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Manuscript Draft

Manuscript Number: HAZMAT-D-17-01637R1

Title: Metabolism of carbamazepine in plant roots and endophytic rhizobacteria isolated from Phragmites australis

Article Type: Research Paper

Keywords: endophytic bacteria; hairy roots; glutathione conjugate; acridine pathway; phytoremediation

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First Author: Andrés Sauvêtre

Order of Authors: Andrés Sauvêtre; Robert G May, Dr.; Rudolf Harpaintner; Charlotte Poschenrieder, Prof. Dr.; Peter Schroeder, Prof. Dr.

Abstract: Carbamazepine (CBZ) is a pharmaceutical frequently categorized as a recalcitrant pollutant in the aquatic environment. Endophytic bacteria previously isolated from reed plants have shown the ability to promote growth of their host and to contribute to CBZ metabolism.

In this work, a horseradish (Armoracia rusticana) hairy root (HR) culture has been used as plant model to study the interactions between roots and endophytic bacteria in response to CBZ exposure. HRs could remove up to 5% of the initial CBZ concentration when they were grown in spiked Murashige and Skoog (MS) medium. Higher removal rates were observed when HRs were inoculated with the endophytic bacteria Rhizobium radiobacter (21%) and Diaphorobacter nitroreducens (10%). Transformation products resulting from CBZ degradation were identified using liquid chromatography–ultra high-resolution quadrupole time of flight mass spectrometry (LC-UHR-QTOF-MS). CBZ metabolism could be divided in four pathways. Metabolites involving GSH conjugation and 2,3-dihydroxylation, as well as acridine related compounds are described in plants for the first time.

This study presents strong evidence that xenobiotic metabolism and degradation pathways in plants can be modulated by the interaction with their endophytic community. Hence it points to plausible applications for the elimination of recalcitrant compounds such as CBZ from wastewater in CWs.

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18/07/17

Dear Dr. Li Puma,

Please find enclosed our revision to the manuscript „Metabolism of carbamazepine in plant roots and endophytic rhizobacteria isolated from P. australis" for publication in your journal.

We have responded to all comments of the reviewers and improved the manuscript.

As requested, please find the list of responses added to this letter.

We hope that you find our manuscript (4861 words) by now suitable for publication by "Journal of Hazardous Materials" and are awaiting constructive reviews.

Sincerely yours,

Peter Schröder (for the authors)

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Response to the reviewers´comments:

The authors wish to thank reviewers for their positive opinion about the manuscript and the constructive comments.

In detail:

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"However, the nature of the metabolic pathways established in real conditions needs to be further investigated in inoculation experiments in CWs systems."

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A: sonication conditions were specified: were sonicated for 10 min in a sonicator bath at 35 kHz.

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A: The aim of the conjugation was to obtain the elution and fragmentation patterns of the conjugate, to identify this one in the biological samples. The conjugate were not purified, in order to elute in one TOF run the three compounds (substrates and product) with their corresponding m/z ratios and retention times. Since the synthesis of the conjugate was done following a published method and the fragmentation patterns obtained fit with this work (Bu et al., 2005) (identity of the conjugate verified by NMR spectroscopy), authors did not estimate necessary to realize a purification of the conjugate.

p8, line 22: R2: Recommend mentioning the basic details about the method (mobile phases, etc...) at least on the SI. In the manuscript, for sure LOD and LOQ values.

A: Details about the method have been added in the SI section. A sentence in the manuscript was added with the additional info requested: "In all analyses, the LOD and LOQ were 131 and 397 µg.L-1 respectively".

p9, line 3: R2: I believe this is the first time where this acronym appears. Spell Transformation Products on first use.

A: The acronym for TPs (Transformation products) has been added as it is mentioned for the first time. The title of the section has been modified as well, avoiding the use of the abbreviation.

p9, line 7: R2: Was this the same set-up mentioned above?

A: Yes. The sentence has been modified to clarify this: "TPs were identified after LC-QTOF-MS/MS separation (as described in the previous section) using different collision energies (15, 24 and 30 eV)"

p9, line 9: R2: If standards for the different expected TPs were available, was a local database created for the qualification of new unknowns? Was retention time set as one of the qualification variables.

A: The authors thank the reviewer for this valuable comment: the section has been modified including this information: "When analytical standards were available, a local database with retention time and fragmentation pattern was created. When analytical

standards were not available, fragmentation patterns were predicted using the competitive fragmentation model by Allen et al. 2015 [26]. Only TPs with a retention time and fragmentation pattern matching standards or fragmentation pattern matching predicted patterns were considered.

