ROLE OF DECARBOXYLASES IN THE BIOSYNTHESIS OF BIOGENIC AMINES OF PEA GROWING IN SOIL CONTAMINATED WITH LOMEFLOXACIN

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Abstract. Lomefloxacin is an antibiotic used in human and animal medicine that is excreted to the environment, where it is taken up by plants. In the present study, we identified a new, universal parameter of lomefloxacin's toxicity to pea. We showed that activity of arginine decarboxylase and putrescine, which is synthetised in this pathway is a very good toxicity parameter, as confirmed by the calculated EC indices. The lomefloxacin toxicity resulted in a complete inactivation of ornithine decarboxylase and increase of activities of arginine decarboxylase, and S-adenosylmethionine decarboxylase. We propose that putrescine is not synthesised directly from ornithine. Instead, we suggest that the biosynthesis pathway proceeds from arginine to agmatine and is catalysed by arginine decarboxylase, which is followed by the formation of N-carbamoylputrescine with the participation of agmatine imniohydrolase. N-carbamoylputrescine amidohydrolase is involved in the synthesis of putrescine with N-carbamoylputrescine. The activity of S-adenosylmethionine decarboxylase in the roots and stems increased almost 5-fold, arginine decarboxylase in the roots increased up to 18-fold, while in the stems it increased 6-fold, and ornithine decarboxylase in the roots and stems increased 3.5-fold. The highest biogenic amines content was found in seedlings growing in soil contaminated with 50 $mg \times kg^{-1}$ of soil lomefloxacin. We also recommend quantifying the activity of decarboxylases by measuring the secreted CO_2 volume with a multi-gas analyser equipped with an infrared detector. It is a simple, reliable, and cheap technique.
Keywords: *morphological parameters of plants, decarboxylases activity, toxicity, infrared detector*

Abbreviations: Lomefloxacin – LOM; Effect concentration for the inhibition of growth rate at the level of x % (x = 10, 25, 50, 90) – EC_x; Fresh mass – FM; Dry mass – DM; Biogenic amines – BAs; Polyamines – PAs; Cadaverine – Cad; Putrescine – Put; Spermidine – Spd; Spermine – Spm; Tyramine – Tyr; Agmatine – Agm; Arginine – Arg; S-adenosylmethionine decarboxylase – SAMDC; arginine decarboxylase – ADC; ornithine decarboxylase – ODC

Introduction

The compounds most frequently contaminating soil include: heavy metals, pesticides, aromatic hydrocarbons, solvents (Ashraf et al., 2014) and, recently, drugs used in human and veterinary medicine (Arnold et al., 2013). Fluoroquinoles, including lomefloxacin (LOM), are one of the numerous chemical groups of drugs used in the therapy of humans and animals (Andreu et al., 2007). LOM is used in the therapy of bacterial infections, including bronchitis, urinary tract infections and in preoperative prophylaxis to prevent a urinary tract infection. In the body, the administered LOM dose

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is not completely absorbed and metabolised. Therefore, a minimum of five metabolites of this drug are detected in urine. After the completion of drug administration, LOM is excreted within 72 hours: 65% in the parent form and 9% in the form of urine-soluble glucuronides (as its glucuronide metabolite) (DrugBank: Lomefloxacin 2005). The maximum LOM concentration in the urine after five hours amounts to 9 $mg \times l^{-1}$, and after twelve hours to 3.9 $mg \times l^{-1}$ (Wei et al., 2007). Veterinary drugs eventually reach farmlands, along with solid and liquid manure as well as slurry (Förster et al., 2009; Karci and Balcioğlu, 2009; Martinez-Carballo et al., 2007), where they are detected. Concentrations of fluoroquinolones - ciprofloxacin, enrofloxacin and norfloxacin, range from 0.1 to 288 $\mu g \times kg^{-1}$ in the topsoil within an area under intensive vegetable production in northern China (Xie et al., 2012). In Austria, the maximum ciprofloxacin concentration in farmlands reaches 370 $\mu g \times kg^{-1}$ (Martinez-Carballo et al., 2007). On the other hand, in farmlands of the northern part of Marmara region (Turkey), enrofloxacin content ranges from 20 to 50 $ng \times kg^{-1}$ (Karci and Balcioğlu, 2009).

The drugs present in soils are taken up by plants (Jørgensen and Halling-Sørensen, 2000; Jjemba, 2002; Baciak et al., 2016b), disturb plant metabolic balance and cause numerous morphological changes during the germination, growth and development (Bártíková et al., 2016). Biochemical responses of plants to drugs include, the accumulation of soluble hydrocarbons (Sikorski et al., 2014) and biogenic amines (BAs) (Bouchereau et al., 1999) as protective compounds.

The BAs most frequently occurring in plants are putrescine (Put), spermidine (Spd) and spermine (Spm) (Tiburcio et al., 2014). Cadaverine (Cad), histamine (His) and tyramine (Tyr) may occur naturally in certain plant tissues and their distribution is a useful characteristic in chemotaxonomic studies (Bouchereau et al., 1999). Agmatine (Agm) is another intermediate of the putrescine synthesis from arginine in plants (Fuell et al., 2010). In mature seeds, a significant, species-dependent variability in BAs contents occurs (Shalaby, 2000). For example, in lucerne seeds, the total content of BAs, including Put, Cad, His, Tyr, Spd and Spm amounts to 107.18 $mg \times kg^{-1}$ d.m., while the total content of the same BAs in radish seeds is two times lower and amounts to 48.24 $mg \times kg^{-1}$ d.m. (Frías et al., 2007; Martínez-Villaluenga et al., 2008). The BAs content is also a varietal characteristic (Glória et al., 2005). In the seeds of various broccoli varieties, the total BAs content amounts to 24.21, 32.49 and 44.96 $mg \times kg^{-1}$ d.m., respectively (Martínez-Villaluenga et al., 2008).

BAs are basic compounds (Silla-Santos, 2001). They occur in plants in a free form (Casal et al., 2004) or as positively-charged molecules. At a physiological pH they can interact with nucleic acids, phospholipid membranes and proteins (Martin-Tanguy, 2001). When binding to phospholipid membranes, BAs affect their permeability and when binding to proteins, they stabilise their structure (Lightfoot and Hall, 2014). BAs modulate the activity of certain ion channels and affect DNA and RNA molecules (Groppa and Benavides, 2008; Alcazar et al., 2010). BAs are also associated with the growth and development of plants, e.g. in roots and flowering shoots, they also play a role in fruit ripening and plant ageing (Bouchereau et al., 1999). In recent years, there has been growing interest in BAs as compounds involved in the protection of plants against environmental factors which frequently modify BAs' content (Chai et al., 2010; Calzadilla et al., 2014). BAs content, maintained within the limits typical of a plant species or variety, is primarily determined by the activity of enzymes participating in their biosynthesis.

