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Ametryn removal by *Metarhizium brunneum*: Biodegradation pathway proposal and metabolic background revealed

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HIGHLIGHTS

- Biodegradation pathway of ametryn by the fungal strain is proposed.
- Targeted metabolomics revealed significant changes in microbial metabolism.
- Ametryn and its metabolites induced oxidative stress conditions.

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ABSTRACT

Ametryn is a representative of a class of s-triazine herbicides absorbed by plant roots and leaves and characterized as a photosynthesis inhibitor. It is still in use in some countries in the farming of pineapples, soybean, corn, cotton, sugar cane or bananas; however, due to the adverse effects of s-triazine herbicides on living organisms use of these pesticides in the European Union has been banned. In the current study, we characterized the biodegradation of ametryn (100 mg L^{-1}) by entomopathogenic fungal cosmopolite *Metarhizium brunneum*. Ametryn significantly inhibited the growth and glucose uptake in fungal cultures. The concentration of the xenobiotic drops to 87.75 mg L^{-1} at the end of culturing and the biodegradation process leads to formation of four metabolites: 2-hydroxy atrazine, ethyl hydroxylated ametryn, S-demethylated ametryn and deethylametryn. Inhibited growth is reflected in the metabolomics data, where significant differences in concentrations of L-proline, gamma-aminobutyric acid, L-glutamine, 4-hydroxyproline, L-glutamic acid, ornithine and L-arginine were observed in the presence of the xenobiotic when compared to control cultures. The metabolomics data demonstrated that the presence of ametryn in the fungal culture induced oxidative stress and serious disruptions of the carbon and nitrogen metabolism. Our results provide deeper insights into the microorganism strategy for xenobiotic biodegradation which may result in future enhancements to ametryn removal by the tested strain.

1. Introduction

Pesticides are powerful substances used in modern agriculture for adequate food production. Their use may minimize economic losses, and ensure the quality of crops and their protection against pests, weeds and diseases (Tejada et al., 2011; Peters et al., 2014). But, constant development of agricultural chemistry in the past decades has contributed to increasing pollution of the environment with these xenobiotics residues, especially surface water, groundwater and soil (Peters et al., 2014; Szewczyk et al., 2015; Długoński, 2016).

Triazines are chemical compounds belonging to the wider group of azines, characterized by a structure of a hetero-cyclic ring containing three unsaturated nitrogen atoms. Triazine herbicides are 1,3,5-triazines commonly called s-triazines (Elbashir and Aboul-Enein, 2015). S-triazines are very stable compounds and this allows them to accumulate in the environment for several months to several years in soil or water sediments (Prosen, 2012). Due to their physicochemical properties such as relatively high water solubility, low sorption coefficient or long half-life time, s-triazine pesticides are susceptible to leaching from the soil (Borges et al., 2009; Shah et al., 2011; Sandoval-Carrasco et al., 2013). Additionally, under

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environmental conditions these compounds can undergo slow degradation to water-soluble by-products. These facts points to them as being one of the major risks to aquatic ecosystems (Sandoval-Carrasco et al., 2013; Elbashir and Aboul-Enein, 2015).

Literature data indicate various adverse effects of triazine herbicides on living organisms, including: carcinogenicity, endocrine disrupting activity, neuroendocrine disruptors, growth and size disruptions or the aquatic ecosystems imbalance (Chen et al., 2009; Hayes et al., 2010; Abigail et al., 2013; Liu et al., 2016; Velisek et al., 2017). Due to the adverse effects of s-triazines on the environment, the use of these herbicides in agriculture within the European Union (EU) has been banned – for ametryn from the year of 2002 (Liu et al., 2014). Despite the withdrawal of these pesticides in EU countries, they are still present in water and soil samples, even in areas where their usage is limited or far from agricultural areas (Borges et al., 2009; Allan et al., 2017).

Ametryn is a systemic s-triazine herbicide absorbed by plant roots and leaves and characterized as a photosynthesis inhibitor. It is still in use in some countries in the farming of pineapples, soybean, corn, cotton, sugar cane or bananas to protect against broad-leaved weeds and grass (Grillo et al., 2011; Abigail et al., 2013; Peters et al., 2014). This xenobiotic has been classified by the Environmental Protection Agency (EPA) as a Class III herbicide moderately toxic to fish, large mammals and humans, while being highly toxic to crustaceans and molluscs (Navaratna et al., 2012). In summary, there is a need to develop and characterize effective methods for removing ametryn and other s-triazine residues from the environment.