Results and discussion

p 15, line 26: R2: Why do you think this is?

A: As it has been discussed in section 3.1 (p10, lines 43-47), CBZ moves into root tissues by simple diffusion, limiting the overall metabolism to enzymatic processes occurring in the cell compartment. The inoculation of HRs with beneficial endophytic bacteria carrying additional genes for CBZ metabolism would increase the overall turnover, resulting in an increase of CBZ uptake into the symplast. This effect is reflected by the elevated amount of CBZ found in root extracts of inoculated HRs.

A sentence explaining this hypothesis has been added:

The content and composition of TPs identified in the media was investigated in root extracts after 21 days of incubation (fig. 4). The amount of CBZ found in root extracts inoculated with endophytic bacteria was twice as high as in HR extracts. This result confirmed the observations performed in experiments with concentrations 10 and 25 µM (fig. 1) and can be explained by an increase in the overall CBZ metabolism causing elevated CBZ diffusion into the symplast of inoculated HRs.

Conclusion

p15, line 55: R2: I am missing a brief paragraph on the extrapolation to whole organism, or even organism-soil/sediment scenarios. Perhaps reference to other compounds for which studies in HR, and whole organism are available and compared. How would the observed results change under those conditions? Would you expect translocation of the compound to other areas of the plant limit the ability of root-associated organism to increase metabolic degradation of the compound?

A: A paragraph has been added at the end of the conclusion (p 17, line 12):

However, the nature of the metabolic pathways established in real conditions needs to be further investigated in inoculation experiments in CWs systems. Few compounds have been studied using both, HRs and whole macrophyte plants. The metabolites identified were conserved in both systems in the case of oxybenzone (Chen et al, 2016 and 2017) and diclofenac (Huber et al, 2012). Similar results are expected for CBZ when extrapolating to whole organisms in CWs, however, the importance of each sub-pathway may be different. Plants in CWs have high transpiration rates, and hence a big part of the parent compound is expected to be translocated to aerial parts, where further metabolism (especially phase II plant metabolism) could result in a storage of the compound and its conjugates, eventually in vacuolar compartments. In addition, endophytic bacteria inhabiting aerial plant parts could enhance breakdown of translocated compounds. From the point of view of phytoremediation, both scenarios would fulfil the main purpose, namely to remove the parent compound and its reactive metabolites from polluted water. Of course, plant biomass should be harvested and used for bioenergetic purposes, avoiding a re-entry of metabolites and parent compound in the water bodies.

Response to reviewers´comments

The authors wish to thank reviewers for their positive opinion about the manuscript and the constructive comments. The revised version of the ms covers all comments, and is significantly improved. Page and line numbers used here to answer reviewers' comments correspond to those of the PDF file generated when clicking "View submission" and named "HAZMAT-D-17-01637"

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A: sonication conditions were specified: were sonicated for 10 min in a sonicator bath at 35 kHz.

p8, line 1: R2: What was the percent "success" of the conjugation? where theses purified to eliminate leftover unconjugated substrates?

A: The aim of the conjugation was to obtain the elution and fragmentation patterns of the conjugate, to identify this one in the biological samples. The conjugate were not purified, in order to elute in one TOF run the three compounds (substrates and product) with their corresponding m/z ratios and retention times. Since the synthesis of the conjugate was done following a published method and the fragmentation patterns obtained fit with this work (Bu et al., 2005) (identity of the conjugate verified by NMR spectroscopy), authors did not estimate necessary to realize a purification of the conjugate.

p8, line 22: R2: Recommend mentioning the basic details about the method (mobile phases, etc...) at least on the SI. In the manuscript, for sure LOD and LOQ values.

A: Details about the method have been added in the SI section. A sentence in the manuscript was added with the additional info requested: “In all analyses, the LOD and LOQ were 131 and 397 $\mu g.L^{-1}$ respectively”.

p9, line 3: R2: I believe this is the first time where this acronym appears. Spell Transformation Products on first use.

A: The acronym for TPs (Transformation products) has been added as it is mentioned for the first time. The title of the section has been modified as well, avoiding the use of the abbreviation.

p9, line 7: R2: Was this the same set-up mentioned above?

