the strips were equilibrated in a buffer containing 6 M urea, 2% SDS, 0.375 M Tris-HCl with pH 8.8, 20% glycerol, 130 mM DTT (Bio-Rad) for 10 min and then in a buffer containing 6 M urea, 2% SDS, 0.375 M Tris-HCl with pH= 8.8, 20% glycerol, 135 mM jodoacetamid (Bio-Rad) for another 10 min.

In the second stage of electrophoresis, the separation of proteins was performed with 10% polyacrylamide gels (10.0 cm x 7.0 cm) in a Mini-PROTEAN Tetra System apparatus for electrophoresis (Bio-Rad). Electrophoresis was run for 60 min at a constant voltage of 200 V. Gels were stained in a 0.1% Commassie Brillant Blue G-250 solution (Sigma) overnight and then visualization with a Gel Doc EZ Imager (Bio-Rad) and an analysis with PDQuest (Bio-Rad) were performed.

MALDI TOF/TOF Analysis

Spots were excised out of the gels with a scalpel, placed in test tubes, washed and destained (Shevchenko *et al.*, 1996). Proteins were dehydrated in a vacuum centrifuge and subsequently digested overnight in a solution of 15 ng µL^{-1} trypsin (Promega) in 25 mM ammonium bicarbonate (Sigma). Incubation was conducted at 37°C. The sample was sonified for 5 min and dehydrated in a vacuum centrifuge and 1 µL of a previously prepared solution of α-cyano-4-hydroxy-cinnamic acid (Sigma) was then added and dissolved in 50% acetonitrile (SIGMA) with 0.1% trifluoroacetic acid (Sigma). The entire sample was then applied onto a steel MALDI plate. Mass spectra of the peptides were taken with a MALDI TOF/TOF Autoflex III SmartBeam mass spectrometer (BruckerDaltonics). The PMF search was conducted in the NCBI database. The statistical probability of the PMF was calculated in the MASCOT database. Results exceeding 46 were taken into account ($p<0.05$).

Results

The impact of incremental tetracycline doses, i.e. 3, 15, 30, 50 and 150 mg kg^{-1} of soil, on the growth of stems in 14-days-old pea seedlings, was investigated on the first, second and third day after the application of the drug in comparison with the control.

The 14-days-old plants took up the drug from soil only on three days, i.e. on day 15 (24 h after the application of the drug), day 16 (after 48 h) and on day 17 (after 72 h). After a 24 h hazard assessment, no clear impact of the drug on the length of stems was recorded. On day 2 (after 48 h), there was a reduction in the length of stems only at concentrations of 15 and 30 mg of tetracycline/kg of soil and higher by 7%, on average. The higher tetracycline doses (50 and 150 mg kg^{-1} of soil) in soil inhibited the growth of stems by 15% and 59%, respectively. An increase of the time of drug uptake to 72 h resulted in a reduction of stems by 16%, 46%, 57% and 35% at the concentrations of 15, 30, 50 and 150 mg of tetracycline/kg of soil, respectively. The hazard assessment for tetracycline after 48 and 72 h demonstrated an inhibition of stem growth (Fig. 1A).

The content of chlorophyll, osmotic pressure, peroxidase activity and protein content were measured in over-ground parts of 17-days-old pea seedlings (after 72 h hazard assessment). It was found that together with incremental doses of the drug in soil and increasing the time of its uptake, the content of chlorophyll in leaf tissues decreased. Chlorophyll content in leaves of the controls was 40 SPAD and decreased successively up to the lowest value (38 SPAD) at 150 mg of the drug/kg of soil (Fig. 1B).

The inhibition of seedling stem growth in soil contaminated with tetracycline is confirmed by measurements of osmotic pressure. The osmotic potential of pea seedling stems was determined after 72 h of exposing the plants to an uptake of the drug by the root system. The measured value in control stems was 84 PSI. The osmotic potential of stems was inhibited by 3%, 13%, 16%, 19% and 49% by incremental concentrations of the drug in soil: 3, 15, 30, 50 and 150 mg kg^{-1} of soil, respectively (Fig. 1C).

Table 1: The proteins identified in aboveground parts (stem and leaves) of pea seedlings (*Pisum sativum* L.)