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Therefore, the aim of our study was to determine the toxicity of LOM towards the activity of S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50), arginine decarboxylase (ADC, EC 4.1.1.19) and ornithine decarboxylase (ODC, EC 4.1.1.17) in the BAs biosynthesis pathway in the roots and stems of pea seedlings growing in soil contaminated with LOM, as well as to find out which BAs and how much of them are present in pea seedlings growing in soil, both uncontaminated and contaminated with increasing concentrations of LOM. The literature discussing the effects of drugs on the composition and amount of BAs is scarce. The data is also lacking on drug affected activity of decarboxylases and their role in the BAs biosynthesis pathway in the roots and stems of pea seedlings. In the study, we used for the first time in decarboxylases activity analysis a multi-gas analyser equipped with an infrared detector.

Material and Methods

Phytotoxkit

Seeds of pea (*Pisum sativum* L.) cv. Pomorska were germinated for 9 days in plastic test containers of the Phytotoxkit test (MicroBio Test Inc., Belgium). The germination was carried out under controlled conditions: 8 hours (temperature of 16°C – night) and 16 hours (temperature of 20°C, 140 μmol photons $m^{-2}\times s^{-1}$ PAR – day). The control soil was soaked with 27 ml of distilled water and the other samples were soaked separately with LOM at the following concentrations: 0.2, 0.5, 2, 5, 20, 50, 200, 500, 2000 $mg\times kg^{-1}$ of soil. On the 9th day, the percentage of germinated seeds, the length of roots and stems and the fresh and dry mass content were assessed according to ISTA (International Society of Testing Analysis) (1999). The osmotic potential of the stems was determined using a plant water status console (Soil Moisture Equipment Corp, Santa Barbara, CA, USA).

Chemicals

Lomefloxacin (*Fig. 1*) CAS Number: 98079-52-8, ≥98.0% was purchased from Sigma Aldrich (St. Louis, Mo, USA).

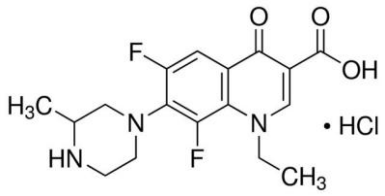

Figure 1. *Chemical structure of lomefloxacin (LOM)*

Biogenic amines assay

BAs content was determined in seeds, as well as roots and stems of 4- and 9-days old pea seedlings. BAs were extracted from plant material with cold 5% hydrochloric acid (Bouchereau et al., 2000). The extracted plant material was shaken for 1h and then centrifuged at 16.000 g for 30 min at temperature 4°C. The supernatants were filtered through a 0.22 μm pore nylon membrane syringe filter (Filter-Bio, Nantong City, China) and stored at -20°C. The filtrate was analyzed by ion-exchange chromatography using amino acids analyzer AAA400 (Ingos, Prague, Czech Republic). BAs were

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separated at 76°C on a 70×3.7 mm column filed with Ostion Lg ANB (Ingos, Prague, Czech Republic) and then eluted from the ion-exchange column with two pH 5.65 sodium citrate buffers with the addition of 1.0 and 2.6 M sodium chloride. The quality and quantity of the BAs were assayed with post-column ninhydrin derivatization and photometric detection (λ=570 nm). The standards of Sigma Aldrich (St. Louis, Mo, USA) for chromatographic analysis of the BAs were used. Quantities of BAs were expressed as mean ± SD for 3-5 replications of each treatment.

Enzyme activity

In roots and stems of 9 days old pea seedlings activity of the following BAs biosynthesis enzymes was determined: SAMDC (EC 4.1.1.50), ADC (EC 4.1.1.19) and ODC (EC 4.1.1.17). Plant homogenates, prepared by grinding in liquid nitrogen, were treated with 50 mM phosphate buffer (pH 8.0), containing 1 mM 2-mercaptoethanol, 50 μM pyridoxal phosphate. The extraction was carried out at 4°C (Chattopadhyay et al., 1997). The extracts were centrifuged at 4°C, 12.000 g for 15 min. The supernatants were transferred to chromatography vials (1.5 ml), next L-ornithine (40 mM L-ornithine monohydrochloride), L-arginine (40 mM L-arginine), S-adenozylometionine (15 mM S-(5′-adenosyl)-L-methionine chloride dihydrochloride) were used as substrates for ODC, ADC, and SAMDC, respectively. Chromatography vials were tightly closed with silicone septa (UltraClean Closure). The samples were shaken and incubated at 37°C for 2 h. The reactions were stopped by cooling down the samples to 0°C (*Fig. 2*).

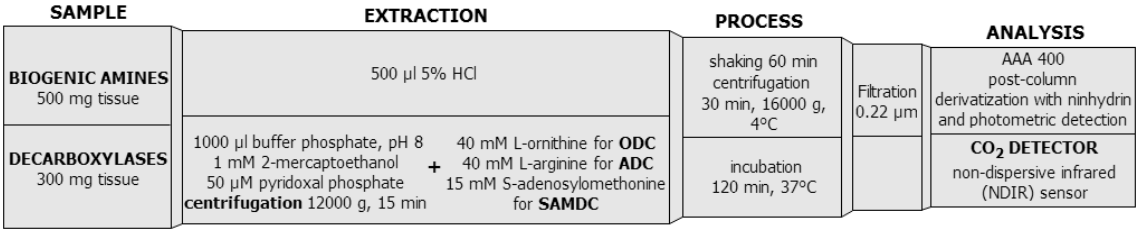

Figure 2. *Scheme of biogenic amines and decarboxylases activity sample preparation and analysis*

The amount of released CO_2 (ppm) was measured with a multi-gas analyzer (*Fig. 3*) equipped with infrared detector (MultiRAE IR One-to-Five Gas Monitor, from 0 to 20000 ppm measurement of CO_2 using non-dispersive infrared (NDIR) sensor).

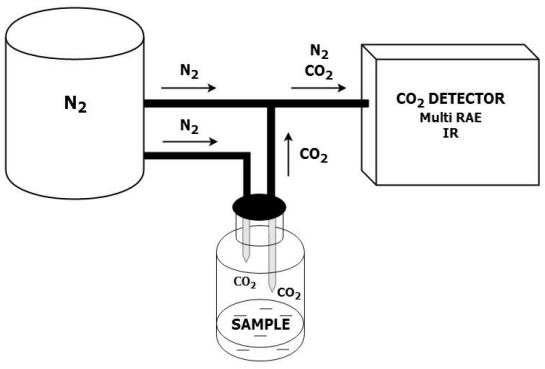

Figure 3. *Scheme of measurement activity decarboxylases (released CO_2) using the analyzer Multi RAE*

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Decarboxylase activity was expressed in μmol CO_2 released per gram protein. The protein content w was determined according to Lowry et al. (1951).