A: Yes. The sentence has been modified to clarify this: "TPs were identified after LC-QTOF-MS/MS separation (as described in the previous section) using different collision energies (15, 24 and 30 eV)"

p9, line 9: R2: If standards for the different expected TPs were available, was a local database created for the qualification of new unknowns? Was retention time set as one of the qualification variables.

A: The authors thank the reviewer for this valuable comment: the section has been modified including this information: "When analytical standards were available, a local database with retention time and fragmentation pattern was created. When analytical standards were not available, fragmentation patterns were predicted using the competitive fragmentation model by Allen et al. 2015 [26]. Only TPs with a retention time and fragmentation pattern matching standards or fragmentation pattern matching predicted patterns were considered.

Results and discussion

p 15, line 26: R2: Why do you think this is?

A: As it has been discussed in section 3.1 (p10, lines 43-47), CBZ moves into root tissues by simple diffusion, limiting the overall metabolism to enzymatic processes occurring in the cell compartment. The inoculation of HRs with beneficial endophytic bacteria carrying additional genes for CBZ metabolism would increase the overall turnover, resulting in an increase of CBZ uptake into the symplast. This effect is reflected by the elevated amount of CBZ found in root extracts of inoculated HRs.

A sentence explaining this hypothesis has been added:

The content and composition of TPs identified in the media was investigated in root extracts after 21 days of incubation (fig. 4). The amount of CBZ found in root extracts inoculated with endophytic bacteria was twice as high as in HR extracts. This result confirmed the observations performed in experiments with concentrations 10 and 25 μM (fig. 1) and can be explained by an increase in the overall CBZ metabolism causing elevated CBZ diffusion into the symplast of inoculated HRs.

Conclusion

p15, line 55: R2: I am missing a brief paragraph on the extrapolation to whole organism, or even organism-soil/sediment scenarios. Perhaps reference to other compounds for which studies in HR, and whole organism are available and compared. How would the observed results change under those conditions? Would you expect translocation of the compound to other areas of the plant limit the ability of root-associated organism to increase metabolic degradation of the compound?

A: A paragraph has been added at the end of the conclusion (p 17, line 12):

However, the nature of the metabolic pathways established in real conditions needs to be further investigated in inoculation experiments in CWs systems. Few compounds have been studied using both, HRs and whole macrophyte plants. The metabolites identified were conserved in both systems in the case of oxybenzone (Chen et al, 2016 and 2017) and diclofenac (Huber et al, 2012). Similar results are expected for CBZ when extrapolating to whole organisms in CWs, however, the importance of each sub-pathway may be different. Plants in CWs have high transpiration rates, and hence a big part of the parent compound is expected to be translocated to aerial parts, where further metabolism (especially phase II plant metabolism) could result in a storage of the compound and its conjugates, eventually in vacuolar compartments. In addition, endophytic bacteria inhabiting aerial plant parts could enhance breakdown of translocated compounds. From the point of view of phytoremediation, both scenarios would fulfil the main purpose, namely to remove the parent compound and its reactive metabolites from polluted water. Of course, plant biomass should be harvested and used for bioenergetic purposes, avoiding a re-entry of metabolites and parent compound in the water bodies.

Metabolism of carbamazepine in plant roots and endophytic rhizobacteria isolated from *Phragmites australis*

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Abstract

Carbamazepine (CBZ) is a pharmaceutical frequently categorized as a recalcitrant pollutant in the aquatic environment. Endophytic bacteria previously isolated from reed plants have shown the ability to promote growth of their host and to contribute to CBZ metabolism.

In this work, a horseradish (*Armoracia rusticana*) hairy root (HR) culture has been used as a plant model to study the interactions between roots and endophytic bacteria in response to CBZ exposure. HRs could remove up to 5% of the initial CBZ concentration when they were grown in spiked Murashige and Skoog (MS) medium. Higher removal rates were observed when HRs were inoculated with the endophytic bacteria *Rhizobium radiobacter* (21%) and *Diaphorobacter nitroreducens* (10%). Transformation products resulting from CBZ degradation were identified using liquid chromatography−ultra high-resolution quadrupole time of flight mass spectrometry (LC-UHR-QTOF-MS). CBZ metabolism could be divided in four pathways. Metabolites involving GSH conjugation and 2,3-dihydroxylation, as well as acridine related compounds are described in plants for the first time.

This study presents strong evidence that xenobiotic metabolism and degradation pathways in plants can be modulated by the interaction with their endophytic community. Hence it points to plausible applications for the elimination of recalcitrant compounds such as CBZ from wastewater in CWs.

