SPARFLOXACIN – A POTENTIAL CONTAMINANT OF ORGANICALLY GROWN PLANTS?

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Abstract. Pharmaceuticals in water and soil ecosystems increasingly become a problem for environmental protection and agriculture. They may be transferred to soil and plants even in organic production systems as they often occur in manure. This paper presents assessment of sparfloxacin effect on protein profile and activity of guaiacol peroxidase in pea roots. The obtained results show that sparfloxacin modifies pea root proteome. In proteomic profiles of roots from seedlings grown at the highest sparfloxacin concentration (800 mg kg^{-1}) there were 41 proteins more than in protein separations of roots of control seedlings, in which 176 proteins were identified. A higher number of proteins, compared to control roots (21 more), were also obtained on separations of root proteins from plants grown in substrate „contaminated" with 40 mg kg^{-1} sparfloxacin. With increasing sparfloxacin concentration the expression of root proteins involved in cell bioenergetics decreased, while isoflavone reductase, responsible for free radicle scavenging, became up-regulated. Moreover, the presence of sparfloxacin in soil resulted in increased activity of guaiacol peroxidase. The sparfloxacin content in roots was directly proportional to the level of this pharmaceutical in soil. At the highest concentration of sparfloxacin in soil (800 mg kg^{-1}) the level of this compound in roots reached 247 μg g^{-1} fresh weight. Soluble carbohydrate contents in pea tissues correlated with seedling growth (root and stem elongation), which decreased parallel to increasing concentrations of soil sparfloxacin.
Keywords: *2D electrophoresis, drug uptake, pea, protein profile, antibiotics*

Abbreviations: Fluoroquinolons – FQ; Guaiacol peroxidase – POX; Reactive oxygen species – ROS; Sparfloxacin – SPX

Introduction

Common use of antibiotics in human and veterinary medicine results in their increased presence in water and soil (Sukul and Spiteller, 2007). In 1999 total use of antibiotics in European Union and Switzerland amounted to 13288 tons, of which 8637 tons were used in human medicine (65%), 3854 tons in veterinary medicine (29%) and 797 tons were applied as growth stimulators (6%). However, in 2006 in France only, 1300 tons of antibiotics were used (Awad et al., 2014). It is estimated that global use of antibiotics in recent years amounted to, on average, 100 – 200 thousand tons/year (Chen et al., 2012; Liu et al., 2009). During medication and after its completion, most of the applied antibiotics (30 – 90%) are released to the environment, in an unmodified form or transformed to active metabolites (Liu et al., 2009; Chen et al., 2012; Pinheiro et al., 2013). For farmaceuticals the main route of entry to soil is through animal waste, used as organic fertilizers. Fluoroquinolons (FQ) are a group of antibiotics characterised by slow degradation in the environment, related to their strong adsorption to organic matter. Decay time of antibiotics and their active metabolites in manure and soil

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depends on both environmental conditions and chemical properties of the antibiotic (Seo et al., 2010). FQs are chemotherapeutics commonly used for treatment of bacterial infections (Jia et al., 2012), which results in their presence in environment at rather high levels (Khetan and Collins, 2007). FQs contents in wastewater amount to 120 μg dm^3, while in groundwater and surface water it occurred at 1.3 μg L^{-1}, and it was even detected in drinking water (Jia et al., 2012; Wammer et al., 2013).

In municipal wastewater among FQs the highest concentrations are reached by ofloxacin (1.3 μg L^{-1}) and norfloxacin (77-87 ng dm^3). Moreover, levofloxacin, pipemidic acid, enrofloxacin and sparfloxacin were also detected (Jia et al., 2012). However, by far the highest concentrations of antibiotics were found in manure, in which they were mainly represented by tetracyclines. According to Martínez-Carballo et al. (2007) chlorotetracycline, oxytetracycline and tetracycline concentrations in manure were 46 mg kg^{-1}, 29 mg kg^{-1} and 23 mg kg^{-1}, respectively. High concentrations of tetracyclines were found in soils of Turkey and Spain, amounting to 0.5 mg kg^{-1} and 0.2 mg kg^{-1}, respectively (Andreu et al., 2009; Karci and Balcioglu, 2009). In soils fertilised with manure the tetracycline content may even reach 7 mg kg^{-1} (Zhang et al., 2008).

FQs are taken up and accumulated in plants and their levels in plants depend on drug concentration, time of exposure and properties of the plant (Lillenberg et al., 2010; Ziółkowska et al., 2015). The mechanism of antibiotics action on plants is still unknown. Antibiotic concentrations in plants are not documented. It is not clear if antibiotic accumulation may modify plant metabolism and affect plant quality. There have been very few reports assessing antibiotic contents in plants, in contrast to drug levels in animals.

The purpose of this work was to determine the level of sparfloxacin in pea and study the effect of this drug on selected physiological-biochemical parameters of pea (peroxidase activity, protein content and 2D electrophoretic profile, as well as soluble carbohydrate contents).