Statistical analysis

The results were processed in the Statistica application, version 12.5 (www.statsoft.com) with the use of Newman-Keuls tests $P < 0.01$ for all comparisons. Standard deviation and homogenous groups (A, B, C) identifying significant differences between means were determined (means denoted by the same letter are not significantly different at $P = 0.01$). EC_x data were analyzed using the regression model with different significant was selected to calculate the effective concentrations at 10, 25, 50 and 90% levels.

Results

The impact of increasing LOM concentrations: 0, 0.2, 0.5, 2, 5, 20, 50, 200, 500 and 2000 mg×kg^{-1} of soil and their toxicity towards the activity of decarboxylases and BAs' content were determined in 9-day-old pea seedlings. To evaluate the LOM toxicity, we measured the parameters recommended by the OECD (2006) i.e. the germination of seeds, the length of roots and stems, their fresh and dry mass and osmotic potential. Additionally, BAs content in seeds, 4-day and 9-day-old pea seedlings was measured. BAs' content ratios and $EC_{10, 25, 50, 90}$ indices for studied morphological and biochemical features were calculated.

LOM toxicity towards morphological parameters

We showed that none of the soil LOM concentrations (ranging from 0.2 to 2000 mg×kg^{-1} of soil) inhibited the germination of pea seeds. However, it was found that with an increase in the LOM concentration in soil, the length of the roots and stems of the seedlings steadily decreased. The basic observed morphological changes in this study include the inhibition of root and stem elongation (*Fig. 4*). The pea roots exposed to the highest LOM concentration (2000 mg×kg^{-1} of soil) were shorter by almost half compared to the control roots (103 mm). The pea stems responded similarly: in the control sample, their length was 40 mm, and at the highest concentration of the drug, it was only 23 mm (*Fig. 4A*). The elongation inhibition resulted in decreased fresh mass of pea seedlings. The fresh mass of control roots and stems averaged 267 mg, while roots and stems grown in LOM contaminated soil (2000 mg×kg^{-1} of soil) were lower by 175 mg on average (*Fig. 4B*). LOM did not affect the dry mass of roots and stems which on average was 8.40% (*Fig. 4C*).

The reduction in root and stem elongation in pea, resulting from LOM application resulted in decreased seedling fresh mass but increased osmotic potential. Even the lowest concentration of LOM (0.2 mg×kg^{-1} of soil) increased the osmotic potential of stems by 8 psi to the level of 24.08 psi as compared with the control sample (16.27 psi). The highest concentration of the drug (2000 mg×kg^{-1} of soil) increased, the osmotic potential of stems 7-fold on average (*Fig. 4D*).

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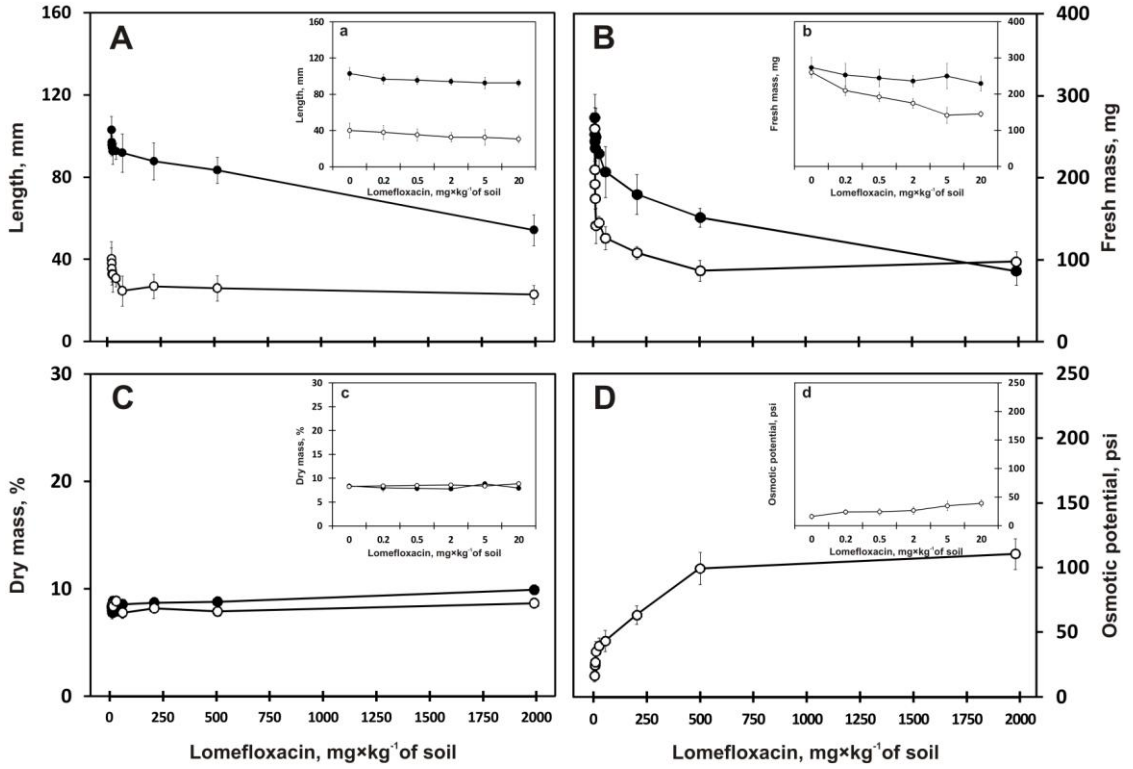

Figure 4. *Roots (●) and stems (○) length A, roots (●) and stems (○) fresh mass B, roots (●) and stems (○) dry mass C, stems osmotic potential D of pea growing in soil contaminated with different lomefloxacin concentration 0.2, 0.5, 2, 5, 20, 50, 200, 500, 2000 mg×kg^{-1} of soil (above-mentioned qualities of pea in low lomefloxacin concentration 0-20 mg×kg^{-1} of soil a, b, c, d). Data points represent the means and standard deviation (± SD) for 9 replicate samples.*

LOM toxicity towards BAs content, and activity of decarboxylases

The following BAs were identified in pea seeds: a monoamine – Tyr, diamines: Put and Cad, a triamine – Spd and a tetramine - Spm. Control seeds contained 78, 9, 1, 250 and 12 μg×g^{-1} FM of Tyr, Put, Cad, Spd and Spm, respectively (*Fig. 5A*). The main BA in pea seeds was Spd, which resulted in a low value of Put/Spd content ratio, equal – 0.04 (*Figs. 5A, 5B*).

