

# Phytotoxicity of Enrofloxacin Soil Pollutant to Narrow-Leaved Lupin Plant

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## Abstract

Drugs used in human and veterinary medicine belong to a new class of environmental pollutants. Antibiotics and chemotherapeutics may produce biological effects at very low concentrations. Moreover, the continuous exposure of bacteria to even small concentrations of antibiotics or active metabolites could lead to the emergence of resistant bacteria strains. Antibiotics may also have high biological activity against non-target organisms as plants. Biotests, opposite of instrumental (chemical) methods, allow estimation of whether very low levels of active substance residues in the soil can be phytotoxic to crop plants. The aim of this study was to evaluate the biogenic amines content in lupin seedlings as affected by different enrofloxacin concentrations in soil. With increasing enrofloxacin concentrations the root growth was inhibited more severely and dry mass increased slightly but steadily. At the highest enrofloxacin concentration, the dry mass of both roots and stems did not exceed 15% fresh mass. The lowest content of raffinose family oligosaccharides (RFOs) was observed in seedlings growing in the soil without enrofloxacin, the highest in those growing in soil containing 50 mM of the antibiotic. The speed of RFO mobilization during germination was a good indicator of elongation in seedlings. A lowered RFO content during germination was correlated with an increase in glucose, galactose, and sucrose content. Spermine, spermidine, and putrescine were detected in roots. The total biogenic amine content ranged 7-105  $\mu\text{M}\cdot\text{g}^{-1}$  fresh mass. The results showed, that the biogenic amine profiles seem to be good parameters for the qualification of plant products.

**Keywords:** soil, enrofloxacin, lupin seeds and seedlings, soluble sugars, polyamines, biotests

## Introduction

Drugs used in human and veterinary medicine belong to a new class of environmental pollutants capable of producing biological effects at low concentrations. Moreover, the continuous exposure of bacteria to even small concentrations of antibiotics or active metabolites could lead to the emergence of resistant bacteria strains. Fluoroquinolones (FQs) like ciprofloxacin, lomefloxacin, ofloxacin, and

enrofloxacin are a class of antibiotics. They are commonly used in both human and veterinary medicine, and are not readily biodegradable by microorganisms [1]. At low concentrations, FQs are phytotoxic and toxic to most plants and animals. At the same time, while water plants respond to FQs with chlorosis, due to chlorophyll synthesis inhibition fish are not affected even by high concentrations [2]. The most important veterinarian-used FQs are enrofloxacin, danofloxacin, sarafloxacin, orbifloxacin, marbofloxacin, and difloxacin. They are commonly detected in water and soil, and their ecotoxic activity in the environment depends

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on the concentration, type of organisms affected, and exposure time. High levels of FQs (norfloxacin, ciprofloxacin, lomefloxacin, and andenrofloxacin) were reported in tap water in Guangzhou and Macao: 1.0 to 679.7 ng·L<sup>-1</sup>, and 2.0 to 37.0 ng·L<sup>-1</sup>, respectively [3].

In soil, drugs can be detected at concentrations exceeding 1 µg·kg<sup>-1</sup> soil. The drug content in soil depends on the frequency of manure fertilization. The sulphonamides content in soil fertilized with manure was documented even three months after application [4]. According to European Agency for the Evaluation of Medicinal Products (EMA) recommendations, an analysis of environmental risk of drug application should be conducted when estimated concentration in surface water exceeds 0.01 mg·L<sup>-1</sup> [5, 6]. Such threshold values are not defined for manure, farm animal faeces, or sludge that are introduced into fields. Broad range analyses carried out in the USA, Germany, and the UK confirmed that drugs are present as pollutants in the environment [7, 8]. Fluoroquinolones are strongly adsorbed by soils, [9] and have been detected in untreated raw sewage sludge at levels between 1.40 and 2.03 mg·kg<sup>-1</sup> dry matter (dm); similar levels were found in digested sludge, from 2.13 to 2.42 mg·kg<sup>-1</sup> dm. Sludge is used as fertilizer, for irrigation of fields, watering of crops, or can be brought into the soil as contamination from sewage-treatment plants. The enrofloxacin content in soil decreases with increases of depth [10].