Materials and methods

Germination and growth

Seeds of pea (*Pisum sativum* L.) cv. Cysterski were germinated for seven days in Phytotoxkit plates (MicroBio Test, Inc., Belgium). Germination was carried out under controlled climatic conditions with temperature set at 25° C and 90% relative humidity (RH), under photoperiod conditions (16/8 day/night, light intensity 3.4 klx). Ninety millilitres of soil (sand:vermiculite:peat, 1:0.3:1, v/v/v) were placed in each plastic microbiotest plate. The soil was covered with Whatman No. 1 filter paper and watered with 27 ml distilled water supplemented with sparfloxacin (5-Amino-1-cyclohexyl-7-(*cis*-3,5-dimethylpiperazino)-6,8-difluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid; Sigma-Aldrich) at final concentrations of 0.08; 0.4; 0.8; 4; 8; 40; 80; 200; 400; 800 mg kg^{-1} of soil. The control plants were watered with pure distilled water. The root length was estimated after seven days of germination using Image Tool for Windows. Fresh weight of seedlings was determined according to standard seed testing recommendations ISTA (2011). The experiment was carried out with four replicates, each containing 40 seedlings.

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Sparfloxacin content in roots

The content of sparfloxacin in 7-days old roots was determined. Plant sap was squeezed from fresh plant material with mortar and pestle. Roots (500 mg) of were homogenized with 1 ml of methanol and centrifuged for 10 minutes at 13200g. The obtained supernatant was transferred onto nylon filters (mesh size 0.22) (Sigma). 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 analysed 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 40^{o} C. The MS–MS analyser 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 a flow rate of 45 ml min^{-1}. Validation of the method included the assessment of selectivity, linearity (1 to 11 µg ml^{-1}), limits of detection (8 ng ml^{-1}) and quantification (26 ng ml^{-1}). Chromatographic system and data collection were controlled with a MassLynx 4.1. chromatographic software interfaced to a personal computer.

Activity of guaiacol peroxidase

The activity of guaiacol peroxidase in the root tissues of seven day old pea was measured. Pea roots (500 mg) were homogenized (30 min) in a porcelain mortar at 4^{o}C 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 supernatant was passed through a nylon filter(mesh diameter 0.45). The extract was centrifuged for 30 minutes at 4000g at 4^{o}C. Next, the amount of isolated protein was determined by Lowry et al. (1951).

Guaiacol peroxidase activity was determined spectrophotometrically (CECLI, CE2021 2000 series). 50 µl of the plant extract and 20 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.

Soluble carbohydrate contents

Soluble carbohydrate contents in roots were analysed by gas chromatography according to Piotrowicz-Cieślak et al. (2007). Roots (500 mg fresh mass) were homogenised in ethanol:water 1:1 (v/v) containing 100 µg xylitol as internal standard. The homogenate and the wash were combined in a 1.5 ml microfuge tube, heated at 75°C for 30 min and centrifuged at 13 200 g for 10 min. The supernatant was passed through a 10 000 MW cut-off filter (Lida, Kenosha, WI, USA). Aliquots of 0.3 ml filtrate were transferred to silylation vials and evaporated to dryness. Dry residues were derived with 200 µl of silylation mixture (trimethylsilylimidazole : pyridine, 1:1, v/v) in silylation vials (Thermo Scientific) at 80°C for 45 min, and then cooled at room temperature. One µl of carbohydrate extract was injected into a split-mode injector of a

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Thermo Scientific gas chromatograph equipped with flame ionisation detector. Soluble carbohydrates were analysed on a DB-1 capillary column (15 m length, 0.25 mm ID, 0.25 μm film thickness, J&W Scientific). Soluble carbohydrates were identified with internal standards, and their concentrations were calculated from the ratios of peak area, for each analysed carbohydrate, to the peak area of respective internal standard. Quantities of soluble carbohydrates were expressed as mean ± standard deviation (SD) for 3-5 replications of each treatment.

Two-dimensional gel electrophoresis

Proteins were isolated from roots growing in the presence of water, or sparfloxacin at medium and the highest dose (0, 40 and 800 mg of the drug/kg of soil). The roots frozen in liquid nitrogen (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 minutes. The extract was centrifuged at 4°C for 10 minutes at 5500g. 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 minutes at 3200g. 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 hours at -20°C. After 12 hours, precipitated proteins were centrifuged at 4°C/3200g/15 minutes and the obtained pellet was washed two times in cold acetone (Chempur) centrifuging it each time at 4°C/ 5500 g for 5 minutes. The pellet was dried at room temperature.

The dried pellet was dissolved in 9 M urea (POCH), 20g/dm^3 CHAPS (Sigma), 0.3% DTT (Sigma) and incubated for one hour at room temperature. Next, the samples were sonicated in an ultrasonic ice bath for 15 seconds and they were then centrifuged at 14000g/5 minutes. The procedure was repeated three times. The prepared solution of proteins was used in further analyses.

IPG, 7 cm, pH 4-7 strips (ReadyStrip™, Bio-Rad) were actively rehydrated for 12 hours in a rehydration buffer (Bio-Rad) with 70 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 6M urea, 2% SDS, 0.375 M Tris - HCl with pH 8.8, 20% glycerol, 130 mM DTT (Bio-Rad) for 10 minutes 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 minutes.

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 minutes 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

Protein spots were excised out of the gels with a scalpel, placed in test tubes, washed and destained. Proteins were dehydrated in a vacuum centrifuge and subsequently

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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 sonicated for 5 minutes and dehydrated in a vacuum centrifuge and 1µl 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 72 were taken into account ($p<0.05$).