In 4-day-old roots and stems of pea seedlings Agm was also identified. Tissues of control roots contained 91, 209, 1020, 222, 19 and 12 μg×g^{-1} FM of Tyr, Put, Cad, Spd, Agm and Spm, respectively (*Fig. 5A*), but tissues of control stems contained 180, 157, 1444, 474, 52 and 83 μg×g^{-1} FM of Tyr, Put, Cad, Spd, Agm and Spm, respectively (*Fig. 5A*). The roots and stems contained the most Cad 1020 μg×g^{-1} and 1444 μg×g^{-1} FM, respectively (*Figs. 5A*)**.** The Put/Spd content ratio in the pea roots amounted to almost 1, while in the stems, it was lower by half on average and did not exceed 0.53 (*Fig. 5B*).

In 9-day-old pea roots and stems, the number of identified BAs was the same as in 4-day-old seedlings (Tyr, Put, Cad, Spd, Agm and Spm), but it was different than that in the seeds (Tyr, Put, Cad, Spd and Spm). 9-day-old control tissues of roots contained 24, 60, 356, 71, 2.8 and 1.5 μg×g^{-1} FM of Tyr, Put, Cad, Spd, Agm, Spm, respectively (*Fig.*

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5A), but control tissues of stems contained 4.3, 37, 103, 70, 4.6 and 2.8 $\mu g \times g^{-1}$ FM of Tyr, Put, Cad, Spd, Agm, Spm, respectively (*Fig. 5A*). Similarly to 4-day-old seedlings, 9-day-old seedlings contained the most Cad (*Fig. 5A*).

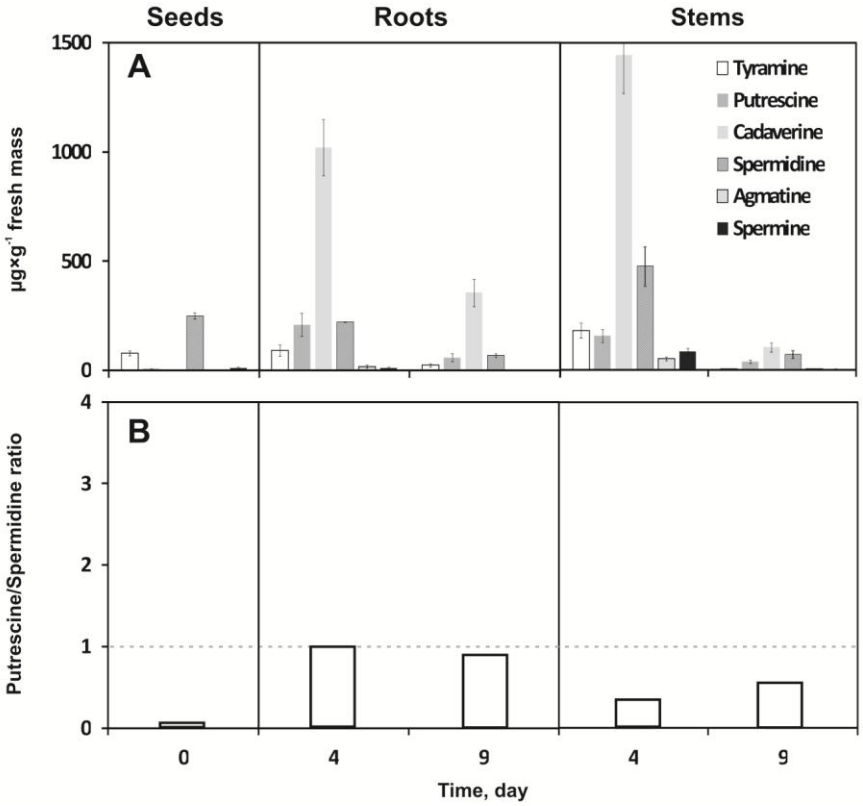

Figure 5. *Tyramine, putrescine, cadaverine, spermidine, agmatine and spermine in control seeds, roots, stems A of pea after 4 and 9 days. Putrescine/spermidine ratio in control seeds, roots, stems B of pea after 4 and 9 days. Data points represent the means and standard deviation (± SD) for 9 replicate samples.*

Increasing soil LOM concentrations resulted in elevated BAs contents in roots and stems. Roots and stems of 9-day-old seedlings, exposed to the highest drug concentration (2000 $mg \times kg^{-1}$ of soil) contained, on average, 2- and 4 times more Tyr, respectively, compared which the same organs in control plant (*Fig. 6A*). On the other hand, the greatest increase in Spm content, i.e. 3.4-fold (in roots) and 5.6-fold (stems) was found in seedlings growing in soil contaminated with the highest of the tested LOM concentrations (*Fig. 6F*).

The LOM concentration of 20 $mg \times kg^{-1}$ of soil significantly increased the content of BAs in the roots and stems, except Spm. However, the greatest increase in BAs was noted in the stems, and was 10-, 3.4-, 6-, 1.7- and 5-fold for Tyr, Put, Cad, Spm and Agm, respectively. The contents of Tyr, Put, Cad, Spm in the roots of seedlings growing in soil containing LOM at 20 $mg \times kg^{-1}$ of soil increased on average 2-fold, except Agm whose content increased 4-fold (*Figs. 6A, 6B, 6C, 6E, 6F*). In pea seedlings growing in LOM contaminated soil (20 $mg \times kg^{-1}$ of soil), the highest value of the Put/(Spd+Spm) content ratio was 1.11 and 0.95 in roots and stems, respectively (*Table 1*).

