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

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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.

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

Keywords

triazines, xenobiotics, pathway, fungi, metabolomics, oxidative stress

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 et al., 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 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 et al., 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 at 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 et al., 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 (Fuji 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 transferred to a new 1.5-mL Eppendorf tube, evaporated until dry under a vacuum and stored at −20°C for future analysis. Frozen samples were thawed at 4°C for 30 min followed by 15 min at room temperature, resuspended in 1 ml of water:ACN 98:2 (v/v), sonicated and vortexed occasionally for 2 min. Finally, the samples were diluted to the working range of the analytical method with water and transferred to a closed glass vials for LC-MS/MS analysis.

2.7. LC-MS/MS analysis

2.7.1. Glucose quantitation

Glucose determination was performed in flow injection analysis mode (FIA) according to a method described previously (Różalska et al., 2010) on an LC-MS/MS system consisting of 3200 QTRAP (AB Sciex, USA) and 1200 HPLC System (Agilent, USA). All samples were analyzed in triplicate. The working range of the method covered 1–10 μg mL^{-1}, r = 0.99892.

2.7.2. Ametryn and atrazine-2-hydroxy quantitation

The analysis was conducted on a 4500 QTRAP mass spectrometer (SCIEX, USA), coupled with a microLC 200 System (Eksigent, USA). The chromatography was performed on an Eksigent Halo C18 column (0.5 × 50 mm, 2.7 μm, 90 Å) with temperature set to 50°C and a total constant flow of 50 μL min^{-1}. The eluent consisted of 0.1% of formic acid (FA) in water (A) and 0.1% FA in ACN (B). Pre injection equilibration lasted for 0.5 min. After injection of 10 μL (2 μL on a column), the gradient separation was conducted as follows: linear increase from 98% A to 98% B after 1.2 min., hold 98% from 1.2 to 2.4 min., reverse to initial conditions from 2.4 to 2.5 min., and hold until the end of the method in 3.0 min. The MS/MS detection was conducted in positive ionization multiple reaction monitoring (MRM) mode. The optimized electrospray ion source (ESI) parameters were as follows: CUR: 35; IS: 5000 V; TEMP: 400°C; GS1: 28; GS2:40 and ihe:ON. Compound-dependent MRM parameters are presented in Table S-1. Quantitation method curves used terbuthylazine as internal standard and satisfied the criteria of limit of quantitation (S/N ≥ 10) and linearity (r ≥ 0.995) within the following working ranges: ametryn – 0.1–10 ng mL^{-1}, r = 0.99954; atrazine-2-hydroxy – 1–100 ng mL^{-1}, r = 0.99958. All samples were analyzed in triplicate.

2.7.3. Ametryn metabolite screening

The screening analysis was performed on a microLC-MS/MS system (4500 QTRAP mass spectrometer (SCIEX, USA), coupled with a microLC 200 System (Eksigent, USA)) in a very similar chromatographic condition to the ones used for ametryn quantitation. The major differences were: total constant flow set to 10 μL min^{-1}, sample injection of 10 μL (5 μL on column) and the gradient set up as follows: 98% A until 0.5 min., linear increase to 98% B in 12 min. and maintained until 19 min., reverse to initial conditions from 19 to 19.1 min and hold until 20 min.
The MS/MS scanning used the Information Dependent Acquisition (IDA) method built on predicted MRM (pMRM) pairs triggering Enhanced Product Ion (EPI) mass spectra in positive ionization mode. The optimization included tuning of ion source settings, compound dependent parameters, IDA filters and EPI settings (Table S-2). The optimized ion source parameters were: CUR: 25, IS: 5000 V, TEMP: 300°C, GS1: 20 psi, GS2: 30 psi.

The pMRM-based methods were constructed with LightSight 2.3 software (SCIEX, USA) based on characteristic fragmentations occurring in the ametryn mass spectrum: 68 m/z, 144 m/z and 186 m/z (Fig. 2A) and covered major possible biological phase I and II transformations that may occur on the substrate in the living cell. Initial methods were further optimized in Analyst 1.6.2 software (SCIEX, USA).
The collected EPI spectra were further used for identification of potential metabolites. The initial identification was conducted within LightSight 2.3 software (SCIEX, USA), where all samples were analyzed as pairs of control samples (without ametryn addition) corresponding to the tested sample (with ametryn addition), for a quick identification of unique or intensive signals in the ametryn supplemented samples. From manual mass spectra analysis in Analyst 1.6.2 (SCIEX, USA) and PeakView 2.2 software (SCIEX, USA) the LightSight 2.3 results were reviewed to confirm or reject the hits or new chemical patterns proposed. The identification workflow was similar to one presented previously (Szewczyk et al., 2015). All samples were analyzed in triplicate.

