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Root response in *Pisum sativum* under naproxen stress: Morpho-anatomical, cytological, and biochemical traits

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**Highlights**

- NPX content in root biomass corresponded with the treatment level.
- Total root length and lateral roots count were reduced by 1 and 0.1 mg/L NPX.
- Proportion of stele and xylem area in maturation zone increased under NPX treatment.
- Oxidative stress reflected in cell membrane integrity was detected under 1 mg/L NPX.
- Antioxidant defence capacity culminated at 0.5 mg/L NPX.

**Abstract**

Non-steroidal anti-inflammatory drugs as an important group of emerging environmental contaminants in irrigation water and soils can influence biochemical and physiological processes essential for growth and development in plants as non-target organisms. Plants are able to take up, transport, transform, and accumulate drugs in the roots. Root biomass in ten-days old pea plants was lowered by 6% already under 0.1 mg/L naproxen (NPX) due to a lowered number of lateral roots, although 0.5 mg/L NPX stimulated the total root length by 30% as against control. Higher section area (by 40%) in root tip, area of xylem (by 150%) or stele-to-section ratio (by 10%) in zone of maturation, and lower section area in zone of lateral roots (by 18%) prove the changes in primary root anatomy and its earlier differentiation at 10 mg/L NPX. Accumulated NPX (up to 10 mg/g DW at 10 mg/L) and products of its metabolization in roots increased the amounts of hydrogen peroxide (by 33%), and superoxide (by 62%), which was reflected in elevated lipid peroxidation (by 32%), disruption of membrane integrity (by 89%) and lowering both oxidoreductase and dehydrogenase activities (by up to 40%). Elevated antioxidant capacity (SOD, APX, and other molecules) under low treatments decreased at 10 mg/L NPX (both by approx. 30%). Naproxen was proved to cause changes at both cellular and tissue levels in roots, which was also reflected in their anatomy and morphology. Higher environmental loading through drugs thus can influence even the root function.

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1. Introduction

Drugs deserve the same attention as other pollutants because of their increasing entry into the environment and hazardous potential based on their possible high toxicity for non-target organisms (López-Pacheco et al., 2019) including plants (Ahmed et al., 2015; Bartrons and Penuelas, 2017). Obsolete or insufficient technologies for wastewater and sewage processing represent the main source of contamination by various mixtures of drugs and products of their transformation with possible synergistic or antagonist effects for both aquatic and terrestrial ecosystems (Bellver-Domingo et al., 2017). Nevertheless, to understand the combined effects, it is essential to assess each compound separately.

In connection with the therapeutic possibilities, availability of drugs, and currently increased consumption not only due to the Covid-19 pandemic, the attention is focused on non-steroidal anti-inflammatory drugs (NSAID). Naproxen ((S)-(+-)2-(6-methoxy-2-naphthyl)propionic acid, NPX) is one of the most commonly used and currently monitored drugs throughout the world (López-Pacheco et al., 2019; Marsik et al., 2017). NPX is removed from wastewater with relatively high efficiency (e.g., by photolysis; Szymonik et al., 2017), but even its sorption onto activated sludge is significant (up to 75 ng/g dry weight of sludge; Martínez-Alcalá et al., 2017). Nevertheless, some residual NPX concentrations were found even in drinking water (Szymonik et al., 2017).

Plants can play an essential and positive role in removing pollutants, including drugs, resulting from direct phytoremediation and indirectly enhancing sorption and biodegradation. Many works have found applicability of several plant species, both wetland (He et al., 2018; Phragmites australis; Nivala et al., 2019; P. australis; Zhang et al., 2018; Canna indica), and terrestrial (Marsidi et al., 2016; Vetiveria zizanioides) for wastewater and sludge decontamination. These works mostly do not deal with the direct effect of drugs on these plants. Depending on the physicochemical properties, concentration or exposure period, drugs and products of their transformation can cause responses in, e.g., protein profile (Yan et al., 2016), gene expression (Landa et al., 2018), cell division (Lutterbeck et al., 2015; Svbodniková et al., 2019), activity of plant growth regulators (Carter et al., 2015), changes in root meristems (Yu et al., 2017) or membrane permeability (Kummerová et al., 2016).