Antibiotics may also have high biological activity against non-target organisms like plants. Research on drug effect on plants has been done only to a limited extent. It has been shown that plants absorb antibiotics from soil, e.g. sulfadimetoxin in barley [11]; sulfametazine, in corn, lettuce, and potato [12]; chlortetracycline, tetracycline, tylosin, sulfamethoxazole, sulfamethazine, and trimethoprim in sweet oat, rice, and cucumber [13]; sulfamethazine in yellow lupin, pea, lentil, soybean, beans, and alfalfa [14]; and enrofloxacin and ciprofloxacin in lettuce, common barley, and cucumber [15].

In plants exposed to chemical stress, biochemical and physiological processes are perturbed, which leads to reduced seed germination and affects seedling development [16]. Hemicelluloses, RFOs, and galactosyl cyclitols are the storage substances of lupin seeds [17]. During the germination process RFOs in lupin seeds undergo hydrolysis to simple compounds in order to provide easily accessible energy and carbon skeletons at the early stages of seed germination [19, 20]. Raffinose family oligosaccharides (RFO) and polyamines (PAs) are an important component in plant responses to stress, and play a significant role in counteracting stresses [21, 22]. Polyamines (mainly diamine putrescine (Put), triamine spermidine (Spd), and tetraamine spermine (Spm)) are polycationic compounds of low molecular weight that are present in all living organisms. In plants they are responsible for distribution, growth, development, and productivity [23]. It is known that antibiotics present in soil inhibit plant root and stem elongation. The aim of the current study was to determine the role of polyamines in narrow-leaved lupin plants growing in soil contaminated with enrofloxacin.

## Material and Methods

### Seed Germination and Root Growth Test

Seeds of narrow-leaved lupin (*Lupinus angustifolius*) cv. Karo were germinated for seven days in PHYTO-TOXKIT™ plates (MicroBio Test Inc., Belgium). Germination was carried out under controlled climatic conditions with temperature set at 25°C and 90% relative humidity (RH %), in darkness. Ninety ml of soil (sand, vermiculite, peat 1:0.3:1, v/v/v) were placed in plastic micro-biotest plates. The soil was covered with Whatman No. 1 filter-paper and watered with 27 ml distilled water supplemented with different enrofloxacin (Sigma-Aldrich) at final concentrations: 0.5 mM, 1 mM, 10 mM, and 50 mM. The control plants were watered with pure distilled water. Enrofloxacin were determined in triplicate on each sample. The root length was estimated using Image Tool for Windows. Dry and fresh mass roots and stems were determined.

### Soluble Carbohydrate Contents

Soluble carbohydrate (sucrose, glucose, galactose, raffinose, stachyose, and verbascose) content in seedlings were analyzed using GC chromatography according to Piotrowicz-Cieślak [18]. Tissues (100 mg fresh mass) were homogenized in ethanol: water mix, 1:1 (v/v) containing 300 µg phenyl- $\alpha$ -D-glucose as internal standard. The homogenate and the wash were combined in a 1.5 ml microfuge tube, heated at 75°C for 30 min to inactivate endogenous enzymes, and centrifuged at 15,000 g for 20 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 under a stream of nitrogen. Dry residues were derived from 300 µl of silylation mixture (trimethylsilylimidazole: pyridine, 1:1, v/v) in silylation vials (Thermo Scientific) at 70°C for 30 min, and then cooled at room temperature. One µl soluble carbohydrate extract was injected into a split-mode injector of a Thermo Scientific gas chromatograph equipped with flame ionization detector. Soluble carbohydrates were analyzed on a DB-1 capillary column (15 m length, 0.25 mm ID, 0.25 µm film thickness, J&W Scientific) and identified with internal standards as available. Concentrations were calculated from the ratios of peak area, for each analyzed cyclitol, to the peak area of respective internal standard. Quantities of cyclitols were expressed as mean±SD for 3-5 replications of each treatment.