Statistical analysis

The experiment was conducted in four replicates. The results were statistically evaluated using analysis of variance (F test) for two factor experiments (split-plot). The mean values of the plots were compared using q SNK test (Student-Newman-Keuls).

Result

Germination and growth

The effect of sparfloxacin (SPX) on elongation of roots of garden pea, cv. Cysterski, was studied after seven days of plant culture on control substrate or substrate supplemented with SPX (0.08; 0.4; 0.8; 4; 8; 40; 80; 200; 400; 800 mg kg^{-1} of soil). The longest roots were found in control seedlings and measured 84 mm. With increasing drug concentration in soil the length of roots decreased (*Fig. 1A*). In seedlings grown on soil contaminated with SPX at 40 mg kg^{-1} the rate of root growth decreased by 27% compared to control roots. Seedlings grown on soil with the highest SPX concentration (800 mg kg^{-1} of soil) had the shortest roots. The rate of root growth in these seedlings was lower by 54% compared to control roots (*Fig. 1A*). The length of pea seedlings stems also decreased with increasing antibiotic concentration (*Fig. 1A*). The longest stems (35 mm long) were found in control seedlings and those grown at the lowest SPX concentrations (0.08, 0.4, 0.8, 4, 8 mg kg^{-1}). A slight decrease in stem length (by 6 and 14%) was found in seedlings grown on soil with SPX at 40 and 80 mg kg^{-1}, respectively. Seedlings growing in soil contaminated with SPX at 400 and 800 mg kg^{-1} had the shortest stems. Stem growth inhibition was 32%, 37% and 46% for plants grown with SPX at 200, 400, 800 mg kg^{-1}, respectively (*Fig. 1A*).

Seedling fresh weight was also analysed, as well as their dry weight. With increasing concentration of SPX the seedling fresh weight decreased (*Fig. 1B*). Dry weight was lowest in control seedlings (24%) and in seedlings grown at the highest SPX concentrations in soil (400 and 800 mg kg^{-1}) it was highest (30 and 32%, respectively).

Sparfloxacin content in roots

In 7-days old pea seedlings, grown in soil contaminated with increasing SPX levels, the concentrations of the content of this antibiotic in roots was 0.015 to 248 ng g^{-1} fresh weight and increased parallel to incremental doses of SPX in soil (*Fig. 1C*). In seedlings grown at SPX soil concentration of 40 mg g^{-1} the root content of SPX was 15 times higher than in seedlings affected by SPX at 800 mg g^{-1}.

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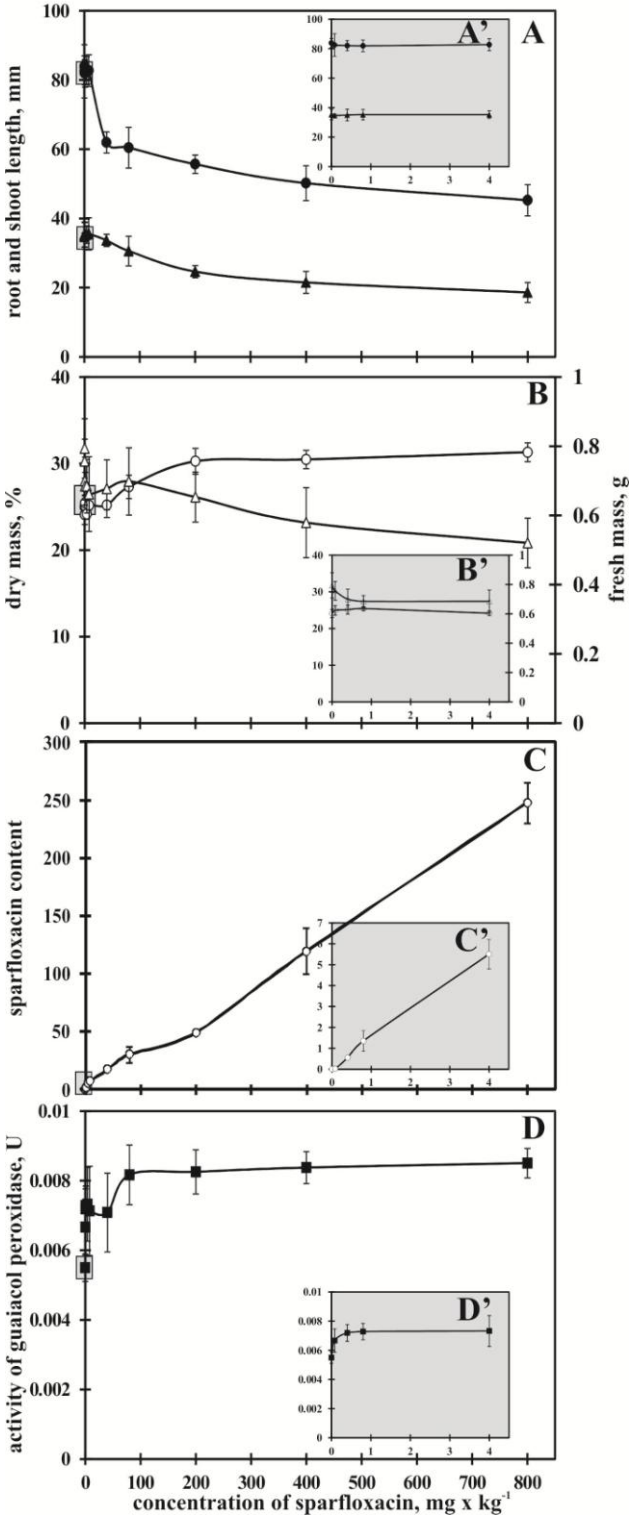