2.7.4. Metabolomics

A 4500 QTRAP mass spectrometer (SCIEX, USA), coupled with a microLC 200 System (Eksigent, USA) were used for the analysis. Chromatography separation was conducted on an Eksigent 3C8-EP (0.5 mm × 150 mm × 3 µm, 120 Å) column: temperature 45°C, injection volume 5 µL with 2 µL metered on the column. The eluent consisted of 0.1% FA in water (A) and 0.1% FA in ACN (B). The gradient used had a constant flow of 50 µL min^{-1} with 0.5 min of preflush conditioning, followed by 0.2 min in which 98% of eluent A was used and then a decrease to 2% of eluent A after 2.2 min and hold until 3.4 min. Initial conditions were restored from 3.5–4.0 min. The MS/MS detection was performed in positive ionization scheduled MRM mode. The optimized ESI ion source parameters were as follows: CUR: 25; IS: 5000 V; TEMP: 400°C; GS1: 28; GS2: 40 and ihe:ON. Compound-dependent MRM parameters, retention time, linearity and working range for the 48 determined compounds are presented in Table S-3. All samples were analyzed in triplicate.

2.8. PCA and heat map analysis

Principle Component Analysis (PCA) was performed with the MarkerView™ 1.2.1 software (SCIEX, USA) on the quantitation data from all replicates from the metabolomic study. Mean center scaling was applied for PCA calculation. Excel 2013 (Microsoft Corporation, USA) was used to report averaged quantitation data in the form of a heat map.

3. Results

3.1. Ametryn biodegradation

The process of ametryn (100 mg L^{-1}) removal by the tested fungal strain starts after 5 days of culturing. The major derivative formed during biodegradation is 2-hydroxy atrazine, the concentration of which starts to increase from 1.65 mg L^{-1} on the 5th day of incubation to 8.65 mg L^{-1} on the 17th day of experiment. At the same time, the content of ametryn gradually drops to 87.75 mg L^{-1} at the end of culturing (Fig. 1A).
The qualitative pMRM IDA LC-MS/MS analysis of culture homogenate extracts revealed the presence of three additional metabolites identified from mass spectra analysis as presented in Figure 2. Triazine pesticides follow a typical fragmentation of substituted secondary amines leading to formation of characteristic fragment ions and neutral losses (NL). As an example, the major fragmentations of ametryn includes: NL 43 or NL 29 responsible for the side chain removal that leads to formation of 186 m/z and 158 m/z ions, heterocyclic aromatic ring cleavage leading to formation of 144 m/z, 85 m/z, 116 m/z and 68 m/z ions and characteristic for all triazine fragmentation forming a 68 m/z ion ring substructure (Fig. 2A). All the other metabolite fragmentation patterns followed the same or similar rules, as presented and interpreted in Figure 2B-D. As shown in Figure 2, the metabolites were identified as: 2-hydroxy atrazine (4-(ethylamino)-6-[(propan-2-yl)amino]-1,3,5-triazin-2-ol), ethyl hydroxylated ametryn (1-({4-(methylsulfanyl)-6-[(propan-2-yl)amino]-1,3,5-triazin-2-yl}amino)ethan-1-ol), S-demethylated ametryn (4-(ethylamino)-6-[(propan-2-yl)amino]-1,3,5-triazine-2-thiol) and deethylametryn (6-(methylsulfanyl)-N^2-(propan-2-yl)-1,3,5-triazine-2,4-diamine). The trend analysis of chromatographic pMRM peak areas of the identified metabolites showed that their formation also starts after 5 days of incubation. S-demethylated ametryn occurs rapidly and reaches its maximum on the 5th day of culture, then its content is lower with every measurement, while 2 other metabolites after gradual concentration increase from the 5th day of the experiment reaches their apex in 10th (ethyl hydroxylated ametryn) or 10–13th (deethylametryn) days of incubation. After reaching the apex, their amount drops slowly until the end of the culture (Fig. 1B).

3.2. Strain growth and metabolomics of ametryn biodegradation

The growth rate of the tested strain in the applied liquid mineral medium is relatively slow in comparison to other fungal strains (Bernat et al., 2013; Szewczyk et al., 2015; Soboń et al., 2016). The adaptation phase lasts 5–7 days in the control and ametryn