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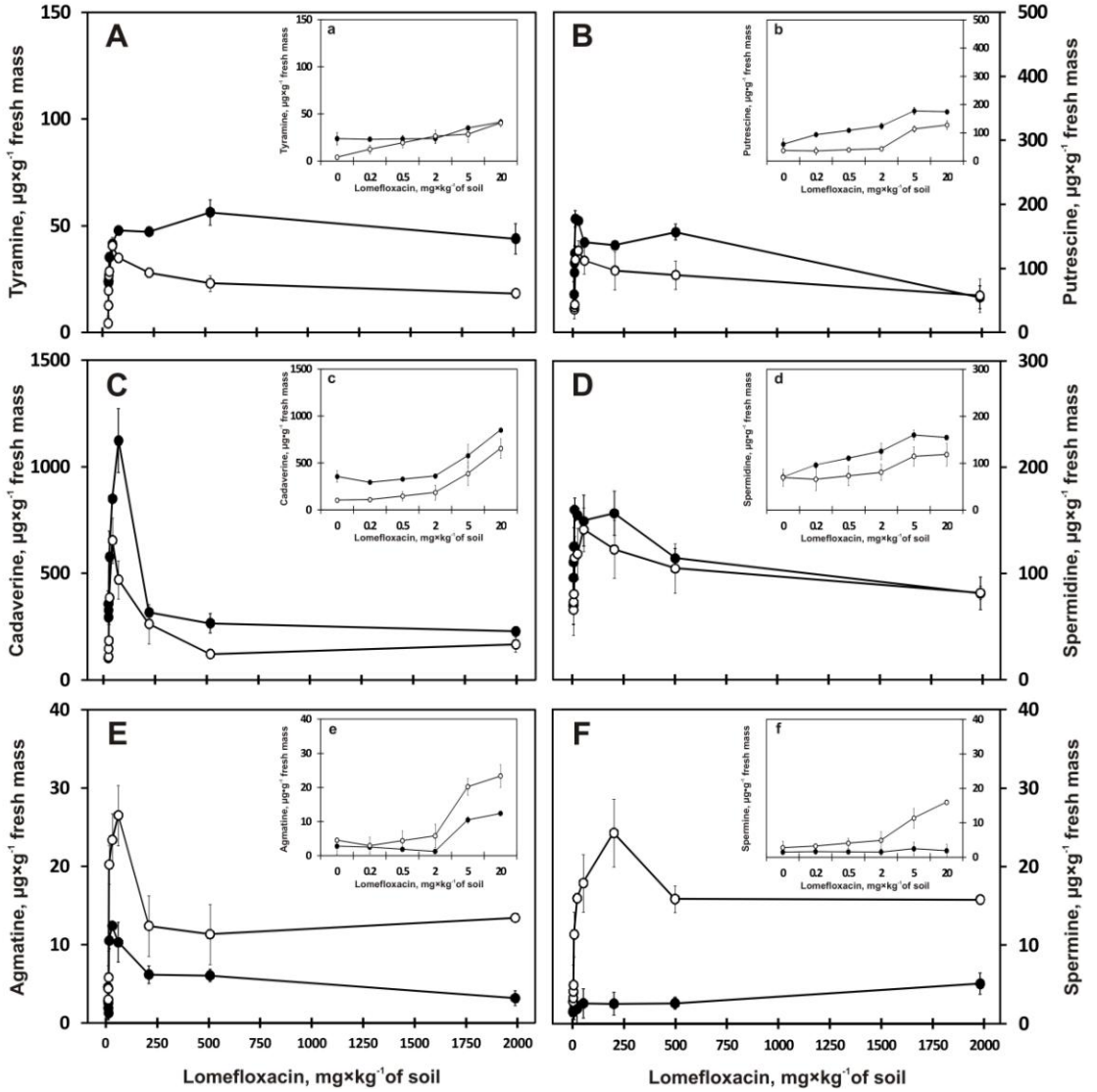

Figure 6. *Tyramine A, putrescine B, cadaverine C, spermidine D, agmatine E and spermine F in roots (●) and stems (○) of pea growing in soil contaminated with different lomefloxacin concentration 0.2, 0.5, 2, 5, 20, 50, 200, 500, 2000 mg×kg^{-1} of soil (above-mentioned qualities of pea in low lomefloxacin concentration 0-20 mg×kg^{-1}of soil a, b, c, d, e, f). Data points represent the means and standard deviation (± SD) for 9 replicate samples.*

Table 1. *Spermine/Putrescine, Putrescine/(Spermidine+Spermine) ratio in roots and stems pea growing in soil contaminated with lomefloxacin.*

Ratio	Lomefloxacin, mg×kg^{-1} of soil									
	0	0.2	0.5	2	5	20	50	200	500	2000
ROOTS										
Spermine/Putrescine	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.09
Putrescine/(Spermidine+Spermine)	0.83	0.96	0.97	0.98	1.09	1.11	0.93	0.85	0.89	0.64
STEMS										
Spermine/Putrescine	0.07	0.09	0.10	0.11	0.10	0.12	0.16	0.25	0.18	0.27
Putrescine/(Spermidine+Spermine)	0.52	0.52	0.52	0.51	0.90	0.95	0.70	0.66	0.74	0.59

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Activity of SAMDC, ADC, and ODC was determined in roots and stems of 9-day-old seedlings. In control roots the following activity values were obtained: 1.83; 0.86; 0.21 μmol $CO_2 \times g^{-1}$ protein$\times h^{-1}$, for SAMDC, ODC, and ADC, respectively. In control stems the decarboxylase activity, given in the same enzyme order as above, was 1.91, 0.54 and 0.54 μmol $CO_2 \times g^{-1}$ protein$\times h^{-1}$. Different concentrations LOM in soil resulted in the maximum activity of each decarboxylase, and these were 500, 200 and 20 mg LOM$\times kg^{-1}$ of soil for SAMDC, ODC, and ADC respectively. The maximum increase in decarboxylase activity in roots (*Fig.7*), resulting from LOM presence in soil, was 5-fold, 18-fold and 6-fold for SAMDC, ODC, and ADC, respectively.