### Polyamine Contents

The polyamines were extracted from lyophilized plant material with cold 5% perchloric acid in accordance with Bouchereau et al. [24]. The lyophilized plant material was shaken with 25 cm<sup>3</sup> of 5% HClO<sub>4</sub> solution for 30 min and then centrifuged at 16,000 g for 30 min at 4°C. The supernatant was evaporated and the residue was re-dissolved in

3 ml of a 5% HClO<sub>4</sub>. The extract was analyzed by using the AAA 400 amino acid analyzer (Ingos, Praha, Czech Rep.). The polyamines were separated at 70°C on a 7.0 × 0.37 cm column filled with Ostion Lg ANB 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 of sodium chloride. The quality and quantity of the polyamines were assayed with a spectrophotometric detector, following their reaction with ninhydrin, and expressed in  $\mu\text{M}\cdot\text{g}^{-1}$  of fresh weight.

### Statistical Analysis

The experiment was conducted in nine 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).

### Results

Seeds of narrow-leaved lupin var. Karo, irrespective of the concentration of enrofloxacin (0, 0.5, 1, 10, and 50 mM), germinated in the range between 90% (10 mM) and 100% (control) after 7 days (Fig. 1). The seeds were considered germinated when the radicle penetrated the seed coat. Enrofloxacin 0.5, 1, and 10 mM concentrations inhibited germination by 10% on average. A dramatic decrease in germination (by 95%) appeared at 50 mM concentration. The effect of enrofloxacin on growth after 7 days is shown in Fig. 1 as mean length of root and stems. Enrofloxacin altered morphological organs of the examined plants already at a level of 0.5 mM, but a dramatic decrease in root and stem elongation was observed at 10 mM. The highest concentration of enrofloxacin (50 mM) inhibited root elongation by 98% on average, and stem elongation was stopped completely (100%) (Fig. 1). The fresh mass of roots and stems decreased as enrofloxacin concentration grew. The dry mass roots and stems increased slightly but steadily together with the increase of enrofloxacin concentration.

At the highest enrofloxacin concentration, the dry mass of both roots and stems did not exceed 15% fresh mass (Fig. 1).

The level of putrescine increased from 0.4 to 7  $\mu\text{M}\cdot\text{g}^{-1}$  of fresh mass, in control and at the highest concentration, respectively. The level of spermidine and spermine increased slightly, but the content of these polyamines was higher than that of putrescine - spermidine 8 times and spermine 6 times higher.

In the germinating seeds of narrow-leaved lupin the main soluble carbohydrates were: glucose, galactose, sucrose and RFOs (Figs. 2 and 3). The content of RFOs was the lowest in the seeds germinating in soil without enrofloxacin. In those which did not germinate at all (soil with 50 mM of enrofloxacin), RFOs were mobilized and appeared in smaller amounts than in dry seeds. Moreover, the lowered content of RFOs resulted in an increase in content of glucose, galactose, and sucrose (Fig. 3).

### Discussion

The sensitivity of indicator plants to environment contamination is often used to estimate the degree of environment degradation. Plants respond to many kinds (morphological and biochemical) of toxic substances in different manners. Plant sensitivity to human and veterinary pharmaceuticals can be used to evaluate environmental degradation (soil) by these compounds [14, 25]. Phytotoxicity of enrofloxacin on plants generates both phytotoxic effect and hormesis related to plant drug uptake [26].

Biotests, in contrast to instrumental (chemical) methods, allow simple and inexpensive estimation of very low levels of active substances in soil that can be phytotoxic to crop plants [27]. Germination inhibition after the application of other toxic compounds (glyphosate, sulfamethazine) was observed [14, 16, 28]. However, enrofloxacin 50 mM inhibited narrow-leaved lupin seeds germination by 95% (Fig. 1). Antibiotics found in the soil are absorbed by plants and inhibit their elongation [15]. It was proven that the root elongation test is better than the seeds germination test [14, 22] when assessing the soil contaminated with some antibiotics.