Spot No.	Accession No.	Protein	Plant	Score	MW (kDa)	pI (exp.)	pI calculated (Mascot)
1	gi | 100380	Ribulose- bisphosphate carboxylase activase (fragment)	*Nicotiana tabacum*	79	40.6	6.05	5.69
2	gi | 399942	Heat shock- related protein hsp 70. chloroplastic	*Pisum sativum*	62	75.8	4.85	5.22
3	gi | 131384	Oxygen- evolving enhancer protein 1. chloroplastic. 33kDa subunit of oxygen evolving system of Photosystem II	*P. sativum*	135	39.5	5.18	6.25
4	gi | 3243234	Isoflavone reductase related protein	*Pyrus communis*	98	35.2	6.26	8.37
5	gi | 357492793	Ferritin- 2	*Medicago truncatula*	73	43.1	5.35	5.35
6	gi | 392056685	Putative NAD- dependent dehydrogenase 2	*Erythroxylum coca*	63	26.8	6.08	5.64
7	gi | 17224763	ATP- synthase. beta-subunit	*Burmania longifolia*	56	57.1	5.46	5.10
8	gi | 131390	Oxygen- evolving enhancer protein 2. chloroplastic. 23kDa subunit of oxygen evolving system of photosystem II	*P. sativum*	79	21.7	5.96	8.29
9	gi | 225449132	ATP synthase. subunit d. mitochondrial	*Vitis vinifera*	46	19.0	5.15	5.34
10	gi | 6996529	Ethylene- responsive enolase	*Glycine max*	54	53.6	6.05	4.75

The activity of guaiacol peroxidase increased with the increments of drug concentrations in the plants. The lowest activity of this enzyme (0.6 U) was detected in control seedlings. The highest activity, by 33% than in control seedlings, was recorded in the plants taking up the drug from soil at the concentration of 150 mg kg^{-1} of soil (Fig. 1D).

The content of tetracycline was determined in pea stems divided into three parts (a 40 mm long bottom of stem, a 40 mm long upper stem and leaves) and taking up the drug for 24, 48 and 72 h. It was found that pea seedlings took up tetracycline from soil and the lowest amount of drug was accumulated in the 40 mm bottom of stem whereas the highest in leaves. It was also found that a prolonged uptake time (up to 72 h) resulted in a higher content of tetracycline in the tested pea tissues. After the first day of treatment, the presence of drug was detected in all three analyzed above-ground plant parts. On day two, the content of the antibiotic increased by 10% in the 40 mm bottom of stem, by 60% in the upper stem, and two-fold (100%) in leaves of seedlings growing on soil added with 150 mg of the drug. On the third day, the content of tetracycline increased proportionally to the time of exposure, reaching the maximum values in the 40 mm bottom of stem, 40 mm upper stem and leaves of 7, 18 and 24 µg g^{-1} of fresh weight, respectively (Fig. 2).

A 2-D electrophoresis was performed for proteins with pH 4–7 from aboveground parts of pea seedlings, three days after tetracycline was applied (150 mg kg^{-1} of soil weight) and from the control sample. There were 145 spots of proteins from samples treated with antibiotics and 194 in the control sample (Fig. 3). The number of spots common to both samples was 70. Proteins with a molecular weight of 25–37 kDa accounted for 40% of all spots; there were only 8% of separated proteins with the molecular weight of < 20 kDa. The smallest number of proteins had a molecular weight within the range 75–100 kDa; which accounted 2% of all proteins.

Ten proteins were identified by mass spectrometry (Table 1). Molecular weights of the analysed proteins ranged from 19–75.8 kDa. The analysed samples were found to contain proteins involved in protection against the effects of oxidative stress: ferritin, NAD-dependent dehydrogenase 2, isoflavone reductase (found in seedlings growing with tetracycline). Moreover, proteins of chloroplasts were identified: ribulose-bisphosphate carboxylase activase, heat-shock - related protein HSP 70,

