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Poplar and diclofenac pollution: A focus on physiology, oxidative stress and uptake in plant organs

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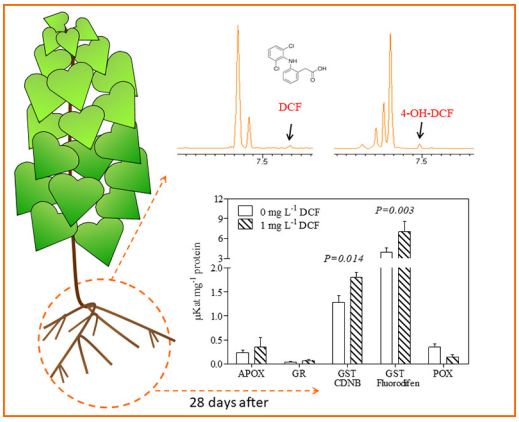
HIGHLIGHTS

- *Populus alba* L. Villafranca clone was successfully tested for removal of diclofenac from water.
- LC-MS/MS detected Diclofenac and 4-OH-Diclofenac metabolites in poplar roots.
- Stress enzymes response is involved in maintaining poplar healthy physiological traits.

GRAPHICAL ABSTRACT



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ABSTRACT

Poplar plants may have an important role in the removal of pharmaceuticals from contaminated waters. In this context, plant uptake of the non-steroidal anti-inflammatory drug diclofenac, as well as physiological response in terms of growth traits and stress enzymes activity was assessed in *Populus alba* Villafranca clone, in order to establish the effectiveness of this species against pharmaceutical active compounds pollution.
This evaluation was conducted in mesocosms with 1 mg L^{-1} of this pharmaceutical for a maximum period of 28 days. Root appears to be the organ with clear uptake of diclofenac (14.76 ± 2.42 ng g^{-1} fresh weight after 1 day of treatment), and presence of products derived from its metabolism. Indeed, 4-OH-diclofenac metabolite was detected in root tissues, indicating diclofenac uptake and metabolism inside the plants, already after 1 day of treatment.
Regarding enzyme activities, glutathione-S-transferases increased in roots after long-term exposure to diclofenac, while an increase in activity of ascorbate peroxidase and glutathione reductase was detected in short and medium-term exposure, as a result of abiotic stress caused by diclofenac. Results suggest the ability of poplar to actively participate in the removal of diclofenac from water when used for phytoremediation purpose.

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1. Introduction

The continuous release of pharmaceutical active compounds (PhACs) into the aquatic environment (Zhang et al., 2014), aggravated by evidences that traditional water treatment plants are not able to achieve the complete removal of these substances (Luo et al., 2014; Schröder et al., 2016), have pose the attention of scientific and public opinion on the relevance of environmental effects caused by PhACs.

Among PhACs, diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl) acetic acid) (DCF) is a non-steroidal anti-inflammatory drug widely used as painkiller across Europe, reaching consumption doses up to 1033 mg per inhabitant per year in Germany (Schröder et al., 2016). The DCF concentrations in municipal wastewaters vary greatly (from ng to mg L^{-1}) between and also within countries, with significantly higher concentrations in hospital or manufacturers wastewaters (Heberer, 2002). A focus on waste water treatment plant effluents reveals that the average European DCF concentration is 49.5 ng L^{-1}, with the highest being 174 ng L^{-1} (Loos et al., 2013). The removal of this PhAC from wastewater has been reported to be extremely variable; Luo et al. (2014) reported a removal efficiency in a range of 0–81%, with an average removal of 35.8%. Among the characteristics that make DCF removal extremely variable, a key role could be associated to its sensibility to photodegradation, since this molecule has been reported to have a half-life of 3 to 10 days (Zhang et al., 2011, 2012; Matamoros et al., 2012) under UV radiation.

Although photodegradation process plays an important part in removing DCF from polluted water new study need to deepen and develop methods to understand the process of biodegradation and therefore improvement of its removal.

Ecological concern about diclofenac arose in the early 2000's, when Oaks et al. (2004) established that the decline of vulture population in India was associated to the widespread use of diclofenac for cattle treatments.

Negative effects of DCF have been observed on aquatic organisms such as loss of cell membrane integrity in duckweed (Kummerová et al., 2016) and tissue-specific oxidative stress in mussels (Gonzalez-Rey and Bebianno, 2014). Accumulation of DCF and its metabolites has been reported in fishes, together with enhanced activity of enzymatic antioxidant systems (Islas-Flores et al., 2014; Guiloski et al., 2015). Regarding terrestrial species, Chen et al. (2015) observed reduction of survival and fertility in soil arthropods.