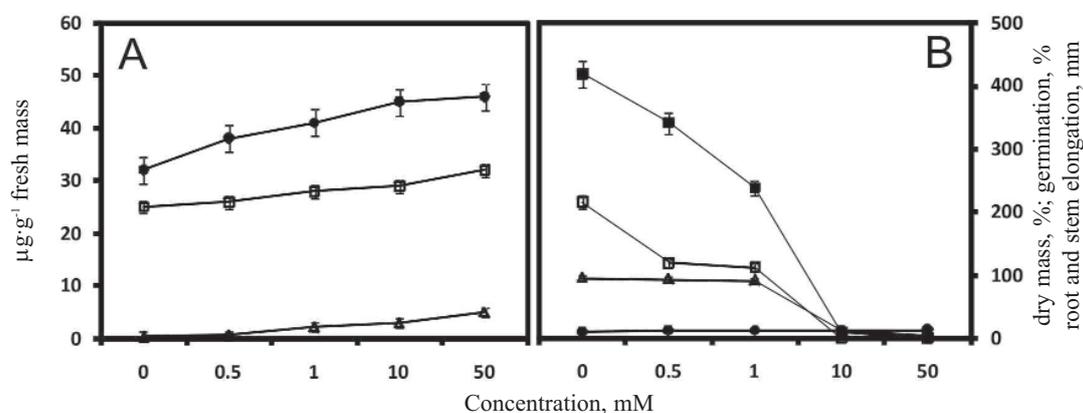


Fig. 1. Panel A) Polyamine (● – spermine, □ – spermidine, ▲ – putrescine) contents [ $\mu\text{M}\cdot\text{g}^{-1}$  fresh mass] and panel B) ● – dry mass, %; ▲ – germination, %; ■ – root elongation, mm; □ – stem elongation, mm; in narrow-leaved lupin seeds germination in soil contaminated with enrofloxacin (0, 0.5, 1, 10, 50 mM).

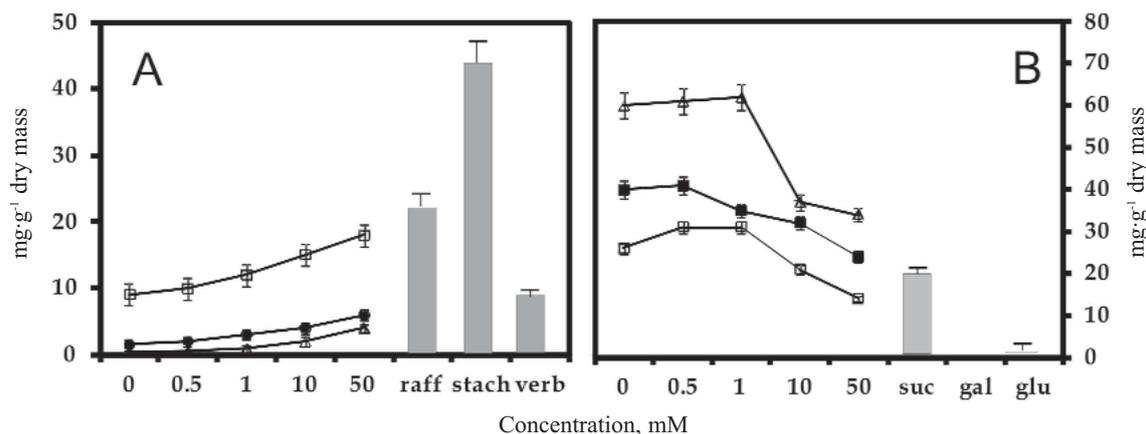


Fig. 2. Panel A) Raffinose family oligosaccharides (● – raffinose, □ – stachyose, ▲ – verbascose) contents and panel B) ▲ – sucrose, ■ – glucose, □ – galactose contents [ $\text{mg}\cdot\text{g}^{-1}$  dry mass] in narrow-leaved lupin seeds germination in soil contaminated with enrofloxacin (0, 0.5, 1, 10, 50 mM). Bars: raff – raffinose, stach – stachyose, verb – verbascose, suc – sucrose, gal – galactose, glu – glucose contents [ $\text{mg}\cdot\text{g}^{-1}$  dry mass] in narrow-leaved lupin seeds.

However, to assess how the soil is contaminated with enrofloxacin, both the root elongation test as well as the seeds germination test are required. During germination, storage carbohydrates in seeds, such as RFOs and galactomannans, undergo hydrolysis [1, 17]. In a typical pattern of legume plants germination, the content of raffinose family oligosaccharides (raffinose, stachyose, and verbascose) decreases while the content of hydrolytic products of RFOs, i.e. glucose, galactose, and sucrose increases [17]. Chromatographic studies of RFO content showed a negative correlation with seedling elongation (Figs. 1 and 2), i.e. the longer the root, the lower the RFO content in the seedling. On the other hand, non-germinating seeds (which grew in the soil with the highest enrofloxacin concentration) RFO hydrolysis started, yet the embryonic axis did not go through the seed coat (Fig. 3). The results definitely show that biochemical changes take place in terms of soluble carbohydrates content in non-germinating seeds (50 mM of enrofloxacin in soil), yet there is no