Figure 1. *Panel A - root (●, mm) and shoot (▲, mm) length; Panel B – dry (Δ, %) and fresh mass (o, mg); panel C - sparfloxacin content (o, ng · g^{-1} fresh weight); panel D – activity of guaiacol peroxidase (■, U one unit of activity equals oxidation 1 μM H_2O_2 per 1 minute) in Pisum sativum after seven days on soil supplemented with different sparfloxacin concentrations (0; 0.08; 0.4; 0.8; 4; 8; 40; 80; 200; 400; 800 mg · kg^{-1} of soil). Panels A', B' C' and D' are a large image a gray square in the main panel (0, 1, 2, 3, 4 i.e. 0, 0.08, 0.4, 0.8, 4 mg · kg^{-1} of soil). Data points represent the means ± SD for nine replicate samples*

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Activity of guaiacol peroxidase

Guaiacol peroxidase (POX) activity in pea roots increased parallel to increasing antibiotic concentrations (*Fig. 1C*). The lowest POX activity was observed in roots of control seedlings and the highest in those grown at the highest dose of SPX 800 mg kg^{-1}). At low antibiotic doses (0.08, 0.4 and 0.8 mg kg^{-1}) the POX activity was by 22, 29 and 33% higher than in control seedlings. In seedlings subjected to soil SPX at 200, 400 and 800 mg kg^{-1} POX activity was higher by 49, 53 and 55 %, compared to control seedlings, respectively.

Soluble carbohydrate contents

Soluble carbohydrates were studied in roots of pea seedlings grown for seven days in soil contaminated with SPX. Both in roots of control plants and plants affected by SPX contaminant the following sugars could be identified: fructose, glucose, *myo*-inositol and sucrose (*Fig. 2*). The highest contents of soluble sugars were found in roots of control seedlings and they amounted to 0.69, 3.14, 5.27, 3.07 mg g^{-1} dry mass for fructose, glucose, *myo*-inositol and sucrose, respectively. On the other hand, it was found that with increasing SPX concentrations the contents of soluble sugars decreased (*Fig. 2*). Sucrose content decreased by 4 and 6 % was found in roots of seedlings grown in soil containing SPX at the two lowest concentrations (0.08 and 0.4 mg kg^{-1}, respectively) whereas SPX at 8 mg kg^{-1} decreased sucrose content by 20%, compared to control roots. The lowest sucrose content 3.57, 3.56 and 3.71 mg g^{-1} dry weight was found in roots of seedlings grown in soil supplemented with SPX at the level of 200, 400 and 800 mg kg^{-1}, respectively.

Two-dimensional gel electrophoresis

Protein profiles of garden pea roots were analysed using two dimensional electrophoresis (2D-PAGE). Roots of seedlings grown in control soil and at the medium and the highest SPX concentrations (40 and 800 mg kg^{-1}) were studied. The obtained gel images were compared with one another using PDQuest^{TM} Basic software (*Fig. 3*). The protein map obtained for control roots contained 176 spots (*Fig. 2A*), whereas for roots of seedlings growing at SPX levels 40 and 800 mg kg^{-1} 197 and 217 proteins (spots) were observed, respectively (*Fig. 2B* and *C*). Of all spots obtained for pea root proteins 92 spots were common for all protein maps. Twelve proteins specific for roots of SPX treated pea plants (at 40 and 800 mg kg^{-1}) were identified based on these maps (*Table 1*). In roots of plants grown at SPX level 800 mg kg^{-1} there were 8 identified up-regulated proteins (*Fig. 4*). Two dimensional maps of control root proteins revealed that 6 SPX-specific proteins, out of all 12 identified, were expressed at the highest level (*Fig. 4*). Increased presence of proteins involved in plant protection from stress factors or xenobiotics was observed in roots of SPX treated plants. Moreover, the amounts of proteins involved in bioenergetic metabolism (as judged by spot intensity) decreased.