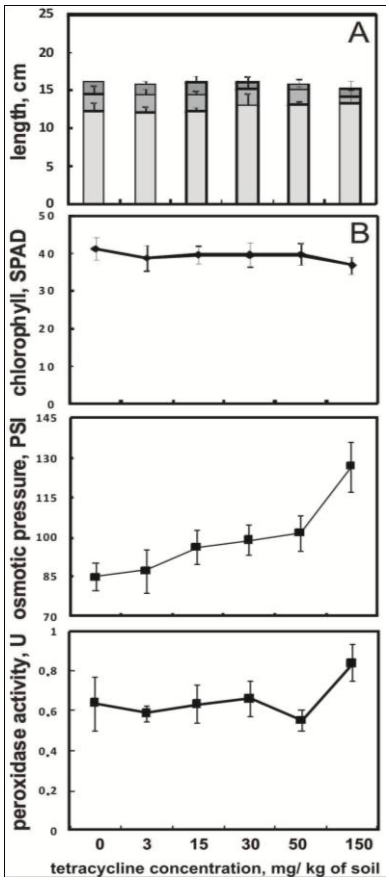

Fig. 1: Seedlings length ((A) □ - 17 days + 24 h; ▨ - 17 days + 48 hours; ■ - 17 days + 72 h), chlorophyll content (B), osmotic pressure (C) and peroxidase activity in *Pisum sativum* after three days on soil supplemented with different tetracycline concentration (0, 3, 15, 30, 50, 150 mg x kg^{-1} of soil). Data points represent the means ± SD from three to nine replicate samples

oxygen-evolving protein 1 as well as oxygen-evolving protein 2 and mitochondrial-ATP synthase, subunit d. The expression of each protein, determined on the basis of intensity measurement (PDQuest BioRad) changed after tetracycline was used (Fig. 4). The greatest differences were found for Hsp 70, whose expression (measured as the intensity of a spot color) increased nearly two-fold in seedlings growing with tetracycline, as well as NAD-dependent dehydrogenase and ethylene-sensitive enolase, whose expression increased by over 30% (Fig. 4). Moreover, the intensity of ferritin 2 in these seedlings was found to increase by 5%. Seedlings growing with water were found to contain an increased (by 5%) amount of ribulose-bisphosphate carboxylase activase and oxygen-evolving enhancer protein 2 (OEE2), whereas expression of oxygen-evolving enhancer protein 1 (OEE1) increased by 8%. The intensity of ATP-synthase subunit d in control samples was higher by 17%, compared to seedlings growing on substrate with tetracycline.

Discussion

The presence of antibiotics and other drugs has been repeatedly detected in the environment. Tetracycline has been found in swine manure, soil fertilized with feces, sewage, sewage sludge, surface waters and even in drinking water (Domínguez *et al*., 2014). Different amounts of tetracycline have been determined in soil: 5 mg kg^{-1} (Hamscher *et al*., 2003) and 98.2 mg kg^{-1} (Chen *et al*., 2012). The applied doses of tetracycline are comparable to their actual/determined concentrations in the soil environment.

Among the studies on the presence of drugs in the environment, there is still insufficient data and information on the uptake capacity, storage and sites of accumulation in the specific parts of plants and on the phytotoxic effects of drugs.

It has been demonstrated that forage plants, agricultural crops and vegetables (barley, carrot) take up and accumulate ciprofloxacin from the soil in different concentrations (Eggen *et al*., 2011). Similarly, it has been shown that cabbage and green onion are capable of taking chlortetracyclines up (Kumar *et al*., 2005), whereas legumes can take sulphametazine up (Piotrowicz-Cieślak *et al*., 2010). In the study reported here, pea seedlings were shown to accumulate tetracycline despite a short exposure time (Fig. 2). Tetracycline was also taken up by *Iberis sempervirens* L. (Marco *et al*., 2013). The concentration of sulphametazine in lettuce and potatoes increased together with an incremental content of this antibiotic in soil (Dolliver *et al*., 2007). The same regularity has been found in present study with tetracycline and pea. It was deliberately attempted to omit an analysis of the content of tetracycline in roots due to their direct contact with the antibiotics deposited in the soil. It was assumed that the analysis of tetracycline concentration in roots would be unreliable (Fig. 2).

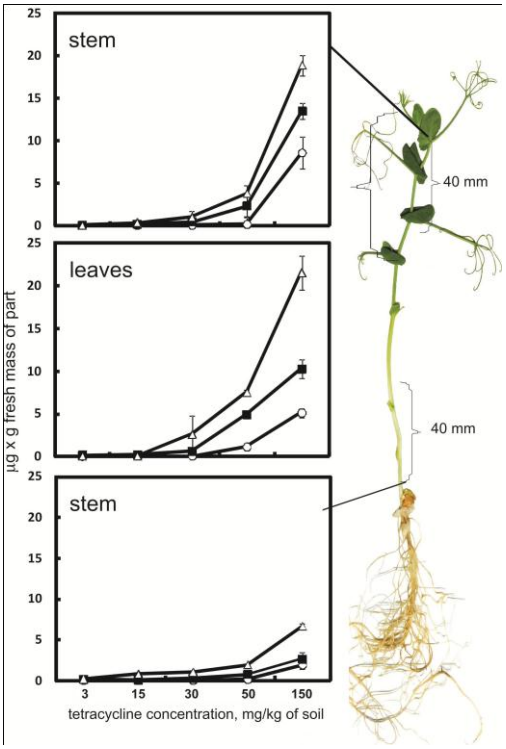

Fig. 2: Tetracycline content in *Pisum sativum* after the first (o), the second (■) and the third (Δ) days on soil supplemented with different tetracycline concentration (0, 3, 15, 30, 50, 150 mg x kg^{-1} of soil). Data points represent the means ± SD for three replicate samples