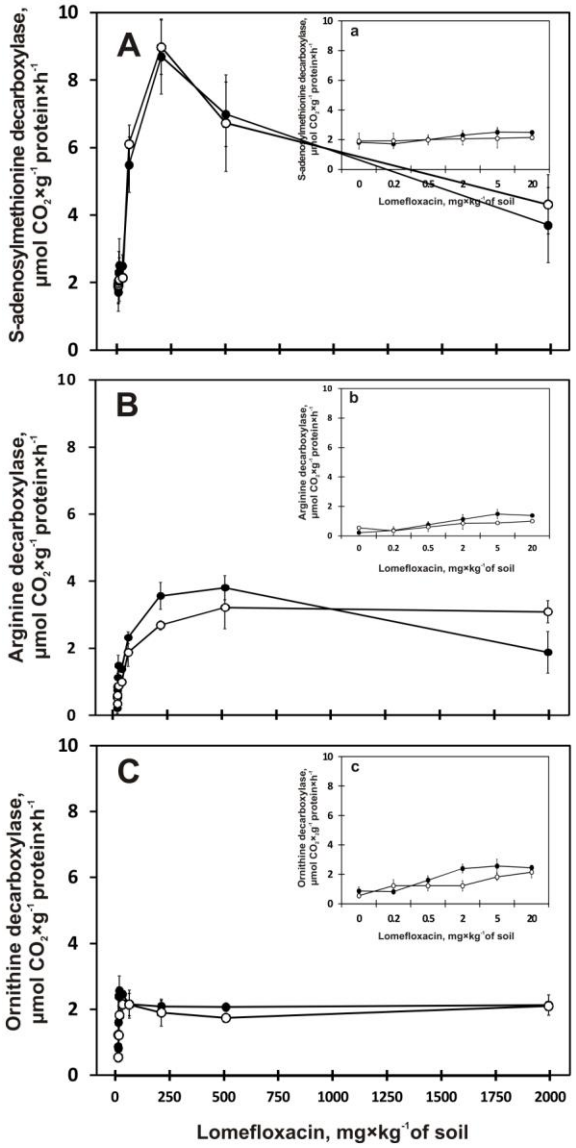

Figure 7. *Activity of S-adenosylomethionine decarboxylase A, arginine decarboxylase B, ornithine decarboxylase C in roots (●) and stems (○) of pea var. Pomorska growing in soil contaminated with different lomefloxacin concentration 0.2, 0.5, 2, 5, 20, 50, 200, 500, 2000 mg×kg^{-1} of soil (above-mentioned qualities of pea in low lomefloxacin concentration 0-20 mg×kg^{-1} of soil a, b, c)*

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The EC_{50} values (soil antibiotic concentrations causing a 50% increase in root BAs content) were 159, 2, 22, 2, 9, above 2000 mg×kg^{-1} LOM of soil for Tyr, Put, Cad, Spd, Agm and Spm, respectively. The most sensitive toxicity endpoints were the activity of ODC followed by the content of Put. Dry mass and Spm concentration were insensitive to LOM. The EC_{50} values in stems were lower, compared to roots (*Table 2*).

Table 2. *Effect of lomefloxacin on growth parameters, tyramine, putrescyne, cadaverine, spermidine, agmatine, spermine contents and SAMDC, ADC, ODC activity. EC_{10}, EC_{25}, EC_{50} and EC_{90} values expressed in mg×kg^{-1}of soil.*

Feature	Lomefloxacin, mg×kg^{-1} of soil			
	EC_{10}	EC_{25}	EC_{50}	EC_{90}
ROOTS				
Lenght	<0.20	202.34	790.38	1731.23
Fresh mass	<0.20	86.03	663.73	1588.06
Dry mass	>2000	>2000	>2000	>2000
Tyramine content	<0.20	20.33	159.41	381.94
Putrescine content	<0.20	0.33	1.84	4.25
Cadaverine content	3.58	10.61	22.33	41.09
Spermidine content	<0.20	<0.20	2.32	13.50
Agmatine content	1.84	4.56	9.09	16.35
Spermine content	>2000	>2000	>2000	>2000
SAMDC activity	9.50	39.46	89.39	169.28
ADC activity	<0.20	1.18	139.76	360.46
ODC activity	<0.20	0.40	1.58	3.47
STEMS				
Lenght	<0.20	<0.20	189.83	1406.28
Fresh mass	<0.20	<0.20	<0.20	1177.85
Dry mass	>2000	>2000	>2000	>2000
Osmotic potential	<0.20	19.97	498.02	1262.92
Tyramine content	<0.20	<0.20	4.90	15.59
Putrescine content	0.30	3.22	8.09	15.89
Cadaverine content	0.72	3.77	8.85	16.99
Spermidine content	<0.20	5.81	18.81	39.60
Agmatine content	<0.20	5.57	17.72	37.16
Spermine content	<0.20	9.79	65.60	154.90
SAMDC activity	13.19	42.25	90.67	166.16
ADC activity	<0.20	63.22	184.89	379.55
ODC activity	<0.20	<0.20	5.53	31.12

Discussion

Pea is a high-protein legume crop cultivated in more than 87 countries worldwide, with approx. imately half of the global production concentrated in Canada, France, China and the Russian Federation (McPhee, 2003), where it is used in human nutrition as well as animal fodder. Therefore, an attention to the quality of the crop should be a priority. Abiotic stresses are the main cause of crop losses (Mantri et al., 2012) resulting in yield reductions of up to 50% (Alcázar et al., 2006). It has been demonstrated that pea is sensitive to soil contamination with drugs and antibiotic toxicity is manifested by morphological and biochemical changes in pea seedlings (Adomas et al., 2013;

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Michelini et al., 2014). Morphological parameters recommended by the OECD (2006) for evaluation of xenobiotics' toxicity showed toxic effects of LOM (*Fig. 4*). However, changes in morphological parameters are a secondary effect of toxic action of xenobiotics. Phytotoxic effects of tetracycline include reduction of the length of wheat roots and stems, and a decrease of their mitotic index (Xie et al., 2011), also lucerne responds identically when exposed to oxytetracycline (Kong et al., 2007). In order to counteract the reduction in water content plants accumulate protective compounds (Yin et al., 2014). Toxic effects of drugs on biochemical process have been shown in plants. In addition to morphological abnormalities, drugs also cause biochemical changes in the content of soluble carbohydrates (Adomas et al., 2013), the activity of cytochrome c oxidase (Piotrowicz-Cieślak et al., 2010), guaiacol peroxidase, catalase (Saidi et al., 2014), and changes in protein profile (Margas et al., 2016). The protein profile is affected by BAs as a result of their interactions with DNA, RNA, proteins, phospholipids and other polyanions. A great variety of chemical structures and functions of BAs has led to multiple classifications. BAs are divided into aliphatic, aromatic and heterocyclic amines. Aliphatic amines include polyamines (PAs) - Agm, Cad, Put, Spd and Spm. Aromatic amines include Tyr. PAs are most often found in the eukaryotes, but recent studies indicate that Spm is found only in angiosperms, whereas thermospermine is found throughout the plant kingdom (Naka et al., 2010; Vera-Sirera et al., 2010). Aliphatic amines are closely correlated with many physiological processes in plants, such as somatic embryogenesis, cell divisions, seed dormancy breaking, rhizogenesis, maturation and aging of leaves, development of reproductive organs, flowering, fruit ripening, responses to stress and many others (Smith, 1985; Galston et al., 1997; Slocum and Flores, 1999; Gill and Tuteja, 2010). BAs are also involved in the regulation direct efflux of free radicals, osmotic regulation, ion balance, stability of the ionic membranes, binding the antioxidants and increasing their activity (Roychoudhury et al., 2011; Tomar et al., 2013). BAs are also active in the regulation of the vascular network development, which is found in vascular plants and serves to conduct water and mineral salts. Thermospermine, a tetramine, play an active role in the regulation of vascular network development. This protective effect of thermospermine has a major impact on the morphology of xylem cells (modelling the cell wall) and cell death and, indirectly, on plant growth (Vera-Sirera et al., 2010).