Some drugs are able to enhance the production of reactive oxygen species (ROS), which represent a group of reactive molecules playing essential physiological, mainly signalling functions. However, their overproduction is associated with the direct damage of various biomolecules and alterations in plant cell redox state. Changes in antioxidant capacity and amounts/activities of both enzymatic and non-enzymatic antioxidant mechanisms (Baxter et al., 2014; Bowler et al., 1994; Di Marco et al., 2014; Farmer and Mueller, 2013; Mhamdi et al., 2010; Passaia et al., 2013; Ziolkowska et al., 2014) are usually the first responses to environmental stress. Alterations in the redox status of the cells may result in cell death or changes in cell proliferation, which may cause changes in anatomy and also the morphology of plant tissues and organs. In the light of all the above-mentioned facts, the question is, if agricultural soils, when irrigated by contaminated water or fertilized by sludge or manure from livestock treated by drugs, are safe for crop production (Gonzales García et al., 2019; Pico et al., 2019).

Because plants are site-fixed, the plasticity of the root system represents one mechanism by which they can adapt to current environmental conditions. Root architecture results from complex and continuous de novo organogenetic processes, consisting of activity of apical root meristem, cell differentiation, initiation and formation of lateral root primordia, and the emergence of lateral roots or processes of vascular bundle elements differentiation and maturation. These processes are regulated not only by endogenous signalling molecules like phytohormones or ROS but also influenced by various external stress factors. Changes in root morphology and anatomy in plants cultivated in soil or nutrient medium contaminated by other organic pollutants were recorded by Alkio et al. (2005), Baidyga et al. (2005), or Kummerová et al. (2013).

This study aimed to characterize the effect of naproxen, which is one of the most abundant non-steroidal anti-inflammatory drug, on (i) growth parameters including morphology and anatomy of pea plant roots documented by quantitative parameters of their tissues, (ii) relevant biochemical markers at the cellular and tissue levels (that early and sensitive indicate the effect of this drug on plants), and (iii) content of naproxen and products of its metabolic transformation in root biomass.

2. Materials and methods

2.1. Chemicals

All chemicals used were obtained from Sigma-Aldrich, USA, unless noted otherwise.

2.2. Naproxen preparation

Naproxen sodium salt (NPX) was dissolved in a mixture of acetone and distilled water in ratio 1:9 (v:v). The stock solution was delivered to a nutrient solution (pH 5.5 ± 0.1; Reid and York, 1958) to final concentrations simulating low environmentally realistic (0.1–0.5 mg/L; López-Pacheco et al., 2019) to higher (1–10 mg/L) loading. Used acetone concentration (max. 0.23 g/L in 10 mg/L NPX) was proved not to affect the growth of various plant species (Kummerová et al., 2016). However, its level was set equal in all treatments, including control.

2.3. Plant material and experimental design

The plant material used was pea (Pisum sativum L., cv. Zázrak). After 3 days of seed germination seedlings were delivered to the dishes with granulated polyethylene and distilled H2O. After next 4 days, the seedlings were transplanted into cultivation vessels with a nutrient solution without (control; 0 mg/L) or with NPX and cultivated for 10 days. The cultivation was done under controlled conditions (23 ± 1 °C, relative air humidity 60%, photoperiod 14/10, and irradiation up to 300 μmol/m²/s of photosynthetic active radiation – PAR provided by white metal-halid lamps, Kanlux, Czech Republic).

2.4. Growth and plant analysis

Dry weight was determined according to Zezulka et al. (2019). Root systems of five plants were acquired by a flatbed scanner, and pictures were analysed using RootNav software (Pound et al., 2013) for determining the length of primary root, total and average length of lateral roots and their number.