Keywords:

endophytic bacteria, hairy roots, glutathione conjugate, acridine pathway, phytoremediation.

1. Introduction

Interactions between rhizosphere-inhabiting bacteria and plants were first studied by Lorenz Hiltner. As early as in 1904, Hiltner defined the concept of the rhizosphere and established the foundations of rhizosphere microbial ecology [1]. Since then, researchers have demonstrated that root associated microbial communities play an important role in plant health and nutrition [2]. Besides the impact on human health through the diet, one of the most promising applications of beneficial microbe-plant interactions is the removal of pollutants. Plant-associated bacteria can directly mediate the degradation of organic pollutants by activating specific catabolic genes [3]. In addition, they can aid plants to cope with stress resulting from exposure to xenobiotics even though they might be devoid of enzymes involved in appropriate catabolic routes. In recent years, hairy root (HR) cultures have been used as a model for the potential of different plant species to remove environmental pollutants [4]. Only now, we start to use them as tools to study bacteria-plants interactions in response to xenobiotic stress [5,6].

Endophytic bacteria harboring both of these qualities (catabolic genes and plant growth promoting traits) can establish successful and durable mutualistic relationships with their hosts, certainly because they do not have to compete with the dense populations of microorganisms present in different soil compartments [7]. Endophytes capable of degrading xenobiotics have been isolated in recent years. Most of the studies focus on organic pollutants such as petroleum derivatives, polycyclic aromatic hydrocarbons (PAHs) like naphthalene and pyrene, organochlorines such trichloroethylene (TCE), or phenolic compounds [8]. However, research on pharmaceutical metabolism by endophytic microorganisms is scarce and available research addresses mainly in vitro degradation without the host, usually in synthetic culture media.

The antiepileptic drug carbamazepine (CBZ) is an emerging contaminant listed in some studies as one of the most recalcitrant compounds during wastewater treatment [9]. Consequently, its occurrence has been reported in groundwater [10] and even in drinking water [11]. CBZ uptake and translocation into the plants has been reported in several species such as cabbage, Wisconsin fast plants, ryegrass, pepper, collard, lettuce, radish, tomato, maize, sunflower, sweet potato and cucumber [12–18].

Extensive studies on CBZ metabolism in humans and animals are available since long [19], and a pathway for CBZ metabolism in human liver has been proposed [20], but comprehensive investigations in plants are still lacking. In mammals, members of the Cytochrome P450 family achieve most of the enzymatic transformations occurring during CBZ metabolism. Predominantly, epoxides and hydroxylated metabolites are found but phase II conjugates involving UDP-glucuronosyltransferases are also considered. So far, a large proportion of these metabolites has not yet been identified in plants. However, in tomato and cucumber metabolism has been scrutinized with 10,11-CBZ-epoxide and 10,11-dihydro-10,11-dihydroxy-CBZ as major metabolites [21].

Endophytic bacteria previously isolated from *Phragmites australis* exposed to CBZ were used for this study. These bacteria have been shown to support plants during xenobiotic stress conditions by promoting their growth and/or by directly degrading the parent compound [22]. Additionally, we have established a hairy root (HR) culture from *A. rusticana* plants that allows us to investigate the metabolism of different organic xenobiotics in plant roots in a rapid manner [23,24]. This work aims to apply this horseradish HR culture as a model to study uptake and metabolism of CBZ in plant roots as well as interactions between roots and endophytic rhizobacteria in response to CBZ exposure. Removal and metabolism of CBZ in HR was characterized by LC-ultra high resolution time-of-flight mass spectrometry. Changes in CBZ uptake and its metabolic pathways when plant roots were assisted by endophytic bacteria are observed and discussed.

2. Materials and Methods

2.1. Plant material and culture conditions

HR cultures of horseradish (*Armoracia rusticana*) had been obtained previously by transformation of nodal segments by *Agrobacterium rhizogenes* strain A4 [24]. HRs were grown in Erlenmeyer flasks filled with 100 mL of full-strength Murashige and Skoog medium (Duchefa Biochemie bv.) containing thiamine (0.32 mg/L) and inositol (0.1 g/L). Cultures were grown aerobically at 23 +/- 2°C in the dark and under slow rotation (75 rpm) in an orbital shaker. HRs were sub-cultured in fresh medium every two weeks.