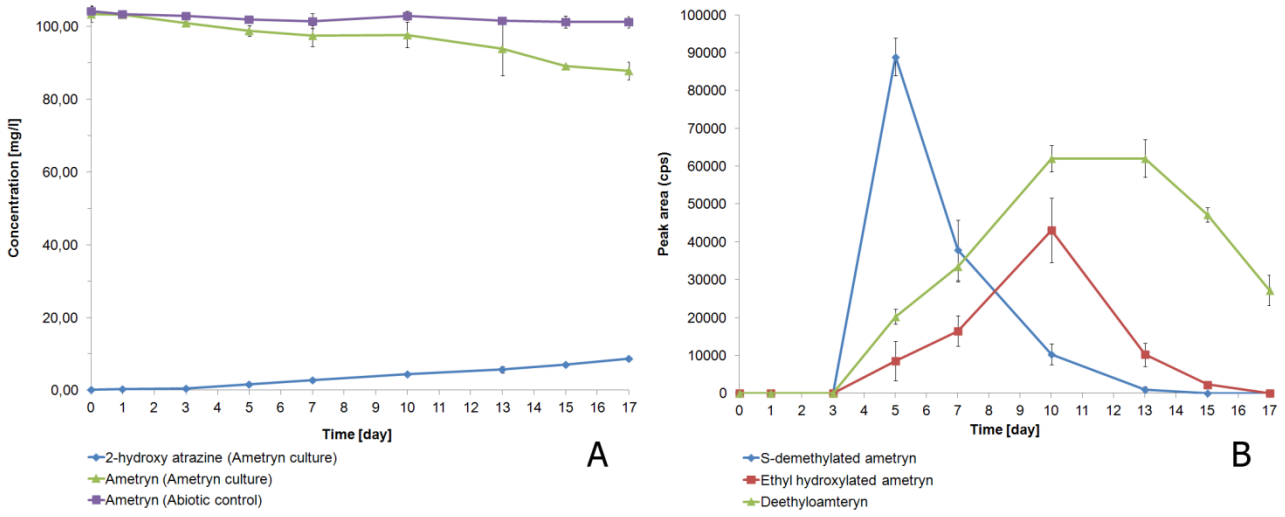

Figure 1. Biodegradation of ametryn (100 mg L^{-1}) during 17-day culture of *Metarhizium brunneum* ARSEF 2107 on mineral medium with 2% of glucose addition. A – ametryn and 2-hyroxy atrazine concentration in the ametryn supplemented cultures, B – trend analysis (peaks areas in counts per second (cps)) of other ametryn metabolites in the ametryn supplemented cultures.

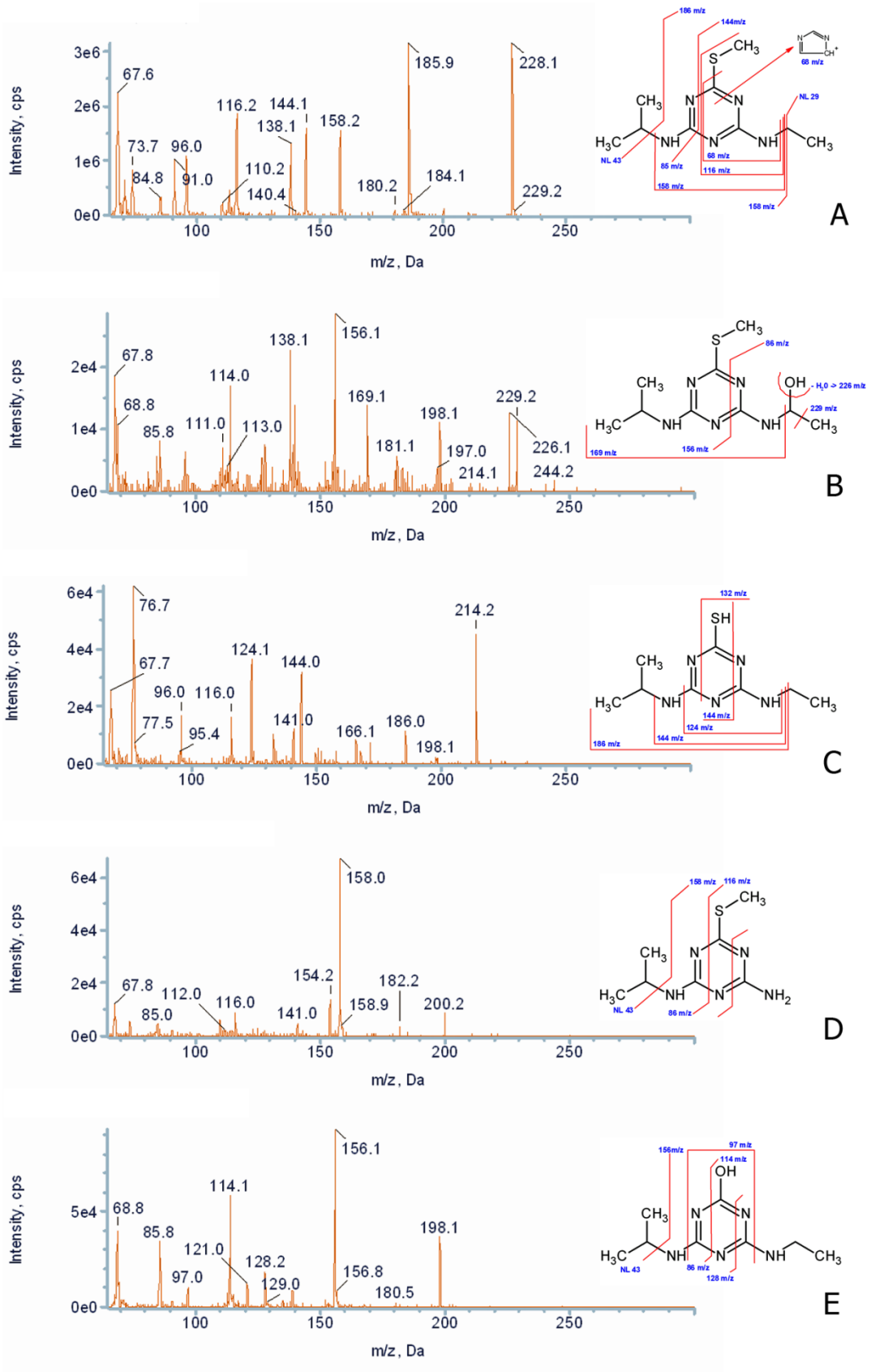

Figure 2. Mass spectra interpretation of ametryn metabolites found in the LC-MS/MS analysis of culture extracts collected during 17-day culture of *Metarhizium brunneum* ARSEF 2107 on mineral medium with ametryn (100 mg L^{-1}) and 2% of glucose addition. A – ametryn, B – ethyl hydroxylated ametryn, C – S-demethylated ametryn, D – deethylametryn, E – 2-hydroxy atrazine.