For anatomic studies, fresh pea roots were fixed in FAA solution (mixture of 100% acetic acid, 40% formaldehyde, and 50% ethanol, 1:1:18, v:v:v). Primary root tip, a zone of maturation (unbranched part of root close to the first appearance of lateral roots), and zone of lateral roots were embedded in Steedman wax (Vitha et al., 2000). Sections (thickness 15 μm) were stained by toluidine blue. Photos were processed in RootScan analysis software (Burton et al., 2012). The following parameters were evaluated: section, cortex and stele areas and their ratios, xylem area, and xylem ratio in the...
2.5. Hydrogen sulphide, hydrogen peroxide, superoxide anion radical, and total thiols

Hydrogen sulphide, hydrogen peroxide, superoxide anion radical, and the content of total thiols were determined spectrophotometrically according to the methodology described in Zezulka et al. (2019).

2.6. Lipid peroxidation and total antioxidant capacity

Lipid peroxidation was spectrophotometrically assessed as malondialdehyde content (MDA) according to Zezulka et al. (2019). Total antioxidant capacity was measured using Total Antioxidant Capacity Assay kit (Sigma-Aldrich, USA) and expressed in mmol of Trolox Equivalents according to manufacturer’s instructions.

2.7. Soluble proteins and enzyme activities

Amount of soluble proteins, the activity of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) were determined according to the methodology described in Zezulka et al. (2019).

2.8. MTT and TTC assays and a loss of the cell integrity

The dehydrogenase and oxidoreductase activities were evaluated spectrophotometrically in MTT and TTC assays according to Babula et al. (2014). Changes in the plasma membrane integrity were analysed using Evans Blue reagent (Babula et al., 2014).

2.9. Determination of NXP and its metabolites

After lyophilisation, the extraction of samples was performed according to Bartha et al. (2014) with HCl/acetonitrile (1:1, v/v) mixture into a volume of 4 ml. The extract was analysed by HPLC-HRMS (Dionex Ultimate 3000 with Hypersil Gold column (150 mm × 2.1 mm, 3 μm)), and LTQ Orbitrap XL with HESI II source (all Thermo Fisher Scientific, USA) in negative polarity mode. Acetonitrile (A) and water with 0.1% of acetic acid (B) were used as mobile phase in following gradients: 0–5 min 10% A + 90% B, 5–20 min 10–90% A, 20–25 min 90% A + 10% B, and then washing back to 10% A + 90% B. The quantification of NXP was performed by external standard calibration. NXP metabolites were identified based on their m/z value and literature sources. Their relative abundance was calculated as a ratio of peak area of one metabolite and sum of all peak areas of NXP and its metabolites.

2.10. Statistics

For statistical evaluation, the software STATISTICA (StatSoft, Inc.;®) was used. In all assessed parameters, the resulting data represent a mean over at least five replications. The significance of the difference was evaluated by the one-way analysis of variance after preceding verification of data normality and homogeneity of data variance (ANOVA, P < 0.05). The comparison of means was made by the Tukey HSD range test.

3. Results and discussion

3.1. Root system growth and morphology

The root biomass can be a reliable external indicator of the internal affection of plant metabolism. Fig. 1A presents that while the growth of pea roots, which were in direct contact with contamination, was limited already under 0.1 mg/L (dry weight lowered by 6% as compared to control), the above-ground part of plants was reduced only by 10 mg/L NXP (by 15%). In contrast, reduced growth of roots in pea under diclofenac (DCF) and paracetamol (PCT) treatments (Zezulka et al., 2019) or in Arabidopsis thaliana treated by NXP (Landa et al., 2018) was found only under higher concentrations (from 5 mg/L and above). Remarkably, under 0.1 mg/L NXP, which can be found in the natural environment (López-Pacheco et al., 2019), the limited growth of roots expressed as a decrease (by 7%) in ratio of root and shoot dry weights (Root-to-shoot ratio, RSR, Fig. 1A) did not affect the growth of shoot.