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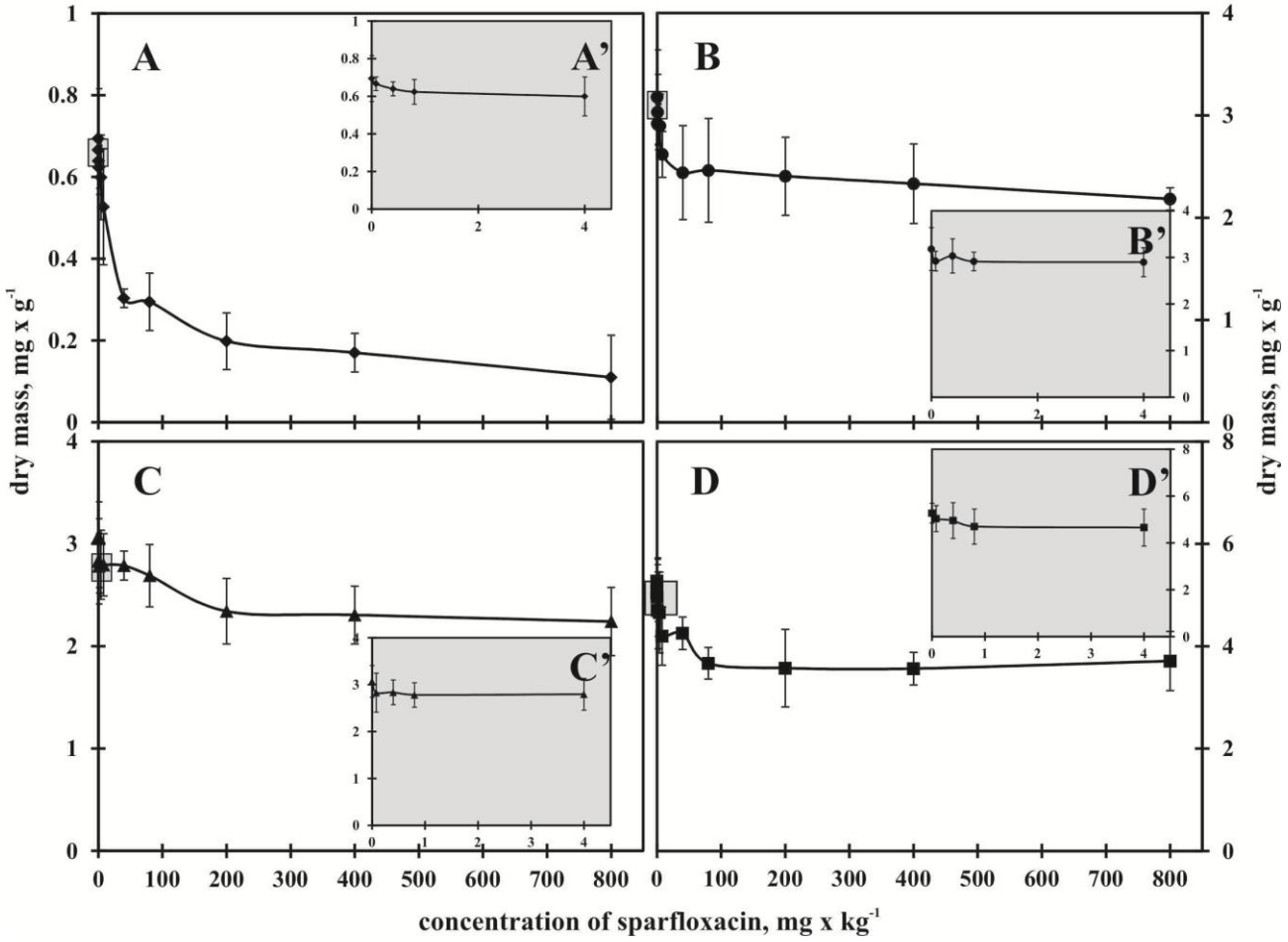

Figure 2. *Panel A - fructose (♦, mg · g^{-1}), Panel B – glucose (●, mg · g^{-1}) panel C – myo-inositol (▲, mg · g^{-1}) ; panel D – sucrose (■,mg · g^{-1}) in Pisum sativum after seven days on soil supplemented with different sparfloxacin concentrations (0; 0.08; 0.4; 0.8; 4; 8; 40; 80; 200; 400; 800 mg · kg^{-1} of soil). Panels A', B' C' and D' are a large image a gray square in the main panel (0, 1, 2, 3, 4 i.e. 0, 0.08, 0.4, 0.8, 4 mg · kg^{-1} of soil). Data points represent the means ± SD for nine replicate samples*

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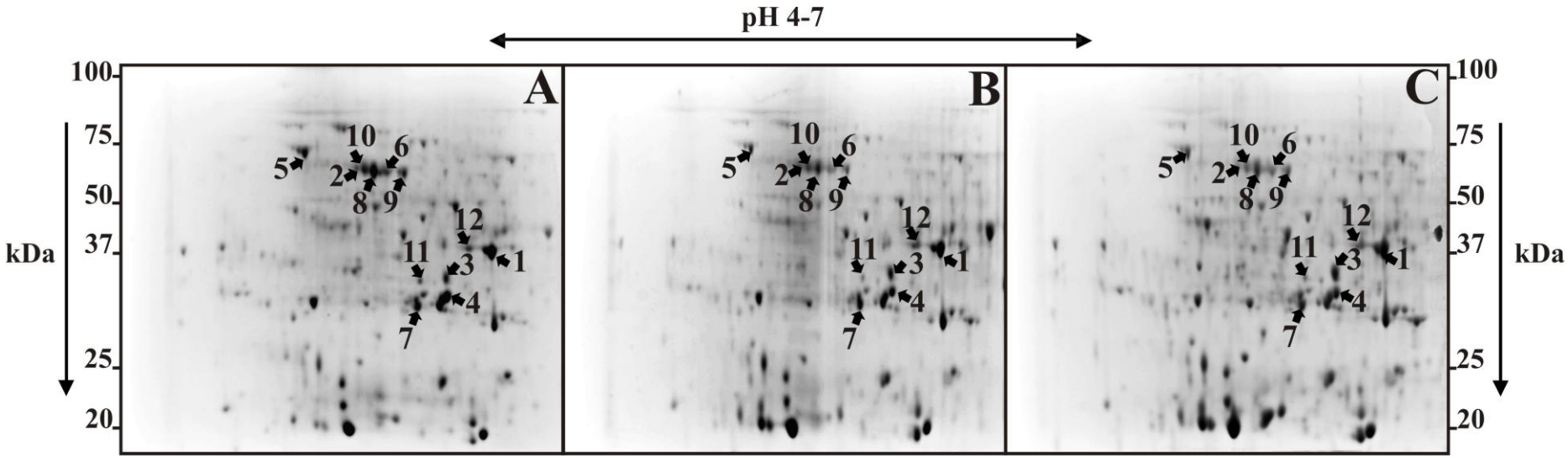