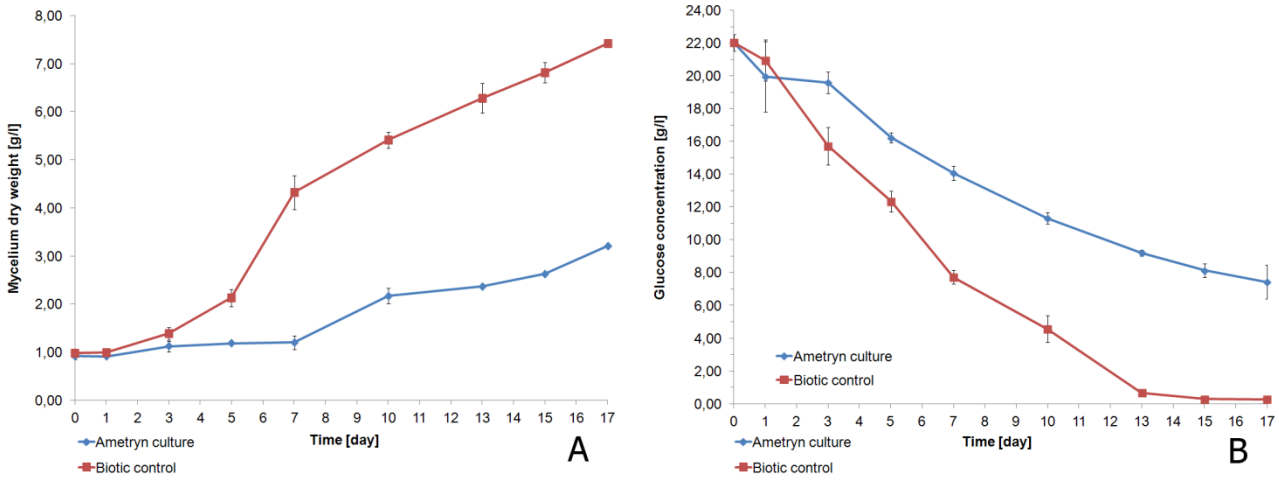

Figure 3. Dry weight (A) and glucose utilization (B) during 17-day cultures of *Metarhizium brunneum* ARSEF 2107 on mineral medium supplemented with 2% of glucose and ametryn (100 mg L^{-1}) addition or biotic controls (without ametryn).

containing cultures, respectively (Fig. 3A). Comparing the growth curves, strong inhibition of the biomass accumulation in the presence of ametryn is observed from day 5 of incubation (Fig. 3A). Maximum growth is reached at the end of the experiment (17th day of incubation), where 3.2 g L^{-1} of mycelium dry weight was measured in ametryn containing cultures and 7.42 g L^{-1} in the control cultures. The growth inhibition by ametryn is reflected by the glucose uptake for the medium. According to the data presented in Fig. 3B, glucose is almost completely absorbed from the medium in the control cultures on the 13th day of culturing, while 7.43 g L^{-1} of glucose is still present in the xenobiotic supplemented cultures at the end of the experiment on the 17th day of incubation.

Deeper insight into the metabolic background of the ametryn biodegradation process conducted by *M. brunneum* ARSEF 2107 was achieved in the targeted metabolomics LC-MS/MS approach focused on the quantitation of free amino acids and their major derivatives in the mycelium extracts. PCA analysis of the whole set of the samples and their replicates (Fig. 4) revealed major differences between the samples. Samples from the beginning of the experiment (0 h) are mostly differentiated by the increased concentrations of betaine and 5-aminopentanoic acid. After one day of culturing, profiles of tested compounds are different. As observed in Figure 4, 1st-day samples are placed far from 0 h-samples. The positions of 1-day-old cultures on the PCA chart are dominated by L-alanine; however, samples supplemented with ametryn are grouped away from the controls. The differentiation between these samples is caused by L-glutamic acid and L-glutamine concentrations in the control cultures when compared to L-valine and guanidinoacetic acid concentrations in the cultures containing ametryn. The last major difference in the PCA chart occurs for gamma-aminobutyric acid (GABA) and L-proline, the relative concentration of which is high in cultures containing ametryn from the 7th to 13th days of the experiment in comparison to all the other samples. Further metabolomic analysis was conducted based on a heat map data analysis (Fig. 5). The averaged values from three replicates and standard deviations are presented in Tables S-4 and S-5. The most common fact observed in both cultures is the relatively high and similar concentration of many tested compounds after the first 24 hours of experimentation (especially for L-proline, L-alanine, L-glutamine, L-glutamic acid, L-lysine, L-valine, GABA and L-threonine), then point of time differentiation in the general metabolic profile occurs.