NXP affected the length of roots. Whereas the biggest total length of roots was found under 0.5 mg/L NXP (by 30% longer than in control), under 1 and especially 10 mg/L NXP its value decreased (by 6 and 80%, respectively; Fig. 1B). This fact relates to changes in both the length of primary root and the number and length of lateral roots (Fig. 1C). The growth of primary and lateral roots is controlled by different mechanisms. We suppose that the increased length of lateral roots found in plants exposed to low NXP concentrations (0.1–1 mg/L; Fig. 1C) could represent one of the adaptations of plants stressed by the exposure to low contamination level. The changes are well visible on root system photographs (Fig. 2). The most substantial effect is evident in pea roots under 10 mg/L treatment simulating a high level of contamination, where mainly the lateral roots were the shortest (by 50%, probably due to the inhibition in cell division as well as elongation) and their number was the lowest (by 36%) as compared to control and other NXP treatments. The occurrence of NXP in the environment and in root tissues (Fig. 1D) reduced the number of lateral roots already from 0.1 mg/L NXP. This finding is in contrast with Baidygova et al. (2005) or Kummerová et al. (2013), who found an increased formation of lateral roots when certain levels of the environmental loading by polycyclic aromatic hydrocarbons (PAHs) inhibited the primary root growth. Formation of lateral root primordia depends on the division of founder cells in pericycle and the formation of lateral root meristem, which can be affected by NXP.

Similarly, specific root length (SRL; Fig. 1B) proved a formation of shorter thick roots under 10 mg/L NXP (values lower by 75% than in control) and longer thin roots especially under 0.5 mg/L NXP (higher by 65%). Possible short-term stimulation effect of low concentrations of xenobiotics, in this case drug NXP, was also proved by e.g. Wild et al. (1992) or Kummerová et al. (2013) under different organic pollutants. It is evident that changes in root system morphology (especially the development of lateral roots), caused by drugs occurring in the environment, can play an important role in both water and nutrient uptake and resistance to the stress conditions.

Lateral roots appear in some distance from primary root tip (Fig. 2) and especially under high treatment (10 mg/L NXP) the shortening of non-branched part of the primary root and the earlier formation of lateral roots was apparent. The distance changes in the dependence of both endogenous (phytohormones, especially the balance between auxin and cytokinins; Casimiro et al., 2001; Reed et al., 1998; Stepanova et al., 2007) and exogenous factors (temperature, water and nutrient availability, salinity or the presence of toxic compounds; Arduini et al., 1994; Pellerin and Taboure, 1995; Soukup et al., 2002). Their effects result in alterations in the activity of primary root apical meristem (RAM), root elongation, and timely differentiation of tissues. The decrease in mitotic activity could be related to the oxidative stress and subsequent apoptotic changes in cells evoked by drugs (Strobodniková et al., 2019). Similar changes were recorded in RAM of Brassica campestris L. seedlings treated by monoterpenoids (Nishida et al., 2005), or in Pisum sativum exposed to ozone (Bambridge et al., 1995).
3.2. Primary root anatomy

Anatomical study of primary root was performed with an accent on the detailed assessment of root tip, zone of maturation, and the zone of lateral roots formation. Besides the portion of cortex and

Fig. 1. Dry weight (g) of roots and shoots and root-to-shoot dry weight ratio (RSR; A), total length of roots (cm) and specific root length (SRL, cm/g dry weight; B), and length of primary root (cm), average length of lateral root (cm), total number of lateral roots (C), and the naproxen content (μg/g dry weight) and bioconcentration factor (BCF, r.u.) (D) in Pisum sativum cultivated in nutrient solution with NPX (0, 0.1, 0.5, 1, and 10 mg/L). Data represent mean over five repetitions. Standard deviations are indicated by errors bars. Different letters above the neighbouring columns show statistically significant differences at P < 0.05 (ANOVA, Tukey HSD test).

Fig. 2. Photos of root systems of Pisum sativum cultivated in nutrient solution with naproxen (NPX; 0, 0.1, 0.5, 1, and 10 mg/L). White bar = 5 cm.
stele, attention was paid even to the vascular bundle, especially its xylem part. Results presented in Table 1 prove that while the cross-section area in root tip zone was smaller by 29% and 26% as compared to control under 0.1 and 0.5 mg/L NPX, respectively, a considerable thickening (visible even by the naked eye) by up to 22% was found under 10 mg/L (Fig. 3A). When pea plant was exposed to PAH fluoranthene, the thickness of its primary root in this zone increased in a good correlation with the increasing concentration of PAH (Kummerová et al., 2013).