2.2. Bacterial strains, culture condition and inoculation

Endophytic bacteria were previously isolated from rhizomes and roots of common reed (*Phragmites australis*) plants exposed to CBZ [22]. Bacterial strains *Rhizobium radiobacter (Cb58), and Diaphorobacter nitroreducens (Cb55),* were maintained at room temperature in Reasoners 2A (R2A) agar plates supplemented with 10 µM CBZ. R2A agar was purchased from Difco Laboratories (Sparks, MD, USA).

For the inoculation of HRs, single colonies were selected and grown in sterile lysogeny broth LB-Lennox medium (tryptone 10g/L, yeast extract 5 g/L and NaCl 5 g/L) at 24°C for one to two days. Forty mL bacterial cultures in logarithmic phase were centrifuged at 8000 x g for 10 min and washed twice with 40 mL Dulbecco´s phosphate-buffered saline (PBS) (Applichem Darmstadt, Germany). The resulting pellets were finally re-suspended in PBS to give a final OD_{600nm} of 0.5. HR cultures of approximately 1.8 g were then incubated in those bacterial suspensions for one hour in an orbital shaker under slow rotation (75 rpm). Every step was performed under sterile conditions in a laminar flow bench.

2.3. Hairy root treatments

After the inoculation, and always under sterile conditions, HRs were individually washed twice with sterile PBS and transferred to a new flask containing 100 mL of MS media, supplemented or not with CBZ (10, 25, and 250µM). Autoclaved HR cultures (121°C for 20 min) were used as controls for exposure experiments void of or inoculated with endophytic bacteria. Three biological replicates were set up for each condition. Incubation with CBZ was

performed in Erlenmeyer flasks containing 100 mL media and lasted for 6 to 21 days, depending on the experiment. Samples from the media were taken at different time points (t0, 1 d, 3 d, 6 d, 14 d and 21 d) and centrifuged for 5 min at 13000 x g. The supernatant was stored at -20°C for further characterization. After the incubation period, root cells were washed twice with PBS, dried on lint-free tissue paper, immediately frozen in liquid nitrogen and stored at −80°C.

2.4. Extraction of CBZ and its metabolites and sample preparation

For the analysis of CBZ and its metabolites, samples from the HR culture media were centrifuged at 13000 x g for 10 min, and the pellet containing cells and debris was discarded. A protein precipitation was carried out before LC-UHR-QTOF-MS analysis. Samples were mixed with 45 mg/mL 5-Sulfosalicylic acid (99.9%, Sigma-Aldrich, Taufkirchen, Germany) (1:10 v:v), vortexed briefly, centrifuged for 5 min at 13000 x g and the protein-free supernatant was used for metabolite identification after filtration trough a 0.22 µm pore size polyvinylidene fluoride filter (Rotilabo®, Carl Roth, Germany). Supernatants were then stored at – 20°C for further analyses.

CBZ and its metabolites were extracted from HRs by grinding the frozen tissue in a glass mortar under liquid nitrogen. From the obtained powder, 0.5 g were mixed with 1 mL of methanol 80%, vortexed and sonicated for 10 min in a sonicator bath at 35 kHz. Samples were then centrifuged at 13000 x g for 10 min and the supernatants were evaporated to dryness. The residues were reconstituted in methanol 10%, filtered through a 0.22 µm pore size polyvinylidene fluoride filter and stored at -20°C until analysis.

2.5. Chemicals and analytical standards

Acetonitrile (HPLC grade), water with 0.1% formic acid (LC–MS grade), acetonitrile with 0.1% formic acid (LC–MS grade) were obtained from Carl Roth (Karlsruhe, Germany). Ultrapure water (MilliQ, Millipore Corporation) was used for sample preparation. Analytical standards of carbamazepine (CBZ), acridine, carbamazepine-10,11-epoxide (CBZE), and 10,11-dihydro-10,11-dihydroxycarbamazepine (all ≥97% purity) were purchased from Sigma-Aldrich (Taufkirchen, Germany). L-Glutathione reduced (≥98.0%) and L-Cysteine (97%) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.6. In vitro synthesis of glutathione related metabolites

The synthesis of the conjugates 10,11-dihydro-10-hydroxy-11-glutationyl-CBZ (CBZE-GSH) and 10,11-dihydro-10-hydroxy-11-cysteinyl-CBZ (CBZE-cys) was achieved according to [25] and is described in detail in the supplementary data.