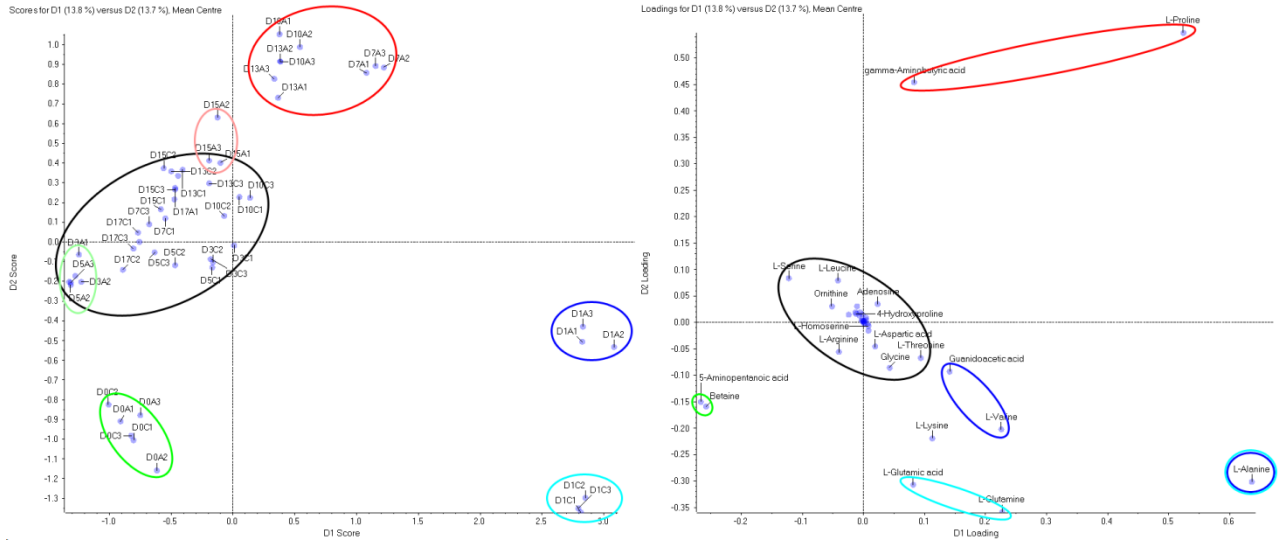

Figure 4. PCA analysis (mean centre scaling) of the quantitation metabolomics data collected during 17-day cultures of *Metarhizium brunneum* ARSEF 2107 on mineral medium supplemented with 2% of glucose and ametryn (100 mg L^{-1}) addition and biotic controls (without ametryn). The colour ovals point the compounds on the loadings plot (right) responsible the most for the samples position and grouping on the scores plot (left). The names of the samples on the scoring plot should read as follows: D – day, 0-17 – day number, A or C – ametryn supplemented culture or biotic control, 1-3 – replicate number.

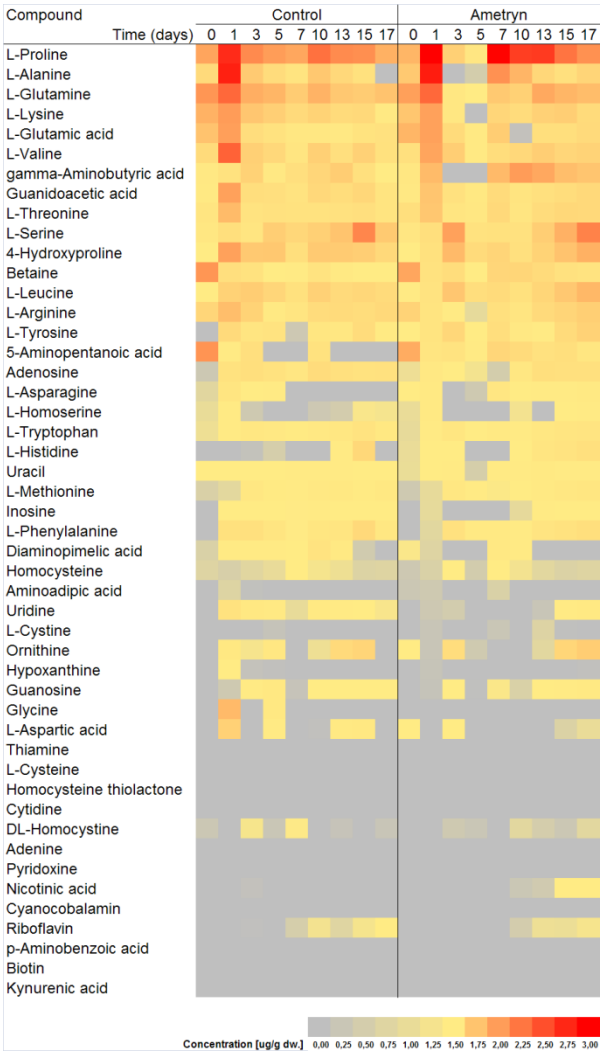

Figure 5. Heat map presentation of the averaged concentrations (n = 3) of the compounds analysed in the metabolomics study during 17-day cultures of *Metarhizium brunneum* ARSEF 2107 on mineral medium supplemented with 2% of glucose and ametryn (100 mg L^{-1}) addition and biotic controls (without ametryn).