Naproxen treatment (0.1–10 mg/L) did not cause any changes in the cross-section area of root maturation and the number of cell layers in the primary cortex was more or less constant. Considerable increase in stele area portion (by 13%) as compared to cortex recorded under 10 mg/L NPX is in good agreement with results stated by Kummerová et al. (2013).

In the zone of lateral roots formation, the cross-section area decreased with increasing treatment level (by up to 18% under 10 mg/L NPX as compared to control). Even the ratio of cortex to stele area was affected, it was higher only under 0.1 and 0.5 mg/L NPX by 10 and 21%, respectively. As shown in Table 1 and visualised in Fig. 3, both the xylem area in the zone of root maturation and its portion in stele was higher in all NPX treatments (by up to 150% and 134% under 10 mg/L, respectively; Fig. 3B). These results prove an earlier stress-induced differentiation of root conductive tissue as presented e.g. by Banon et al. (2004) in roots of Lotus cisticus L. during exposure to drought stress, or by Kummerová et al. (2013) in pea roots treated by PAH fluoranthene. The question is, to what extent this variation in the xylem area is connected with another factor - alterations in growth regulators level, especially auxins (Stepanova et al., 2007). Conversely, in the zone of lateral roots formation, the area of both stele and xylem in NPX-treated roots was smaller as compared to control (by up to 32% under 1 mg/L), but the portion of xylem in stele was more-or-less equal. Interestingly, even though 10 mg/L NPX is quite high concentration, no occurrence of malformed root primordia in the zone of lateral root formation as compared to another organic pollutant fluoranthene was found (Kummerová et al., 2013).

3.3. Biochemical traits

Growth, developmental, structural and morphological changes arise from alteration in biochemical processes running on various levels from subcellular to whole organs levels. Increased accumulation of Evans blue in root tissues treated by NPX (by 89% under 10 mg/L; Fig. 4B) prove the damage of cell membrane and change of its permeability probably because of the lipid peroxidation (increase in content of MDA product by 32%; Fig. 4C) as recorded even by Zezulka et al. (2019) for P. sativum and Zea mays under DCF and PCT treatments. The question is if drugs like NSAID and products of their metabolism (Table 2) are able to affect cell membranes directly via direct damage, or their harmful effect is mediated by oxidation stress and ROS. Our results demonstrate that increased total antioxidant capacity (TAC) and activities of antioxidant enzymes at 0.1 and 0.5 mg/L NPX (TAC by up to 42%, APX by 18%, CAT by up to 34%) may to some extent eliminate the harmful effect of the ROS. At higher loads NPX (1 and 10 mg/L) are these mechanisms deficient and their antioxidant capacity may be partially depleted (TAC decrease by up to 26%, SOD decreased by 12%, and APX decreased by 53% as compared to control; Figs. 5 and 6). This fact is supported by increased production of ROS, in our case hydrogen peroxide (by 33%) and superoxide (by up to 62% as against control) under 10 mg/L NPX (Fig. 5A and B), which are no longer sufficiently eliminated by antioxidant systems. This fact is well evident at highest NPX load, 10 mg/L, where activities of CAT, SOD, and APX dropped below the level of control. However, it is always necessary to consider their production on the one hand and their elimination on the other. Changes in antioxidant mechanisms also corresponded with H2S, which informs about the redox conditions of the cells and plays pivotal roles in plant responses or adaptation under stress conditions (Jin and Pei, 2015). Increase in H2S in the lowest concentrations (0.1 and 0.5 mg/L by 10 and 37% as compared to control, respectively) was followed by a sharp decline in the highest concentrations (1.0 and 10.0 mg/L by 22 and 59%, respectively; Fig. 5E). Both H2S and total thiols, which were reduced (at 1 and 10 mg/L for 10 and 14%, respectively, see Fig. 5E) are involved in redox status of the cell and, in addition, they also influence production of ROS, mainly by NADPH oxidases (Go and Jones, 2013). It is not surprising that amount of cysteine moieties (mainly in proteins) is very important factor influencing not only redox status, but also physiological processes as well as cell surviving or cell death. Production of ROS and changes in thiol status were accompanied by reduced activities of dehydrogenases (MDT) and oxidoreductases (TTC) - by 42% for MTT and 22% for TTC at highest load; Fig. 6A and B). This fact supports the theory about damage of cells of roots by ROS. Glutathione as one of the most abundant low molecular weight thiol compound synthesized in cells also plays critical role in toxicity of xenobiotic electrophiles and their detoxification. Study of Huber et al. (2009) revealed mechanism of PCT

