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Instability of chlorophyll in yellow lupin seedlings grown in soil contaminated with ciprofloxacin and tetracycline

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HIGHLIGHTS

- The deteriorative effect of antibiotics on chlorophyll was measured in lupin seedlings and in a simplified *ex vivo* system.
- The order of chlorophyll breakdown reaction and the reaction constant were determined.
- The relation was established between the content of chlorophyll in plants and soil cyprofloxacin or tetracycline.

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ABSTRACT

With increasing soil concentrations of ciprofloxacin and tetracycline a decrease of leaf chlorophyll content was observed. Tetracycline was more detrimental than ciprofloxacin. The chlorophyll content in plants growing for ten days on a tetracycline containing soil decreased by 68%. The decrease of chlorophyll concentration was even sharper in new leaves that formed after application of the antibiotic (up to 81% drop). The comparison of absorption spectra of commercial, reagent grade chlorophyll, alone and incubated with antibiotics, has shown that ciprofloxacin and tetracycline can react directly with chlorophyll and decrease its concentration by 47.7% and 48.5%, respectively. The changes in fluorescence spectra confirmed the formation of chlorophyll degradation product. The chlorophyll decay was a second order reaction and depended on antibiotic concentration and duration of exposure. Reaction rate constants differed with antibiotics and their soil concentrations. With increasing contents of antibiotics in soil the constant of chlorophyll degradation rate in lupin plants increased from $k = 870\ M^{-1}day^{-1}$ for 3 mg ciprofloxacin to $k = 2490\ M^{-1}day^{-1}$ for 90 mg ciprofloxacin, and in the case of tetracycline the reaction rate constant increased from $k = 1330\ M^{-1}day^{-1}$ to $k = 2910\ M^{-1}day^{-1}$. The sensitivity of chlorophyll to ciprofloxacin and tetracycline was confirmed by determining EC and TU indices.

1. Introduction

Substances derived from solid and liquid industrial and municipal wastes, as well as agricultural fertilizers and plant protection products are the major pollutants of soils (Wuana and Okieimen, 2011). Pharmaceuticals excessively used medicine are an emerging new kind of environmental pollutants. Antibiotic use in animal production is even more extensive. The pharmaceuticals are not completely metabolized in human and animal organisms and they are excreted and contaminate soils and water (Zhang et al., 2015; Calamariet al., 2003). In European Union 9000 tons antibiotics were used in 2014 of which 3000 tons were used in Spain, 1400 t. in Italy, 1400 t. in Germany, 779 t. in France and 581 t. in Poland (EMA, 2016). The highest use of antibiotics worldwide has been in India, China and USA (CDDE, 2015). In 2010 at least 63 200 tons antibiotics were applied to animals, by far more than used in human medicine (Van Boeckel et al., i in., 2015).

A large amount of the applied antibiotic dose (often as much as 70%) passes to faeces and urine completely unmodified or transformed into biologically active metabolites (Winckler and Grafe,

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Abbreviations

Chl	chlorophyll
rgChl	reagent grade chlorophyll
CIP	ciprofloxacin
EC	effective concentration
TC	tetracycline
TU	toxicity units
VAs	antibiotics of veterinary

2001). There are a few routes for antibiotics to enter the natural environments: surface runoff from large animal production farms, contaminating surface waters (Pedersen et al., 2005), waste by-products of pharmaceutical industry (Kümmerer, 2009), the agricultural use of sediments and processed water from wastewater treatment plants (Barber et al., 2009; Lapworth et al., 2012; Navon et al., 2011) and agricultural use of manure and slurry from animal production farms applying antibiotic treatments (Kemper, 2008). Tetracyclines, penicillins and sulfonamides are the antibiotics most frequently used in veterinary medicine and their share in total antibiotic use is approximately 33, 25 and 11%, respectively (EMA, 2016). These substances have been detected nearly all over the world as environmental pollutants – in Africa, America, Europe, Asia. Their wide occurrence results both from their massive use, and their incomplete removal in wastewater treatment systems. In China 16000 tons of antibiotics were applied in 2010 (CDDE, 2015). Between 2000 and 2010, consumption of antibiotic drugs increased by 36% (from approx. 50 billion standard units to over 70 billion standard units). A large part (76%) of this increase was caused by a rise in antibiotic use in Brazil, Russia, India, China, and South Africa (Van Boeckel et al., 2015). The predictions of further growth in antibiotic use worldwide are disturbing and it is estimated that this number will increase by 2030 to 105 600 tons, and China only will account for 30 tons annual usage (CDDE, 2015).