According to the obtained data, the most active pathway in both cultures is 2-oxoglutarate originated that leads to relatively high concentrations of L-proline, L-glutamine and L-glutamic acid. After very low measurements of proline days 3–5 of the experiment in ametryn supplemented cultures, the highest concentrations of this amino acid are reached after the 1st and 7th days of incubation (3.122 and 3.056 mg g^{-1} of dry weight (dw), respectively). A significantly higher amount of L-proline in ametryn containing cultures is maintained from the 7th day until the end of incubation, although the tendency to decrease its content is similar in both cultures, finally reaching 0.86 and 1.215 mg g^{-1} dw in control and ametryn supplemented cultures, respectively. L-glutamine and L-glutamic acid profiles are similar in both cultures; however, in the case of L-glutamine a second apex is reached after 10 (0.759 mg g^{-1} dw) or 13 (0.86 mg g^{-1} dw) days of culturing in control and xenobiotic containing cultures, respectively. L-glutamine concentration in the presence of ametryn is higher in comparison to the control samples from the 13th to 17th days of the experiment. 2-oxoglutarate through L-glutamic acid pathway may also contribute to the formation of ornithine and L-arginine. As observed in the heat map (Fig. 4), both amino acids are synthesized in relatively low concentrations (max. 0.3–0.4 mg g^{-1} dw), but the tendency of their formation is opposite to L-proline, especially in the ametryn containing cultures. Similar behavior is observed for 4-hydroxyproline, where the concentration increases in ametryn containing cultures from 0.16 mg g^{-1} dw on the 10th day of culture to a final concentration of 0.79 mg g^{-1} dw at the end of the experiment.
Several amino acids, including L-alanine, L-serine, L-valine, guanidinoacetic acid, L-leucine/isoleucine, L-tyrosine, L-tryptophan, L-phenylalanine, L-histidine or L-aspartic acid had similar profiling in both cultures, but with significant lower concentrations or time lag in the profiling, while ametryn was present in the cultures. Considering other tested compounds, no significant differences in both profiling or concentration in the presence ametryn were observed.

4. Discussion

Triazine herbicides such as atrazine, ametryn or prometon are some of the most dangerous water pollutants because of their ability to bioaccumulate in the tissues of many aquatic organisms, resistance to degradation and relatively high solubility in water resulting in soil leaching with rain waters. Although the use of triazine pesticides has been banned in many countries, residue analysis revealed the presence of these compounds in soil and surface waters. That is why there is a strong need to find ways to develop successful strategies of triazine removal (Navaratna et al., 2016).
The strain tested in this study *M. brunneum* ARSEF 2107 was chosen from the collection of *Metarhizium* strains available in our department and characterized at the initial screening stage as the only one capable of ametryn removal and the only one resistant to high concentrations of the xenobiotic reaching 100 mg L^{-1} (data not shown). As presented in Figure 1, ametryn (100 mg L^{-1}) biodegradation and strain growth is a process that leads to 12.25 mg L^{-1} of xenobiotic removal after 17 days of the experiment. The complete removal of ametryn (2 mg L^{-1}) was reported in the 5-day cultures of bacterial strain *Nocardioides* DN36 (Satsuma, 2010). The experiments with the use of a biofilm reactor on the 6 strain bacterial mixed culture isolated from sugar cane cultivated soil allowed for 97% removal of the xenobiotic after 50 days of culturing. The initial concentration of ametryn was 31.5 mg L^{-1} (Sandoval-Carrasco et al., 2013). Another example of a mixed culture used for ametryn removal is the usage of microbial consortium from activated sludge. The culture was run for 214 days in the hybrid membrane bioreactor. The initial herbicide concentration was 1 mg L^{-1} and at the end of the experiment 46% of the ametryn was removed (Navaratna et al., 2012). Fungal growth in the control medium is slow, but in the presence of ametryn the growth is strongly inhibited (Fig. 3A). The toxic and inhibiting effect of the herbicide on the tested strain may be a direct cause of the slow biodegradation rate. Nevertheless, the high efficiency of the ametryn removal in the presented bacterial cultures and mixed bacterial cultures is significant. On the other hand, fungi are key players, especially in soil consortia and *M. brunneum* ARSEF 2107 biodegradation of ametryn may be a crucial step for many other organisms including fungi, actinobacteria or bacteria. Additionally, relatively slow conversion of the substrate to its metabolites may be favorable for the adaptation of microbial consortia and higher organisms in the polluted ecosystem.
The qualitative LC-MS/MS analysis of the biodegradation process revealed the presence of four metabolites (Fig. 2). From the quantitative analysis, peak area profiling (Fig. 1 AB) and the compound structures (Fig. 2), the degradation pathway presented in Figure 6 is proposed. The three major courses of the ametryn transformation are observed: two of them are connected with methanethiol substituent modifications leading to demethylation and oxidation resulting in formation of S-demethylated ametryn and 2-hydroxy atrazine; the third route leads to formation of ethyl hydroxylated ametryn and deethylametryn via oxidation of ethanamine substituent. The formation of 2-hydroxy atrazine from S-demethylated ametryn cannot be excluded (Fig. 6); however, we did not find any thiol-oxidized or ring-oxidized next to thiol by-products. To our knowledge, this is the first proposal of the ametryn degradation pathway conducted by a fungal microorganism.
Among the triazine herbicides, atrazine biodegradation is the most studied pathway in both bacterial and fungal strains. The most common reactions observed are hydroxylation and alkyl