In the natural environment, s-triazines may be slowly degraded by photolysis, hydrolysis or red-ox reactions (Prosen, 2012; Elbashir and Aboul-Enein, 2015). Ametryn removal may be conducted via UV radiation combined with oxidation with H_2O_2 (Gao et al., 2009); however, the most promising and environmental-friendly solution, as in many cases of other xenobiotics, is the microbiological degradation of the compound. Among the s-triazine herbicides, biodegradation of ametryn is relatively poorly described when compared, for example, to the numerous articles on atrazine removal. The most favorable method of removal is the complete mineralization of ametryn (2 mg L^{-1}) reported for the bacterial strain *Nocardioides* DN36 (Satsuma, 2010). Partial removal of ametryn (1 mg L^{-1} or 31.5 mg L^{-1}) has been described in mixed bacterial cultures and ranges from 46% to 97%, respectively (Navaratna et al., 2012; Sandoval-Carrasco et al., 2013).

Little is known about the mechanisms and pathways of ametryn biodegradation. A few reports note cyanuric acid and 2-hydroxy atrazine as major by-products of ametryn biodegradation in bacterial cultures (Fujii et al., 2007; Sandoval-Carrasco et al., 2013). Regarding cell homeostasis, previous studies have shown that when microorganisms (bacteria, fungi) are exposed to xenobiotics or their metabolites, a significant increase in reactive oxygen species (ROS) generation may occur and, consequently, this may induce an oxidative stress condition (Peters et al., 2014; Szewczyk et al., 2015; Soboń et al., 2016; Bernat et al., 2014).

In this study, we examined ametryn biodegradation and, via a targeted metabolomics analysis, profiled free amino acids and a few other common small molecules. As a result, we proposed the biodegradation pathway and initial description of cellular response to ametryn and its metabolites in the cultures of cosmopolite fungal strain *Metarhizium brunneum* ARSEF 2107.

2. Materials and methods

2.1. Microorganism and culture conditions

Metarhizium brunneum ARSEF 2107 obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures was examined in this study. The fungus was first cultured on ZT agar slants (Bernat et al., 2013) for 7 days at 28 °C and for another 7 days at room temperature. 14-day-old spores obtained from ZT agar slants were used to inoculate 100 ml of the liquid mineral medium (Lobos et al., 1992) containing 2% glucose in 300 ml Erlenmeyer flasks. The cultivation (with conidia density of 5×10^7 mL^{-1}) was performed on a rotary shaker (120 rpm) for 24 h at 28 °C. After 24 h of cultivation, cultures were either supplemented with ametryn at 100 mg L^{-1} concentration, or left without the xenobiotic in the control cultures. Abiotic controls containing the medium and ametryn were also incubated. The cultures were grown for 17 days under the conditions described above. At certain points of time, samples were collected for analysis of for dry weight, pH, ametryn and glucose quantitation, ametryn biodegradation pathway studies and metabolomics.

2.2. Chemicals

Ametryn, PESTANAL® analytical standard (purity 98.5%), atrazine-2-hydroxy, PESTANAL® analytical standard (purity 98.1%) and glucose pharmaceutical secondary standard grade (purity 99.8%) were purchased from Sigma-Aldrich (Germany). Ethanol, acetonitrile (ACN), QuEChERS extraction method ingredients and the high purity solvents used during sample preparation for liquid chromatography tandem mass spectrometry (LC-MS/MS) analyzes were purchased from Avantor (Poland) (minimal of 98% purity). All other chemicals and ingredients used in LC-MS/MS analyzes were of high purity grade (minimal of 99.8%) purity and obtained from Sigma-Aldrich (Germany), Serva (Germany) or SCIEX (USA).

2.3. Dry weight determination

The samples were filtrated on previously weighed Whatman 1 (Sigma-Aldrich, Germany) drains. Mycelium with the drain was dried under 80 °C until constant weight. All samples were analyzed in triplicate.

2.4. Ametryn and its metabolite extractions

Ten milliliters of the culture was homogenized mechanically with the use of FastPrep24 (MP Biomedicals, USA) with glass beads (1-mm diameter), supplemented with terbuthylazine used as an internal standard for LC-MS/MS analyzes and extracted with ACN according to the QuEChERS procedure (Payá et al., 2007; quechers.cvua-stuttgart.de, 2017). QuEChERS ACN extract clean-up with SPE was omitted.

2.5. Glucose sample preparation

Following separation by filtration on Whatman 1 (Sigma-Aldrich, Germany), the culture medium was separated from the mycelium and diluted with water prior to analysis.

2.6. Metabolomics sample preparation

The sample preparation was conducted according to a procedure described previously (Szewczyk et al., 2015) with minor modifications. Briefly, the mycelium separated by filtration on Whatman 1 (Sigma-Aldrich, Germany) was washed with water and weighed into 3 portions of 100 mg and placed into 2-mL Eppendorf tubes containing 1 ml of 90% cold ethanol. Samples were then mechanically homogenized with parallel extraction under cold conditions three times. The samples were then incubated for further extraction 2 h, −20 °C and centrifuged. The supernatant was