Figure 3. *2D Electrophoresis of proteins of root pea (Pisum sativum L.) without (A) and with the sparfloxacin at the concentration of 40 mg · kg^{-1} of soil (B), 800 mg · kg^{-1} of soil. Protein separation was conducted at pH 4-7*

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Table 1. *Proteins indentified by LC-MS-MS/MS analyses*

Spot No.	Protein name	Function	Species	Protein ID	pI experimental	Mass (kDa)	pI theoretical	Mascot Score
1	Isoflavone reductase related protein	Defense	*Pyrus communis*	Gi:3243234	6.65	36.1	6.02	98
2	F1 ATPase	Provides energy for the cell	*Pisum sativum*	Gi:2116558	5.63	60.3	6.63	119
3	L-ascorbate peroxidase, cytosolic	Detoxification	*Pisum sativum*	Gi:1351963	6.28	28.6	5.52	92
4	Chain A, crystal structure of recombinant ascorbate peroxidase	Detoxification	*Pisum sativum*	Gi:1420981	6.28	27.3	5.52	93
5	Heat shock protein hsp 70	Defense	*Pisum sativum*	Gi:445605	5.0	75.8	5.22	124
6	Enolase	Carbohydrate and energy metabolism	*Ricinus communis*	Gi:255575355	5.90	57.8	5.62	224
7	Chalcone--flavanone isomerase 1B-2-like	Adaptation and protection	*Cicer arietinum*	Gi:502121457	6.0	26.4	5.55	96
8	Ethylene-responsive enolase	Energy metabolism, defense	*Solanum lycopersicum*	Gi:5669648	6.81	57.1	4.75	152
9	S-adenosylmethionine synthethase, partial		*Pisum sativum*	Gi:609557	6.02	43.9	6.60	98
10	ATP-syntase subunit beta, mitochondrial	Carbohydrate and energy metabolism	*Glycine max*	Gi:356536246	5.63	63.2	5.80	221
11	V-type proton ATPase subunit E	Proton pomp, adaptation and protection	*Gossypium hirsutum*	Gi:3334405	6.02	28.1	6.50	66
12	Malate dehydrogenase, mitochondrial-like	Energy metabolism	*Cicer arietinum*	Gi:502155344	6.47	36.6	8.71	102