| NPX (mg/L) | Area (mm²) | Section | Cortex | Stele | Cortex/Stele | Cortex/Section | Stele/Section |
|------------|------------|---------|--------|-------|--------------|---------------|--------------|
| Root tip   |            |         |        |       |              |               |              |
| 0          | 0.22 ± 0.01 |         |        |       |              |               |              |
| 0.1        | 0.14 ± 0.01 |         |        |       |              |               |              |
| 0.5        | 0.14 ± 0.02 |         |        |       |              |               |              |
| 1          | 0.23 ± 0.01 |         |        |       |              |               |              |
| 10         | 0.34 ± 0.01 |         |        |       |              |               |              |
| Zone of maturation | 0.85 ± 0.04 | 0.68 ± 0.05 | 0.17 ± 0.01 | 399.9 ± 27.0 | 79.8 ± 1.8 | 20.2 ± 1.8 | 5.6 ± 1.2 | 3.3 ± 0.3 |
|            | 0.91 ± 0.06 | 0.76 ± 0.05 | 0.15 ± 0.01 | 516.5 ± 35.8 | 83.7 ± 1.0 | 16.3 ± 1.0 | 8.7 ± 2.0 | 5.4 ± 0.5 |
|            | 0.82 ± 0.05 | 0.66 ± 0.05 | 0.16 ± 0.00 | 416.4 ± 27.0 | 80.5 ± 1.0 | 19.4 ± 1.0 | 11.0 ± 1.8 | 7.0 ± 0.6 |
|            | 0.79 ± 0.04 | 0.64 ± 0.04 | 0.15 ± 0.02 | 419.9 ± 29.6 | 80.7 ± 1.1 | 19.3 ± 1.1 | 9.3 ± 0.6 | 5.4 ± 0.4 |
|            | 0.78 ± 0.02 | 0.58 ± 0.02 | 0.20 ± 0.02 | 297.2 ± 26.2 | 74.5 ± 2.7 | 25.5 ± 1.3 | 18.1 ± 1.1 | 9.0 ± 0.7 |
| Zone of lateral roots | 1.70 ± 0.07 | 1.31 ± 0.06 | 0.39 ± 0.03 | 336.7 ± 31.3 | 77.0 ± 1.6 | 23.0 ± 1.6 | 50.1 ± 5.9 | 11.1 ± 0.1 |
|            | 1.65 ± 0.03 | 1.34 ± 0.08 | 0.31 ± 0.04 | 446.4 ± 41.7 | 81.1 ± 3.6 | 18.9 ± 1.7 | 32.6 ± 2.1 | 11.0 ± 0.4 |
|            | 1.68 ± 0.01 | 1.38 ± 0.05 | 0.30 ± 0.02 | 481.6 ± 34.7 | 82.3 ± 3.2 | 17.7 ± 1.4 | 40.8 ± 2.6 | 12.0 ± 0.4 |
|            | 1.44 ± 0.03 | 1.12 ± 0.04 | 0.32 ± 0.02 | 354.7 ± 30.8 | 78.0 ± 1.5 | 22.0 ± 1.5 | 28.9 ± 1.4 | 10.4 ± 0.6 |
|            | 1.24 ± 0.09 | 0.99 ± 0.07 | 0.25 ± 0.03 | 389.9 ± 18.7 | 79.1 ± 3.3 | 20.9 ± 1.7 | 34.0 ± 1.4 | 12.0 ± 0.2 |