The clearest and most rapid growth of tetracycline concentration in pea leaves indicates a special role of leaves in the process of tetracycline deposition, suggesting special role of these organs play in the accumulation of this antibiotic. The highest concentration of tetracycline and sulphonamides in radish leaves, celery leaves, rape seedling and coriander in comparison with their stems and roots was demonstrated by Hu *et al*. (2011). The levels of gentamicin and streptomycin in carrot, lettuce and radish tissues increased with an increasing concentration of antibiotics in manure (1 mg kg^{-1} > 0.5 mg kg^{-1}) (Bassil *et al*., 2013).

The phytotoxicity of an antibiotic to plants may be manifested by inhibition of germination and growth. Even low doses of enrofloxacin have been found to inhibit germination and elongation growth, both in the above-ground part and roots of narrow-leaved lupin (Adomas *et al*., 2013). The conducted hazard assessment for tetracycline at higher concentrations (15, 30, 50 and 150 mg of the drug/kg of soil) demonstrated the inhibition of stem growth only after 48 and 72 h (Fig. 1A). The tested low (3 mg of the drug/kg of soil) concentrations of tetracycline were less phytotoxic to pea than enrofloxacin (Adomas *et al*., 2013) which inhibited the growth of narrow-leaved lupin after

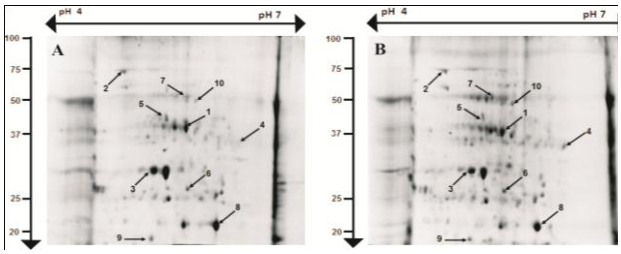

Fig. 3: 2D Electrophoresis of proteins of aboveground parts of pea (*Pisum sativum* L.) without (A) and with the antibiotic (B) at the concentration of 150 mg/ kg of soil. Protein separation was conducted at pH 4-7

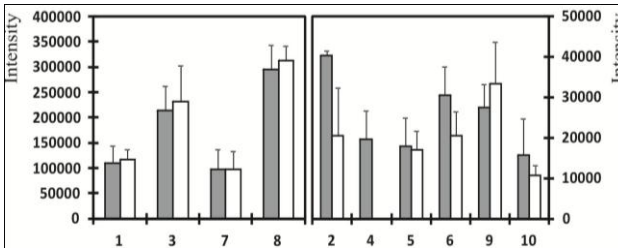

Fig. 4: Intensity of destaining (PDQuest, BioRad) of proteins from control seedlings (■) and from those growing for three days with tetracycline (□). Number of spots 1, 2....10 – see in Table 1

seven days.

The phytotoxicity of tetracycline to pea was also confirmed with measurements of chlorophyll content (Fig. 1). As a relatively unstable compound, chlorophyll is readily degraded and influenced by light, oxygen, dehydration, heating etc. Tetracycline may inhibit the activity of the enzymes involved in the synthesis of chlorophyll molecules (Kasai *et al*., 2004). The applied doses of tetracycline were phytotoxic proportionally to the administered concentration of the drug. A decreased content of both chlorophyll and carotenoids induced by sulphadimetoxine was recorded in studies with *Salix fragilis* L. conducted by Michelini *et al*. (2012). In the present studies, tetracycline significantly impacted the physiological parameters of the plant, such as turgor. An increase in the concentration of the antibiotic reduced turgor in pea, which was manifested as a gradual increase in osmotic pressure of plants. In addition, it was shown that tetracycline found in the tissues of pea plants activated enzymes of oxidative stress, which generate the production of reactive oxygen species (ROS). It was proven that the activity of guaiacol peroxidase (Fig. 1) increased with the increments of tetracycline concentrations in soil, which indicates that tetracycline is a stressor to plants. The toxicity of tetracycline to plants has been also demonstrated in studies with rice, cucumber and oat. However, in comparison with sulphametazine, sulphametoxazole and trimethoprim, the toxicity of tetracycline was significantly lower (Liu *et al*., 2009).