Antibiotics after entering the environment retain their antibacterial properties (Chander et al., 2005). It was found that only 50% of the TC in manure is degraded after 5 months, and it may be detected in soil for periods of several months to years (Hamscher et al., 2005; Rosendahl et al., 2012). Moreover, tetracyclines are characterised by a strong and durable adsorption to soil particles and are resistant to biodegradation (Huang et al., 2013). Fluoroquinolones (FQ) are another group of frequently detected contaminants (Speltini et al., 2010). They are fairly modern synthetic antibiotics with strong bactericidal activity. Ciprofloxacine (CIP) is one of the most often applied FQs. Thank to its broad range of antibacterial activity, it is commonly applied in human and veterinary medicine, however up to 72% of the applied dose can be excreted from the organism in an unchanged form (Daughton and Ternes, 1999).

The uptake of environmental pharmaceuticals by plants has been described by many researchers (Boxall et al., 2006; Calderon-Preciado et al., 2013; Dodgen et al., 2013; Dolliver et al., 2007; Herklotz et al., 2010). It results in plant yellowing and the decrease in leaf chlorophyll content (Lin et al., 2013). The reduction in photosynthetic pigment levels is, in addition to biomass indices, the recommended measure of environmental contamination (US EPA, 2012; ISO, 2006; OECD, 2006). The reason of decrease in chlorophyll content in antibiotic affected plants is unknown. In our research we attempted to establish if chlorophyll decay may result from direct action of antibiotic on this pigment. Moreover, we estimated the stability of chlorophyll and the general plant health condition in yellow lupin seedlings grown in soil contaminated with CIP and TC. Indices of CIP and TC toxicity towards lupin were determined.

2. Materials and methods

2.1. Seedling growth and development

Yellow lupin (Lupinus luteus L. cv. Dukat) seeds were germinated on filter paper for five days, next they were transferred to pots (500 pots with one seedling each) containing 150 ml of perlite (size 2–6 mm; Agro Perlite, Poland). Plants were grown at 19/23 °C night/day temperature with 16 h/day illumination provided by sodium lamps (3 kLx). Each pot was watered every day with 15 ml deionised water. On the 20th day a uniform sample of 300 plants were selected, which had similar size (18–20 cm tall). On the same day aqueous solutions (15 ml) of TC and CIP were added in a single dose to the pots, so as to obtain the following concentrations: 3, 9, 15, 30 and 90 mg $\times$ kg^{-1} of soil. After application of the antibiotics plants were not watered for one day, next they were watered with 15 ml deionised water every day. Seedlings watered with deionised water, but not with antibiotics, were used as controls. Plant material for the first round of analyses was collected 3, 5, 7, and 10 days after antibiotic treatment. Within 10 days on plants grown in antibiotic contaminated soil two to three leaves developed. These leaves were collected and analysed as an additional experimental sample (described below as young leaves). Fresh and dry weight were determined according to ISTA (2011).

2.2. Activity of guaiacol peroxidase

Plant extracts were prepared on ice. The shoot samples (500 mg from five seedlings each) were collected from plants which had grown for 3, 5, 7 and 10 days on soil with an addition of deionised water, CIP or TC and they were homogenised for 30 min in an extraction buffer (0.1 M Tris-HCl Sigma-Aldrich, 8.75% polyvinylpyrrolidone Sigma-Aldrich, 0.1 M KCl, 0.28% Triton X-100 Sigma-Aldrich). The extracts were centrifuged for 30 min at 4000*g* at 4 °C. The supernatant was purified with membrane syringe filters with a pore diameter of 0.45 μm. Protein in the samples was assayed by the Lowry et al. method (1951). The activity of peroxidase was determined with a spectrophotometer (CECLI, CE2021 2000 SERIES) in the reaction mixture containing 100 μl of 1% guaiacol (Sigma-Aldrich), 2 ml 0.1 M KH_2PO_4 (Chempur), 50 μl of the supernatant and 20 μl 0.06% H_2O_2 (Chempur). The absorbance growth rate was measured at room temperature at the wavelength of 470 nm. One unit corresponds to oxidation of 1 μmole guaiacol by within 1 min.

2.3. Activity of catalase

Plant samples were collected as described above and were homogenised in phosphate buffer solution pH 7, which contained 10 g $\times$ L^{-1} polyvinylpyrrolidone (PVP, Sigma-Aldrich), 0.2 mM EDTA (Sigma-Aldrich), 10 ml $\times$ L^{-1} Triton X-100 (Sigma-Aldrich). The samples were centrifuged for 20 min at 12000$\times g$ at 4 °C. The supernatant was then carefully separated from the sediment. Protein content of the samples was determined as described above. The activity of catalase was determined spectrophotometrically in a reaction mixture containing 50 mM phosphate buffer, pH 7 and 15 mM H_2O_2. The absorbance was measured for 10 min at room temperature at the wavelength of 240 nm. One unit corresponds to a reduction of 1 μmole H_2O_2 by within 1 min.