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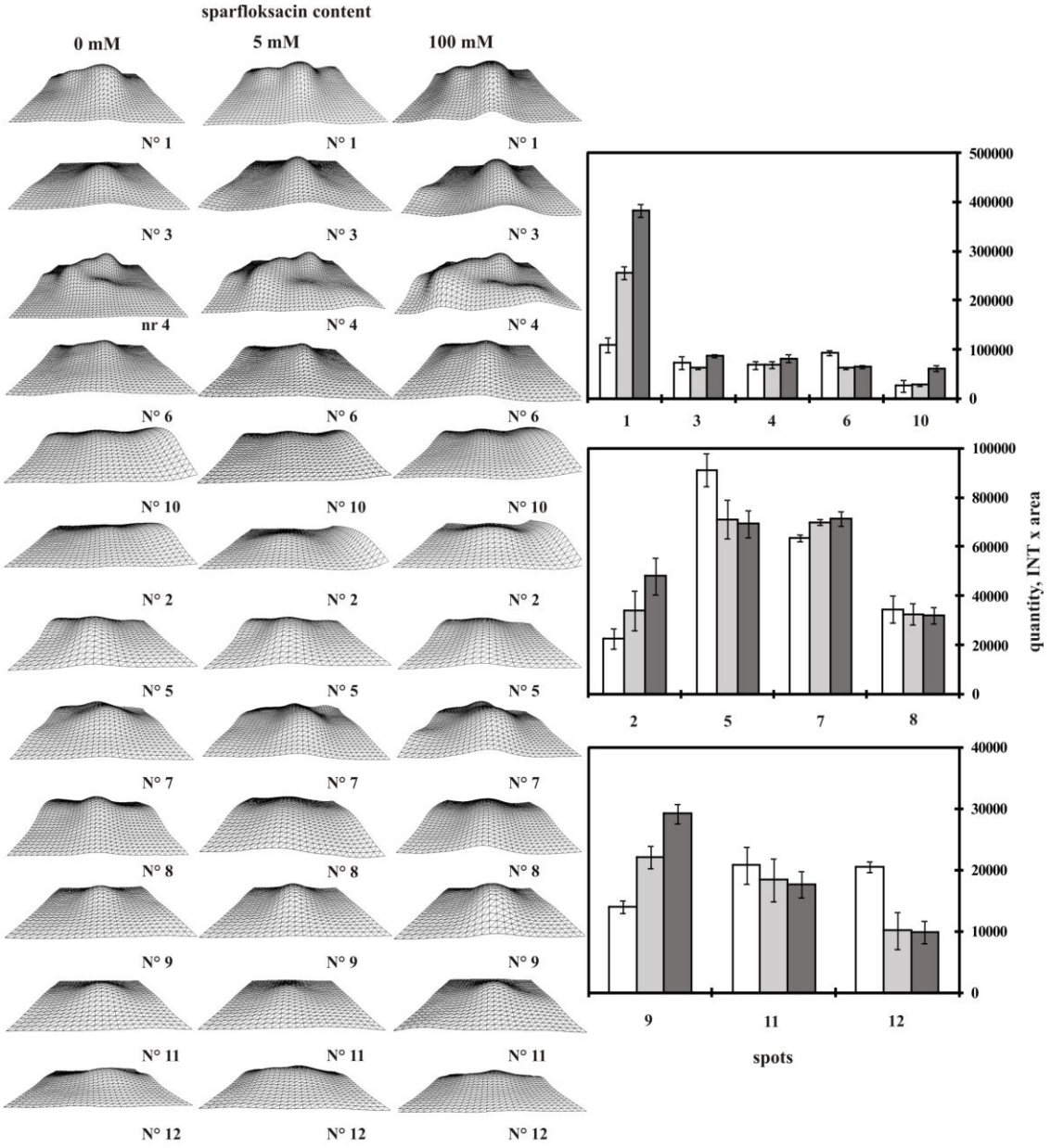

Figure 4. *Intensity of destaining (PDQuest, BioRad) of proteins from control seedlings (□) and with the sparfloxacin at the concentration of 40 mg · kg^{-1} of soil (■), 800 mg kg^{-1} of soil (■). Number of spots 1, 2....10 – see in Table 1*

Discussion

Excellent antibacterial properties of some pharmaceuticals resulted in their wide use in human and veterinary medicine and agriculture (Sarmah et al., 2006). Floroquinolons belong to the most commonly applied drugs, worldwide (Khetan and Collins, 2007). Intensive use of pharmaceuticals results in their increased presence in the environment (Gartiser et al., 2007). Antibiotics are weakly metabolised, so they are passed to the environment unchanged or as active metabolites (Allen et al., 2011). Plants readily take up pharmaceuticals from soil which results in morphological and biochemical anomalies (Liu

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et al., 2013). Plant sensitivity to pharmaceuticals may be exploited for assessment of rate environmental degradation of drugs (Pomati et al., 2004; Adomas et al., 2013).

A biotest, based on seed germination and seedlings elongation measurements, may be applied for determination of the degree of environmental contamination by xenobiotics (Piotrowicz-Cieślak et al., 2010). Increasing soil concentrations of SPX lead to inhibition of root and stem growth in garden pea (*Fig. 1A*). The highest tested SPX concentration (800 mg kg^{-1}) decreased root and stem length by 54% and 46%, respectively, compared to control seedlings. The root and stem length correlated with seedling dry and fresh weight. Fresh weight of roots and stems decreased due to soil contamination with SPX, while dry weight slightly increased (*Fig. 1B*).

Adomas et al. (2013) have described inhibitory effect of enrofloxacin on elongation of narrow leaved lupin seedlings. Root and stem growth inhibition was also documented for legume seedlings (lupin, pea and lentil) grown in soil contaminated with diclophenac (Ziółkowska et al., 2014). The SPX concentrations used in our experiments were higher (5 and 8-fold higher), compared to those used in previous studies, 12 mM diclophenac (Ziółkowska et al., 2014) and 20 mM sulfamethazine (Piotrowicz-Cieślak et al., 2010), respectively. SPX (at 800 mg kg^{-1} i.e. 100 mM) inhibited root growth by 54% and stem growth by 46% (*Fig. 1A*) which suggests that it is less toxic than diclophenac (inhibiting pea root growth by 83% and stem growth by 91% at 12 mM concentration (Ziółkowska et al., 2014)). It is also less toxic than sulfamethazine (Piotrowicz-Cieślak et al., 2010).

Soluble carbohydrate contents in seedlings correlate with their growth (Ziółkowska et al., 2014; Frias et al., 1996). During germination of legume seeds their monosacharide, disaccharide and cyclitol contents increase (Goyoaga et al., 2011). In our experiments a positive correlation was found between seedling length and sugar contents. The following sugars were identified in roots of pea plants, both control or affected by SPX: fructose, glucose, sucrose and *myo*-inositol (*Fig. 2*). It was noted that with increasing sparfloxacin concentration in soil the content of sugars in roots (as organs directly affected by the xenbiotic) decreased. Decreased quantities of soluble sugars were also found in seedlings grown in soil contaminated with diclophenac. Also with this pharmaceutical, like in our current experiments, a negative correlation was observed between plant soluble carbohydrate contents and drug concentrations in soil. We believe, therefore, that sucrose metabolism is a good predictor of seedling growth in contaminated soils, although it does not indicate the level of contamination. Therefore, determinations of soluble carbohydrates in plants may not be used for assessment of the level of soil contamination with antibiotics.