In this study, we used the phenol method for protein extraction. The phenol method provides the highest protein yield and the least contamination of protein samples, which is important since removal of interfering compounds crucial for SDS-PAGE, IEF and 2-D electrophoresis (Pavoković *et al*., 2012). The majority of proteins (~40%) visualized possessed molecular weights between 25 and 37 kDa. Current commercially available 4% total acrylamide IPG gel strips limit the high molecular weight proteins visualized with 2-D electrophoresis (Candiano *et al*., 2002). As a natural consequence of the effect of a stress factor on a tissue, expression of genes of proteins protecting cells from damage is induced. Isoflavone reductase (IFR) plays a key role in the synthesis pathway of isoflavonoids, which play a role in protection against microorganisms and pests (Dakora and Phillips, 1996; Petrucco *et al*., 1996; Lers *et al*., 1998). Moreover, the concentration of IFR in cells was found to be increased by abiotic factors, such as waterlogging in soybean tissues (Iftekhar *et al*., 2010). Tetracycline induced presence of IFR, with simultaneous absence of the enzyme in control samples, which indicates the possibility of IFR involvement in protection mechanisms against harmful effects of the antibiotic (Table 2).

Along with isoflavone reductase, protective functions against the effects of oxidative stress are played by Hsp 70 – a chaperone protein which intensified synthesis has been found to be caused by high and low temperature, dehydration or excessive salinity (Bae *et al*., 2003; Wang *et al*., 2008). A two-fold increase (Fig. 4) in the concentration of Hsp 70 protein in pea seedlings growing with the antibiotic may indicate a protective function of the protein in folding cellular proteins, which may be damaged by free radicals (Agrawal *et al*., 2002; Bohler *et al*., 2007). Free radicals also stimulate the expression of ferritin (Fig. 4). This protein is believed to have a detoxicating capability in case of excessive concentrations of iron, ozone, NO or hydrogen peroxide (Zhao *et al*., 2002; Theil *et al*., 2006; Arosio *et al*., 2009); it may also be able to detoxicate the cell from tetracycline. Data accumulated so far indicate that ferritin is stimulated by dehydration and excessive moisture content (Ravet *et al*., 2009; Alam *et al*., 2010).

The presence of tetracycline resulted in a decreased concentration of RUBISCO activase, OEE1 and OEE2 (Fig. 4), which indicates that photosynthesis is disturbed by tetracyclines. This was confirmed by a decrease in the concentration of chlorophyll in seedlings growing on soil with tetracycline (Fig. 1). Proteins OEE1 and OEE 2 are indispensable in electron transport in the process of photosynthesis (Mayfield *et al.*, 1987). A decrease in RUBISCO activase, OEE1, OEE 2 was demonstrated in oxidative stress (Agrawal *et al*., 2002; Bohler *et al*., 2007; 2010).

A change in the concentration of proteins in seedlings growing with tetracycline compared to the control may result from activation of stress proteins, as well as of diverse activity of cell-metabolism-regulating proteins. Expression

of ATP synthase, necessary in the process of oxidative phosphorylation, was found to have decreased. These reactions are different than those observed during cold or dry periods (Bogeat-Triboulot *et al*., 2007; Badowiec *et al*., 2013). An increase in the expression of ATP synthase was observed in wheat (Wang *et al*., 2008) and in C4 *Aeluropus lagopoides* (*Poaceae*) plants (Sobhanian *et al*., 2010). A small decrease in expression of ATP synthase may indicate that cellular respiration processes may be disturbed, but expression of NAD-dependent dehydrogenase increased rapidly. The enzyme concentration is increased and cellular metabolism probably quickened in order to maintain homeostasis. Therefore, an increase in the concentration of ethylene-sensitive enolase would be justified. The protein participates in metabolism of carbohydrates and its concentration was shown to have increased in cold stress (Badowiec e*t al*., 2013).

Conclusion

The studies demonstrated that pea seedlings took up tetracycline from soil and transported the drug via roots to the over-ground parts. It was found that tetracycline was present in the upper parts of plants with the fastest growing parts: stem and leaves, whereas the bottom part of the stem was responsible for transport. The content proteins in 2-D eletrophorogram spots of pea seedlings decreased when plants were growing in the presence of tetracycline at the concentration of 150 mg kg^{-1} of soil.

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