Aliphatic amines are found in plants at different concentrations. *Citrus sinensis* seedlings contain about 2.5, 3.8 and 3 $\mu g \times g^{-1}$ FM of Put, Spd and Spm, respectively (Wu et al., 2009), while *Arabidopsis thaliana* seedlings contain higher amounts of BAs - 1.2, 7.4 and 3.7-fold more Put, Spd and Spm, respectively (Rambla et al., 2010). On the other hand, in the stems of mature *Arabidopsis thaliana* plants a lower content of BAs is found: 0.66, 4.21 and 2.02 $\mu g \times g^{-1}$ FM of Put, Spd and Spm, respectively (Naka et al., 2010). In our study, the contents of BAs in the seeds, roots and stems also varied. Spm was the dominat BA in the seeds and Cad was dominant in roots and stems (*Fig. 5*). Ohe et al. (2005 and 2010) analysed soy seeds and showed that Spd generally dominates, however, there is more Spm in embryonic axes than in cotyledons. Taking into account the embryonic axis-to-cotyledon ratio, a higher value for Spd is obtained than that of Spm in whole soybean seeds. As is clear from the published data, the year of harvest is also an important factor differentiating the content of BAs in seeds or plants. It should be noted that analyses of the contents of Spd and Spm were performed using a very precise technique based on (^{15}N) nitrogen labeling. It was demonstrated that in the embryonic axes and cotyledons of the same soybean variety in 2010 the

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content of Spd was 6.8 and 7 times higher than in 2005, respectively (Ohe et al., 2005, 2010). Fluctuations in the contents of BAs occur even on a daily basis (Gemperlová et al., 2006). Therefore, our study assessed the content of BAs on the 4th day of pea germination, since the preliminary study (unpublished data) we carried out demonstrated that on the 4th day the profile of BAs changes. On that day, Agm first appeares, although in trace amounts; hence, the tests on the effects of LOM on the content and profile of BAs were performed on 9-day-old seedlings. The content of BAs was determined by the duration of germination and was different in 4-day-old and 9-year-old seedlings. The highest content was found in 4-day-old roots, while the lowest content was in the seeds. Mature pea seeds contained the most Spd (*Fig. 5A*), similar to ripe maize kernels in which Spd and Put account for more than 67% of the identified BAs (Bandeira et al., 1996). However, the content of BAs was also modified by LOM (*Fig. 6*). The dominant compound in dry seeds was Spd, in 4-day-old seedlings – Cad, and in 9-day-old seedlings there was also Cad but 8 times less (3 times less for roots, almost 14 times less for stems) of it compared to the 4th day. In addition, Agm was also found in 4-day-old seedlings (*Fig. 5A*). The highest content of BAs was found in seedlings growing in soil contaminated LOM with 50 $\mu g \times kg^{-1}$ (*Fig. 6*). Analyses of 2-day-old soybean seedlings, showed increased contents of Spd and Spm in cotyledons, and of Put - in roots (Glória et al., 2005). 4-day-old pea roots, as compared to the ungerminated seeds, contained up to 4 times more biogenic amines (and the stems contained even 7 times more of them). However, the rate of increase of the contents of particular BAs, including Put and Spd, was the rate differed (*Figs. 6B, 6D*). There is a relationship between the value of the Put/Spd content ratio and the mitotic activity of cells (Matilla, 1996). In rapidly proliferating meristematic tissues, the value of the Put/Spd content ratio is below one. An increase in the value of that ratio above one characterises cells undergoing intensive elongation growth. It is evident, therefore, that the content of BAs changes during the growth of a seedling and so does the response of pea plants to the presence of LOM in soil. LOM at concentrations of 50 and 20 $mg \times kg^{-1}$ of soil resulted in an increase in the content of BAs to 1473 and 980 $\mu g \times g^{-1}$ fresh mass in the roots and stems (*Fig. 6*), respectively. Enrofloxacin in soil or tetracycline in water also result in the accumulation of BAs in plant tissues (Adomas et al., 2013; Baciak et al., 2016a). BAs are responsible, among other functions, for the control of the osmotic pressure and maintaining membrane stability (Bouchereau et al., 2000). However, an increased content of selected BAs may lead to adverse effects in plant tissues. The accumulation of Put, for example, may result in a loss of potassium, an increase in protein content, membrane depolarisation, or the necrosis of tissues (Tiburcio et al., 1990). Toxic effects of xenobiotics result in changes of BAs content in tissues. It was observed that in plants responding to stress, the Put/(Spd+Spm) content ratio decreases (Bouchereau et al., 1999; Capell et al., 2004). In pea seedlings growing in LOM contaminated soil (20 $mg \times kg^{-1}$ of soil), the highest value of the Put/(Spd+Spm) content ratio was 1.11 and 0.95 in roots and stems, respectively (*Table 1*).

The literature data and our study clearly suggest that the content of BAs in plants is strongly modified, depending on several factors. It seems, therefore, that the qualitative composition of BAs is controlled (through decarboxylases activity) more strictly than the levels of specific BAs. Our study focused on the determination of the activity of the Arg pathway enzymes. The decarboxylases involved in that pathway include SAMDC, ADC, and ODC (*Fig. 8*).

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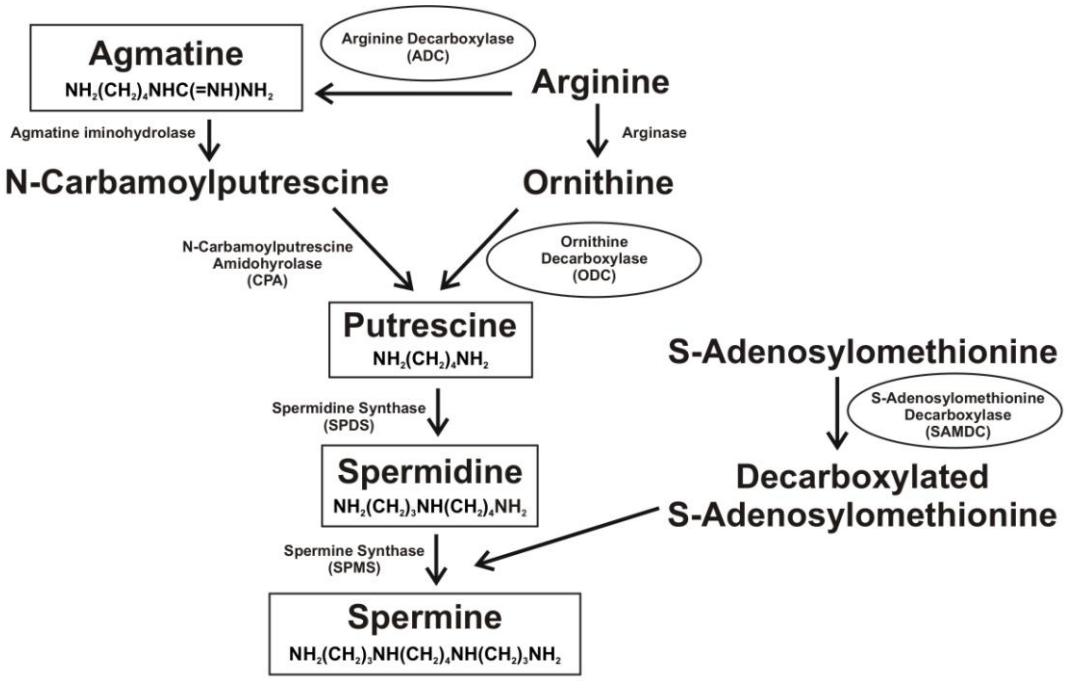