Adverse environmental conditions cause oxidative stress in plants by enhancing production of reactive oxygen species (ROS). To protect tissues and subcellular systems from toxic actions of ROS plants activate adaptive (detoxification) reactions (Caverzan et al., 2012). Ascorbate, glutathione and guaiacol peroxidases participate in ROS scavenging (Faltin et al., 2010). Peroxidases (POX), in spite of their low substrate specificity, are considered good biomarkers of stress in plants. POX contribute to defence/protective reactions but also participate in developmental processes, auxin and ethylene metabolim, cell wall lignification and suberinization (Lepeduš et al., 2004).

The experiments reported here show that with increasing SPX concentrations peroxidase activity in roots of pea seedlings increased (*Fig. 1C*). The results confirm pharmaceutical inducing effect on oxidative stress in plants (Caverzan et al., 2012). Even the lowest concentrations of SPX (0.08, 0.4 and 0.8 mg kg^{-1}) resulted in significant increase of POX activity in seedling roots (by 22, 29 and 33%, respectively). Sharp increase of enzyme activity under the influence of such low antibiotic quantities suggests imminent activation

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of mechanisms protecting plants from ROS. In roots of pea seedlings subjected to the highest SPX concentration (800 mg kg^{-1} of soil) peroxidase activity increased by as much as 55%. Plant adaptive reactions to oxidative stress may vary depending on plant species and chemical properties of the stress inducing substance. Plants may differ in their capability to take up various antibiotics (Pomati et al., 2004). Zabalza et al. (2007) observed decreased activity of guaiacol peroxidase in pea roots resulting from herbicide (imazetapir) action. In addition to antibiotics and herbicides, many other stressors may cause adaptive reactions in plants (Caverzan et al., 2012). Activity of guaiacol peroxidase in roots of common bean (*Phaseolus vulgaris*) was stimulated by salt stress (Jebara et al., 2005).

Legume plants are a rich source of protein for human and animal nutrition. Pea and bean seeds abound in protein, which makes up 20% of their dry mass. Plant tissues are considered a rather challenging material for protein purification (Pavoković et al., 2012). Using the two-dimensional maps of pea root proteins differences in protein profiles of control plants (*Fig. 3A*) and plants treated with SPX (40 and 800 mM, *Fig. 3 B* and *C*) were shown. Much more spots (217) were visualised on the map of proteins from roots responding to the highest SPX concentration (800 mg kg^{-1}) than from control roots (176 spots). More spots were also visible on protein map obtained for pea subjected to SPX at the level of mg kg^{-1}. Increased protein levels result from plant stress response. In 2D-electrophoresis 92 proteins were detected that were common to all samples (from control and SPX-treated plants). Twelve proteins were selected for identification by mass spectrometry. Proteins involved in plant adaptation and protection from detrimental effects of environment were found. The most intense spots on protein maps of roots affected by SPX (40 and 800 mg kg^{-1}) were identified as isoflavone reductase related protein. Isoflavones are mainly identified in legumes. They participate in plant defence against phytopathogens, acting as phytoalexins (Shimada et al., 2000). A key role in isoflavonoid biosynthesis is played by isoflavone reductase (IFR). IFR catalyzes $NADP^+$ reduction in biosynthesis of plant protective metabolites (derivatives of phenylpropanoids) (Franca et al. 2001). Isoflavone reductase, like L-ascorbate peroxidase is involved in detoxication of reactive oxygen species (ROS). Chalcone synthase is another key enzyme in isoflavonoid and flavonoid biosynthesis (Dao et al., 2011). Chalcone-flavanone isomerise, catalysing an early step in flavonoid biosynthesis, was also detected on 2D maps of plant proteins. Chalcone-flavanone isomerase is also involved in plant adaptation and protection from environmental stresses (Shoeva et al., 2014). Induction of this enzyme in roots growing in the presence of SPX (40 and 800 mg kg^{-1}) points to adverse action of this antibiotic on plants. According to the literature changes in content of chalcone synthase and chalcone isomerase in *Arabidopsis* mutants result in plant supersensitivity to UV radiation (Winkel-Shirley, 2002). Adaptive role may also be played in plants by an identified S-adenosylmethionine synthetase and subunit E of V-type proton ATPase. Yet another group of identified proteins are those involved in bioenergetic metabolism and suppressed by increasing concentrations of antibiotic.

Differences in abundancy of proteins identified on proteomic maps of control roots and those subjected to SPX (40 and 800 mg kg^{-1}) indicate a plant protection response activation. Abiotic stresses in plants result in gene activation and modification of metabolite and protein pools. Some of these proteins may be essential for conferring a certain level of plant protection from stressors (Bhatnagar-Mathur et al., 2008).

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