amine side chain oxidative removal which may finally end in formation of cyanuric acid and complete mineralization of the compound (Atrazine Pathway Map, 2017). Ametryn biodegradation resulting in formation of cyanuric acid has been reported for 6-strain microbial consortium in a biofilm reactor cultures (Sandoval-Carrasco et al., 2013).
Complete mineralization is the most favorable way of biodegradation; however, the vast majority of the xenobiotics biodegradation pathways (including triazines) end in the formation of by-products. Formation of ametryn metabolites during the experiments presented in this work is partially in agreement with the reactions of atrazine biodegradation, where the formation 2-hydroxyatrazine has been observed in many species (Atrazine Pathway Map, 2017; KEGG Atrazine degradation – Reference pathway, 2017; Govantes et al., 2009; Solomon et al., 2013; Abigail et al., 2013), while deethylation of ethanamine substituents seems to be characteristic for fungal strains and mammals (Chirnside et al., 2011; Pereira et al., 2013, Sciali et al., 2014).

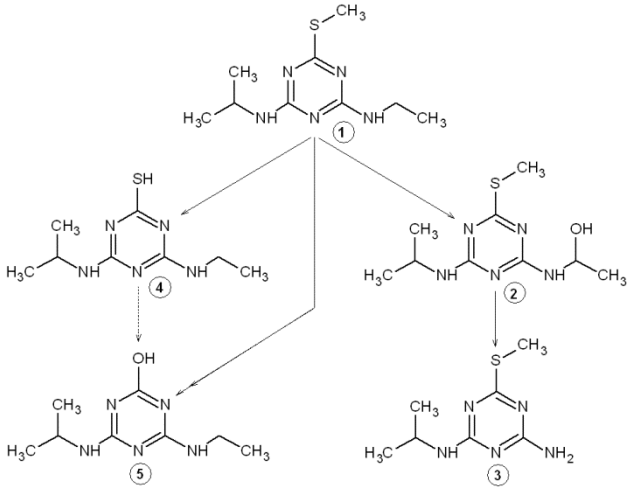

Figure 6. Proposal of the ametryn biodegradation pathway conducted in the 17-day culture of *Metarhizium brunneum* ARSEF 2107 on mineral medium with ametryn (100 mg L^{-1}) and 2% of glucose addition. 1 – ametryn, 2 – ethyl hydroxylated ametryn, 3 – deethylametryn, 4 – S-demethylated ametryn, 5 – 2-hydroxy atrazine.