Figure 8. *Suggested biogenic amines biosynthesis in plants*

In plant metabolism of amino acids, decarboxylases play a double role: they participate in amino acid degradation and they are also responsible for BAs biosynthesis. ODC carries out the reaction of ornithine decarboxylation directly to Put (Fariduddin et al., 2013). The biosynthesis of Put is also preceded by reactions of Arg decarboxylation by ADC to Agm (Lightfoot and Hall, 2014). A synthesis of higher BAs, such as Spd and Spm, involves the binding of aminopropyl radicals to Put with the participation of Spd synthase (SPDS; EC 2.5.1.16), and Spm synthase (SPMS; EC 2.5.1.22). Decarboxylated S-adenosylmethionine (dcSAM) is the donor of aminopropyl groups and it is formed as a result of the activity of SAMDC (EC 4.1.1.50) (Alcázar et al., 2010; Lightfoot and Hall, 2014). Biosyntheses of Spd, Spm/T-Spm are determined by the activity of pyruvoyl-cofactor containing S-adenosymethionine (SAM) decarboxylase (SAMDC), which is the key enzyme limiting the rate of the whole polyamine pathway (Capell et al., 2004). Under osmotic stress, a increase in Put content may occur, which is then not balanced by Put outflow towards the biosynthesis of Spd and as a result a potentially useless pool of Put accumulates. The surplus of Put may also result from the inhibition of the reaction of Put catabolism by diamine oxidase participating in the biosynthesis of Spd, or from the inhibition of the activity of SAMDC (Bouchereau et al., 1999). LOM presence in soil resulted in an increase in the activity of SAMDC in the tissues of pea roots and stems. This decarboxylase was more than twice as active as the other two determined in pea tissues, i.e. ODC and ADC (*Fig. 7*). SAMDC in plants is responsible for the detachment of carbon dioxide from the S-adenosylmethionine molecule (Alcázar et al., 2010; Lightfoot and Hall, 2014). BAs biosynthesis enzymes compete for S-adenosylmethionine with ethylene biosynthesis system, as SAM is the donor of aminopropyl groups for both metabolic pathways (Bouchereau et al., 1999). This metabolic divergence may lead to insufficient supply of substrate for the S-

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adenosylmethionine decarboxylation by SAMDC. The highest activity of SAMDC, on average amounting to 8.84 μmol $CO_2 \times g^{-1}$ protein$\times h^{-1}$, was determined in tissues of pea roots and stems exposed to an LOM concentration of 200 mg$\times kg^{-1}$ of soil (*Fig. 7A*). As demonstrated by earlier studies, mutant thale cress (*Arabidopsis* Heynh.) mutants genetically devoid of SAMDC are bushy and stunted and have an increased root vascular system (Ge et al., 2006), while a double mutant in which the biosynthesis of BAs has been inhibited by the exclusion of ADC is characterised by sticky seedlings, twisted roots and smaller organs (Watson and Malmberg, 1996). ADC is mostly responsible for the biosynthesis of BAs in rape leaves and the osmotic stress increases the activity of lysine and ODC decarboxylases in this plant (Aziz et al., 1997). On the other hand, lupin roots exposed to sodium chloride are characterised by an increase in the activity of ADC (Legocka and Kluk, 2006). The most sensitive endpoints for antibiotic toxicity to stems were the content of Tyr and the activity of ODC. The activity of ADC in pea roots was 90% inhibited (EC_{90}) by LOM at a concentration of 360.46 mg$\times kg^{-1}$ of soil, while in order to inhibit the activity of ODC by 90%, a 100 times lower content of LOM was required (*Table 2*). ODC is the key enzyme in the Put biosynthesis pathway in most plants; on the other hand, during environmental stress, this function is taken over by ADC (Malmberg et al., 1998). Our study revealed that even the lowest concentration of LOM inhibits the activity of ODC (EC_{90} of 3.47 mg$\times kg^{-1}$ of soil) (*Table 2*) and higher LOM concentrations (250 and 500 mg$\times kg^{-1}$ of soil) increase the activity of SAMDC and ADC (*Figs. 7A, 7B*). It can be assumed that the presence of drugs in soil changes the pathway of the biosynthesis of BAs, particularly Put. Its content is correlated to the activity of ADC (EC_{90} of 360.46 mg$\times kg^{-1}$ of soil). We suggest that the Put biosynthesis pathway proceeds from Arg to Agm catalysed by ADC, which is followed by the formation of N-carbamoylputrescine with the participation of Agm imniohydrolase. CPA (N-carbamoylputrescine amidohydrolase) is involved in the synthesis of Put with N-carbamoylputrescine. Therefore, during the exposure of plants to LOM, Put is not synthesised directly from ornithine.

Our study showed that ODC was the enzyme most sensitive to soil contamination with LOM. It was clear from the calculated endpoints for biochemical characteristics of roots and stems were the earliest noticeable symptoms of soil contamination. To the best of our knowledge, this is the first experimental proof of the effect of veterinary drugs on the role and activity of enzymes involved in the biosynthesis of BAs. These results indicate that the activity of ODC may be a good biomarker of soil contaminated with LOM, indicative of exposure before visible damage occurs. Therefore, the role of a biomarker should be the prediction of harmful effects of chemical substances on the environment. We recommend the method employed in the study for the measurement of the released CO_2 using a multi-gas analyser equipped with an infrared detector as a cheap, precise method for the determination of the activity of enzymes without the use of labelled elements.

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