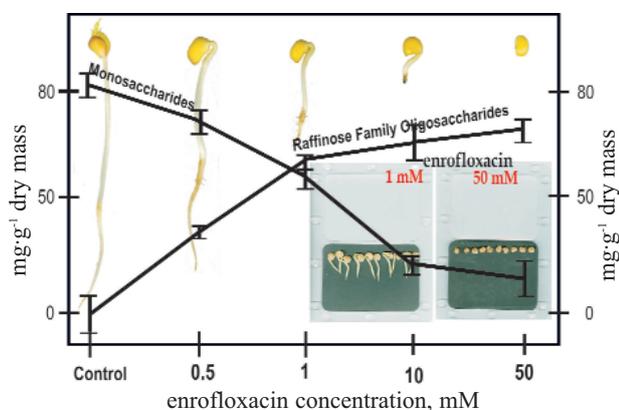


Fig. 3. Raffinose family oligosaccharide and monosaccharide content [ $\text{mg}\cdot\text{g}^{-1}$  dry mass] in narrow-leaved lupin seed germination in soil contaminated with enrofloxacin, and the effects of enrofloxacin treatment (0, 0.5, 1, 10, 50 mM) on lupin seed germination.

root elongation. Enrofloxacin concentration in soil between 50 and 5000  $\mu\text{g}\cdot\text{l}^{-1}$  includes both toxic effects and hormesis in plants (*Cucumis sativus*, *Lactuca sativa*, *Phaseolus vulgaris*, and *Raphanus sativus*) by significantly modifying both length of primary root, hypocotyl, cotyledons, and the number/length of leaves. A toxic effect is induced by high concentrations (5000  $\mu\text{g}\cdot\text{l}^{-1}$ ), while hormesis occurs at low concentrations (50 and 100  $\mu\text{g}\cdot\text{l}^{-1}$ ) [26]. In our research a 50 mM concentration suddenly inhibited root elongation and was 100% phytotoxic for stems (Fig. 1).

On the other hand, organisms react to environmental contamination by distorted biochemical or physiological functions in the cell, tissue or biological fluids. Plants are exposed to biotic and abiotic stress, which affect their distribution, growth, development, and productivity. Polyamines (PAs) are an important component in plant responses to stress and they play a significant role in counteracting stress [22, 29]. Polyamines, mainly diamine putrescine (Put), triamine spermidine (Spd), and tetraamine spermine (Spm), are polycationic compounds of low molecular weight that are present in all living organisms [30]. In our research these polyamines were found (Fig. 1) The content of polyamines varies and depends on the species of the plant, tissues, organs and the developmental stage. It ranges from 2  $\mu\text{M}$  to 2-3 mM and in seeds it reaches even 30 mM [31]. Content of polyamines in *Lupinus angustifolius* seeds cv. Mirela, Ernani, and Trebatsch were different and depended on the cultivar and ranged from 127.7 to 1785.0 spermine, from 429.9 to 718,8 spermidine, and 112.7  $\text{pmol}\cdot\text{mg}^{-1}$  dw putrescine [32].

The content of polyamines in the examined roots ranged from 0.4  $\mu\text{g}/\text{g}$  fresh weight to 47  $\mu\text{M}\cdot\text{g}^{-1}$  fresh weight spermine (Fig. 1). When enrofloxacin concentration was higher, the content of polyamines in lupin roots slightly increased. The research showed that both the narrow-leaved lupin seed germination test as well as the root and stem elongation test are effective when assessing soil contaminated with enrofloxacin.

## Conclusions

In conclusion it is worth emphasizing that for most medicines present in soil it is enough to perform root and stem elongation tests, yet in order to assess enrofloxacin phytotoxicity it is necessary to perform the seed germination test. Moreover, biochemical analysis complements test elongation for soil contaminated with enrofloxacin. The applied statistical analysis allowed for estimation of probability of the phytotoxicity of the lowest drug concentration toward narrow-leaved lupin.

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