Data represent mean ± standard deviation over five repetitions. Different letters mark statistically significant differences at P < 0.05 (ANOVA, Tukey HSD test).
detoxification, which includes creation of glucose or glutathione conjugates. This may relate to changes observed in H$_2$S and total thiols. Finally, even though almost all studies were performed in animal or human models, amount of H$_2$S may increase the synthesis of glutathione, which contribute to detoxification of PCT (and xenobiotics in general) (Parsanathan and Jain, 2018).

3.4. Content of NPX and products of its metabolization

The content of NPX detected in pea roots biomass and calculated

![Fig. 3. Photos of cross-sections in primary root tip (A), zone of maturation (B), and zone of lateral roots formation (C) in Pisum sativum cultivated in nutrient solution with naproxen (NPX; 0, 0.1, 1, and 10 mg/L).](image1)

![Fig. 4. Content of proteins (mg/g FW; A), accumulation of Evans Blue (control = 100%; B), and content of malondialdehyde (MDA; nmol/g FW; C) in Pisum sativum cultivated in nutrient solution with naproxen (NPX; 0, 0.1, 0.5, 1, and 10 mg/L). Statistical evaluation as given in Fig. 1.](image2)
bioconcentration factor (BCF; Fig. 1D) demonstrated that the roots of plants accumulated certain amounts of NPX concerning contamination degree (nearly up to 10 μg/g DW under 10 mg/L NPX). Bioaccumulation is a non-linear process; i.e., bioconcentration factors, indicating the ratio of compound content in fresh mass and its initial concentration in the cultivation medium, are generally high at low concentrations and decrease with increasing environmental loadings (Kummerová et al., 2016). Results given in Fig. 1D have proved this fact as well, showing a nearly four-times higher BCF value found under 0.1 mg/L NPX compared to 10 mg/L.

Besides the intact NPX form even its metabolites were detected in plant root biomass. Metabolic transformation of drugs usually includes hydroxylation, dehydrogenation, or methylation, and conjugation (Klampfl, 2019). Table 2 shows the NPX metabolites of known structures reported by literature sources (Ding et al., 2017; Emhofer et al., 2017; Fu et al., 2017) which were also confirmed by LC-MS based on the same fragmentation patterns. As expected, the
biggest portion of intact NPX and also the highest number of its metabolites including the conjugate with alanine and threonine were found in pea roots grown in medium containing 10 mg/L NPX. The relative abundance of metabolites decreased with the increasing NPX treatment excepting NPX-GLC and two isomers of DMNPX-OH (Table 2). In contrast to Fu et al. (2017) who stated that the main NPX conjugates in Arabidopsis are formed with amino acids, only three NPX conjugates with amino acids were detected in our experiments with pea. The most abundant conjugates were NPX-GLC and NPX-GLC-MAL, which is in agreement with Emhofer et al. (2017).

4. Conclusion

The understanding of the effect of drugs and products of their metabolic transformation on biochemical and physiological processes is essential for explanation of changes in growth and development of plant growing in contaminated environment. As this study proves, enhanced formation of ROS, lipid peroxidation, loss of the plasma membrane integrity, and changes in antioxidant and redox systems modulated the pea root growth and structure in response to increasing NPX treatment. Furthermore, the documentation of anatomical and morphological changes to which no appropriate attention has been devoted yet, shows, that the alterations on subcellular level especially under high treatments express to the level of tissues and organs.

CRediT authorship contribution statement

Lucie Svobodníková: Investigation, Writing - original draft, Visualization. Marie Kummerová: Conceptualization, Writing - original draft, Visualization, Supervision. Stepan Zezulka: Methodology, Formal analysis, Writing - original draft. Petr Babula: Investigation, Writing - original draft. Katarína Sendecká: Investigation, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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