As presented in Figure 6, the deethylation precedes the formation ethyl hydroxylated ametryn. A similar reaction has been postulated in persulfate oxidation of atrazine resulting in 2-chloro-4-acetamido-6-isopropylamino-1,3,5-triazine formation (Ji et al., 2015). The observed hydroxylation of ethanamine substituents of ametryn may partially explain the mechanism of microbial triazine dealkylation as an oxidative process mediated by oxidoreductase; however, further research on transcriptomic and/or proteomic levels is needed to confirm this mechanism.
The product of ametryn's methylthio substituent dealkylation was most probably further transformed to 2-hydroxy atrazine (Fig. 6). The mechanism of biodegradation of methylthio-s-triazines by *Rhodococcus* sp. strain FJ1117YT reported by Fuji et al. (2007) leads to formation of 2-hydroxy atrazine through methylsulfinyl and methylsulfonyl by-products; however, we did not find such compounds in the examined extracts from *M. brunneum* ARSEF 2107 cultures.
Toxicity research was not the aim of the presented article; however, studies on the toxicity of the atrazine and its metabolites have revealed that its major metabolites are less toxic then the herbicide itself (WHO, 2007). Among the identified metabolites of ametryn, 2-hydroxy atrazine is common for both biodegradation pathways. 2-Hydroxy atrazine is considered non-toxic to rat development even at dose levels associated with maternal toxicity 75-125 mg/kg (Sciali et al., 2014). 2-Hydroxy atrazine is reported to induce renal toxicity in rats at 45 mg/kg and higher, but without affecting any of the reproductive or endocrine endpoints in either the male or female pubertal assay (Stoker et al., 2013). To our knowledge, other ametryn metabolites are described for the first time in this article and we did not find any data on their toxicity; however, the ametryn transformation products are structurally similar to the ones occurring during atrazine biodegradation. Therefore, they might be less toxic than the substrate, but this assumption needs further examination.
The biodegradation process is the cause of numerous changes in the intracellular metabolism and may affect genetics, transcriptome, proteome, lipidome or small molecule metabolism. All changes are important in terms of the process of monitoring and optimization; however, the small molecules are useful for initial studies as by-products, co-factors or end products of numerous metabolic pathways. Amino acids and their derivatives are typical end products of carbon and nitrogen biosynthesis and metabolism. They can be used for general metabolism evaluation and understanding as well as markers of the physiological condition of a tested organism (Fang et al., 2002; Noguchi et al., 2006).
The major activated pathway starts at 2-oxoglutarate and leads to the formation of high amounts of L-proline, especially during the biodegradation process (Fig. 1, 4 and 5). In a variety of organisms, stresses such as cold, heat, salt, drought, UV, xenobiotics and heavy metals significantly increase endogenous L-proline concentrations. One common feature from these stresses is the production of ROS and ROS-induced apoptosis (Chen and Dickman, 2005; Krishnan et al., 2008; Lee at al., 2013; Szewczyk and Kowalski, 2016). High amounts of L-proline measured during the removal of ametryn may be explained as a marker of oxidative stress. Oxidative stress has also been confirmed during the biodegradation of tributyltin (TBT) by *Cunnighamella echinulata* (Soboń et al. 2016) and alachlor by *Paecilomyces marquandii* (Szewczyk et al. 2015). In the case of TBT biodegradation, L-proline was one of the major compounds accumulated in the mycelium of *C. echinulata*.
A significant increase in GABA concentration was also observed in cultures containing ametryn during the biodegradation stage (Fig. 4 and 5). The higher concentration of GABA – a direct precursor of L-glutamine reflects its formation profile in the presence of ametryn; however, GABA and L-proline are frequently accumulated in stressed plants. As suggested by Signorelli et al. (2015), the $^{\bullet}$OH radicals lead to decarboxylation of L-proline and final formation of pyrroline which is the major substrate of GABA formation under oxidative stress. GABA and L-proline profiling may suggest a similar mechanism of GABA formation in the tested cultures (Fig. 5).
Another mechanism of ROS scavenging is the oxidation of L-proline by $^{\bullet}$OH radicals resulting in the formation 4-hydroxyproline (Liang et al., 2013). As observed in Fig. 5, an increasing amount of 4-hydroxyproline is observed from the 10^{th} to 17^{th} days of incubation in the cultures containing ametryn.
The concentration of L-arginine and ornithine contrasts with that of L-proline, especially in the cultures containing ametryn. This result support the oxidative stress hypothesis caused by ametryn biodegradation as the 2-oxoglutarate network switches to compounds other than L-proline with less toxic compound formation (Fig. 5). On the other hand, a few reports point to L-arginine as a potential ROS scavenger in mammalian (Tripathi et al., 2013; Rocha et al., 2015) or worm (Ma et al., 2016) cells under oxidative stress conditions. A similar role of L-arginine in ametryn biodegradation by the tested strain cannot be excluded.
The differences in profiling of other amino acids such as L-alanine, L-serine, L-valine, guanidinoacetic acid, L-leucine/isoleucine, L-tyrosine, L-tryptophan, L-phenylalanine, L-histidine or L-aspartic acid in the controls and cultures containing ametryn may be narrowed down to time lags in the concentration apexes or concentration differences. This may reflect the inhibited growth of the fungus in the presence of ametryn (Fig. 3A) and its negative influence on the fungal metabolism.

5. Conclusions

In the presented study, we have focused on the degradation of the s-triazine herbicide – ametryn by entomopathogenic fungal cosmopolite *M. brunneum* ARSEF 2107. The worldwide presence

of *Metarhizium* strains in various ecosystems and its ability to infect insects points to them as good candidates for agricultural application as both natural insecticides and xenobiotic degraders. The growth and biodegradation rate is genus specific and could be enhanced in many ways on physiological or genetic levels. Besides the characterization of the biodegradation course, information on metabolic response may provide a better understanding of any environmentally relevant organism, which would aid the enhancement or modification of a given process for specific purposes (Szewczyk et al., 2015). To our knowledge, this study provides the first data on ametryn biodegradation by a fungal strain as well as the first metabolomic analysis of ametryn biodegradation in any organism.

Ametryn biodegradation by the tested fungal strain leads to formation of 4 metabolites: 2-hydroxy atrazine, ethyl hydroxylated ametryn, S-demethylated ametryn and deethylametryn. The biodegradation of the herbicide is strictly connected with the relatively long growth of the fungus. Additionally, in the presence of the ametryn tested strain reduced culture growth and glucose consumption rates because of the ametryn toxicity. The targeted metabolomic analysis of amino acids and a few other small molecules revealed significant concentration differences in the presence of the xenobiotic when compared to control cultures. The major changes included L-Proline, GABA, L-glutamate, 4-hydroxyproline, L-glutamic acid, ornithine and L-arginine profiling. The obtained data proved that the presence of ametryn in the fungal culture induced oxidative stress and serious disruptions of the carbon and nitrogen metabolism.

The characterization of ametryn removal by the tested fungal strain included xenobiotic pathway research, growth and glucose measurements combined with free amino acid profiling. These results provided deeper insights into the microorganism strategy for the xenobiotic biodegradation. Further research on proteomic and lipidomic levels may reveal other important data about this process. All the obtained data so far, as well as future research may lead to a deeper understanding of the examined process, resulting in highly specific enhancements of ametryn removal by *M. brunneum* ARSEF 2107.

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