As concerns plant uptake and metabolism of DCF, several studies using herbaceous species such as *Scirpus* and *Typha* in mesocosms have been published (Zhang et al., 2012; Bartha et al., 2014), as well as in pots with alfalfa (Christou et al., 2016). *Scirpus validus* has been noticed to be tolerant to concentrations up to 2 mg L^{-1} (Zhang et al., 2012), while oxidative stress responses of the antioxidant enzymes peroxidases, ascorbate peroxidase, and glutathione reductase were observed in *Typha latifolia* (Bartha et al., 2014) and *Medicago sativa*, together with an increase of lipid peroxidation (Christou et al., 2016).

Metabolism of diclofenac has been extensively studied in humans at the stage of discovery and development prior to commercialization (Todd and Sorkin, 1988) while in comparison, little is known about their metabolism in plants. It has been previously demonstrated in herbaceous species that after entering the plant cells, DCF undergoes rapid metabolism, according to the "green liver" model pathway (Sandermann Jr, 1994). The main metabolites deriving from Phase I (activation) and Phase II (conjugation) of plant metabolism were found to be the hydroxylated forms 4-OH-diclofenac and 5-OH-diclofenac and the conjugated forms 4-O-glucopyranosyloxydiclofenac and 4-OH-glutathionyldiclofenac (Huber et al., 2012; Bartha et al., 2014). Moreover, formation of the highly reactive compound diclofenac-2,5-iminoquinone, due to plant peroxidases activity, has been demonstrated (Huber et al., 2016).

Recently DCF has been used as a model compound to unravel its metabolism pathways in *Arabidopsis thaliana* cells (Fu et al., 2017), but not information is actually available on DCF uptake and metabolism in poplar species.

Poplar plants are widely recognized for their great ability to remove contaminants such as heavy metals and organic xenobiotics from soils and waters (Marmiroli et al., 2011). The high transpiration, the deep root system, and the fast growth rate, make poplars an efficient tree in phytoremediation (Capuana, 2011; Pilipović et al., 2015). Moreover, the capability of poplar to absorb and metabolize organic pollutants such as ibuprofen, caffeine and erythromycin has been recently investigated (Iori et al., 2012; Pierattini et al., 2016a, 2016b). Up till now the DCF ability to affect tree growth is poorly understood.

Taking into account the above mentioned studies and poplar characteristics, the study aims to test the success of *Populus alba* L. Villafranca clone to uptake and metabolize DCF. To determine stress effects on plants the responses of the antioxidant enzymes peroxidase, glutathione-S transferases, ascorbate peroxidase and glutathione reductase were recorded over time during 28 days of DCF exposure.

2. Materials and methods

2.1. Plant material and treatments

Populus alba L. Villafranca clone plantlets from *in vitro* culture (Pierattini et al., 2016a) were acclimated (two weeks) to *in vivo* conditions in pots filled with perlite irrigated with Hoagland's solution in a growth chamber (23:18 °C day:night temperature; 50% relative humidity; 14 h photoperiod at 400 μmol m^{-2} s^{-1} photosynthetic photon flux density, supplied by fluorescent lights) before being put in mesocosms (Cui et al., 2015; Zhang et al., 2012) irrigated with Hoagland's solution (Arnon and Hoagland, 1940) at pH 6.8, using perlite as plant support substrate, under controlled greenhouse conditions (23:18 °C day:night temperature; 63% relative humidity; 14 h photoperiod). The pots were in flooded trays such mimicking the conditions of waterlogging as observed in constructed wetlands. After acclimation to greenhouse conditions (two weeks), homogenously grown plants were selected and randomly subjected to two treatments: 1 mg L^{-1} DCF, and control (0 mg L^{-1}). Treatment concentration was chosen according to previous studies in similar experimental conditions (Allam et al., 2015; Bartha et al., 2014; Zhang et al., 2012). Each plant was supplied with 1 L of either control or DCF spiked Hoagland solution; plants ($n = 5$) were sampled after 1, 7, and 28 days of treatment. For each treatment, the nutrient solution was completely replaced after the second sampling time (7 days) and subsequently every three days. DCF-treated poplar plants sampled 28 days after the starting of the experiment received a total of 8 mg DCF each. A schematic overview of the experiment is reported in SM, Fig. S1.

Position of the plant apex was marked at the beginning of the experiment in order to permit distinction between the leaves developed during the experimental period (new leaves) and the pre-existing ones (old leaves), after 28 days of treatment.

A parallel trial with 1 mg L^{-1} DCF Hoagland solution without poplar plants was set up; samples were collected daily for 7 days to determine the poplar-independent depletion of DCF.

2.2. Growth parameters and photosynthetic pigments content

After 1, 7, and 28 days of treatment, five ($n = 5$) plants for each treatment were harvested, the roots were carefully and thoroughly rinsed with deionised water and dried with filter paper, and the plants were separated into roots, stem, and leaves. Stem length, leaves number, fresh weight (FW) and dry weight (DW - oven-dried at 70 °C for two weeks), were recorded.

Leaf (LMR), shoot (SMR), and root (RMR) mass ratios were calculated as the ratio of the corresponding organ dry biomass to the total plant dry biomass. Height mass ratio (HMR) was calculated as the ratio of stem length on stem dry weight.