2.7. CBZ determination

CBZ concentration in nutrient solutions was determined by HPLC (Varian ProStar 210) as described previously [22]. Quantification of CBZ was performed using a standard curve done with dilutions of CBZ in 5% acetonitrile ranging from 1.014 µM to 100 µM. In all analyses, the LOD and LOQ were 131 and 397 $\mu g.L^{-1}$ respectively.

2.8. LC-UHR-QTOF-MS analysis

LC–UHR-QTOF–MS experiments were conducted on an Ultimate 3000 LC system (ThermoFisher) coupled to an ultra-high resolution maXis 4 g plus QTOF mass spectrometer (Bruker) equipped with an electrospray source. The TOF–MS was operated in positive polarity mode with active focus under the following conditions: Capillary voltage, 5000 V; nitrogen dry gas temperature, 225 °C; dry gas flow, 10 L/min; nebulizer pressure, 2 bar. The TOF–MS was calibrated daily with ESI-L tuning mix (Agilent) using the enhanced quadratic algorithm. MS scans were recalibrated using Hexakis (1H, 1H, 4H-hexafluorobutyloxy) phosphazine (Agilent) as a lock mass. The LC conditions were as follows: the column was a Phenomenex Synergi HYDRO-RP column (C18, polar endcapped; particle size 4 µm; 50×2.00 mm). CBZ and its metabolites were separated using a linear gradient of eluents: mobile phase A (H_2O, 0.1% formic acid) and mobile phase B (acetonitrile, 0.1% formic acid). Elution gradient: 0–2 min 97% A (isocratic); 2–10 min 95% B (linearly increasing); 10–12 min 95% B (isocratic); 12–12.5 min 97% A (linearly decreasing); 12.5–17 min 97% A (isocratic). The flow rate was 0.3 mL/min. All solvents used for LC–MS were of the highest grade available.

2.9. Identification of transformation products

Transformation products (TPs) were hypothesized using the software Compound Crawler (Bruker, Bremen). Only compounds whose m/z was within ± 10 ppm of the exact molar mass were considered (table 1). TPs were identified after LC-QTOF-MS/MS separation (as described in the previous section) using different collision energies (15, 24 and 30 eV). Fragmentation patterns were compared to those obtained from analytical standards. When analytical standards were available, a local database with retention time and fragmentation pattern was created. When analytical standards were not available, fragmentation patterns were predicted using the competitive fragmentation model by Allen et al. 2015 [26]. Only TPs with a retention time and fragmentation pattern matching standards or fragmentation pattern matching predicted patterns were considered.

3. Results and discussion

3.1.CBZ removal from nutrient media

CBZ removal by hairy roots and endophytic bacteria was studied in short time experiments with initial CBZ concentrations of 10 and 25 µM spiked to the media. These concentrations, of environmental relevance, represent usual concentrations in WWTP effluents [27]. HRs removed about 5% and 4% of the initial concentration (10 and 25µM respectively) after 6 days of incubation (fig. 1). When HRs were inoculated with *R. radiobacter*, removal significantly increased to 21% and 13% in treatments with 10 and 25 µM respectively. Cultures inoculated with *D. nitroreducens* were also able to remove the CBZ present in the growth media more efficiently than HRs alone, but to a lower extent (10% and 9%). Still, this represented twice the amount of CBZ removed by HRs not assisted by endophytic bacteria. The total amount of CBZ removed increased with the initial concentration of the treatment. Given that the removal of CBZ was strongly correlated with initial concentrations in the media (r= 0.99), we suggest that CBZ migrates into root tissues driven by simple diffusion, limiting the overall metabolism to enzymatic processes occurring into the cell compartment. Generally, it is expected that organic xenobiotics with optimum hydrophobicity (log K_{ow} between 0.5 and 3.5) are readily taken up by plants [28]. Additionally, in absence of a driving force such as transpiration, transformation products with similar physicochemical properties can leave the cell by the same mechanism and are therefore accumulating in the growth media.

3.2. Metabolism of CBZ by HRs and endophytic bacteria

Metabolism of CBZ in hairy roots was studied at initial concentration of 250 µM. In total, 13 TPs were identified in the growth media (table 1). These TPs clustered in four hypothesized pathways, and their evolution during the incubation time was followed.

3.2.1 10,11-diOH pathway

The 10,11-diOH pathway has been extensively described in plants. CBZ is first oxidized to CBZ-10,11-epoxide. This is the main TP identified in root and leaves tissues from sweet potato and carrot as well as in leaves of cucumber and tomato [18,21]. This first oxidation is conserved among different kingdoms, being reported as well in mammals [19], lignolytic fungi [29], and soil bacteria [30]. This first oxidation step is achieved by cytochrome P450 and/or peroxidases and represents the first activation of the parent compound, leading to other sub-pathways. All chromatograms showed multiple peaks corresponding to a theoretical m/z 253.1071 revealing the co-elution of other epoxidized or hydroxylated TPs formed during the incubation. Since no standards were available for most of this hypothesized TPs and some of them showed identical fragmentation patterns, a reliable quantification for this metabolite was not feasible; a fact that had previously been observed in WWTP effluents [31].

The first epoxidation is followed by cleavage and hydroxylation of the epoxy bond to render 10,11-dihydro-10,11-dihydroxy-CBZ, a reaction known to be catalysed by epoxide hydrolases in rat and human liver [32], enzymes obviously present in all kingdoms of life. This metabolite was formed in the media from the first day and accumulated constantly until the last day of incubation in HR cultures (fig 2). In HRs inoculated with endophytic bacteria, accelerated formation of the 10,11-dihydro-10,11-dihydroxy-CBZ could be observed during the first day, followed by a decrease during the next three days. From the fourth day of incubation, the peak area increased with lower rates than in control HR cultures. This trend suggests that the compound was further metabolized by endophytic bacteria, thus accumulating in media in lower amounts.

10,11-dihydro-10-hydroxy-CBZ (10-OH-CBZ) could be identified in media from control HR cultures and HR cultures inoculated with *D. nitroreducens*. In both cases, the peak area increased slowly from the first day on, albeit in higher concentration in non-inoculated HRs. This metabolite has previously been detected in wastewater [33] and fungi [34] but no

enzyme has been postulated for this reaction. In mammals, this metabolite had been identified as a product of oxcarbazepine and not of CBZ [35].

10,11-dihydro-10,11-dihydroxy-CBZ and 10-OH-CBZ were found in negative controls consisting in autoclaved HRs incubated with CBZ (fig S1). The formation of these compounds in absence of biological matrices reveals a chemical oxidative pathway independent from plants or bacteria. However, the total amount accumulated was lower than in biological samples. When autoclaved HRs were inoculated with endophytic bacteria, 10,11-dihydro-10,11-dihydroxy-CBZ was synthesized with a trend similar to the one observed in healthy HRs, albeit in lower amounts. Thus, we can conclude that bacteria and plants can degrade CBZ through the 10,11-diOH pathway although plants are more efficient.

3.2.2 2,3-diOH pathway

A sub-pathway involving successive oxidation reactions at the carbons of the side aromatic benzene group was identified in all samples with biological activity. CBZ was metabolized to 2,3-dihydro-2,3-dihydroxy-CBZ in HR cultures void of or inoculated with endophytic bacteria. This reaction can be achieved by 2,3 dioxygenation, or proceed via the epoxidation at carbons 2 and 3, followed by hydrolysis. The first is typical for bacterial degradation of polycyclic aromatic hydrocarbons [36]. The second would include two steps with the participation of a CYP450 or a peroxidase and an epoxide hydrolase, more representative of plant metabolism. Both could occur in our model, one in HR cultures and the other in inoculated roots. Unfortunately, epoxidation at the 2,3 position of the dibenzazepine ring could not be confirmed, although several peaks corresponding to its theoretical m/z were identified: as mentioned above, this array of metabolites with m/z 253.0971 could correspond to TPs formed by hydroxylation or epoxidation at different carbons on the aromatic ring (1-OH-CBZ, 2-OH-CBZ, 3-OH-CBZ, 4-OH-CBZ, CBZ-1,2-epoxide, CBZ-2,3-epoxide, CBZ-3,4-epoxide, CBZ-1,4-epoxide) or on the central ring (CBZ-10,11-epoxide, oxcarbazepine) of the parent compound. Some of these metabolites are found in wastewater and have shown similar, if not the same fragments under different collision energies [31].

2,3-dihydro-2,3-dihydroxy-CBZ was oxidized to 2,3-dihydroxy-CBZ (2,3-diOH-CBZ). The formation of dihydrodiols and subsequent diol compounds is a conserved mechanism in

bacterial degradation of PAHs such phenanthrene, naphthalene, fluoranthene, pyrene and benzopyrene and is generally achieved by dehydrogenases [36].

2,3-diOH-CBZ was oxidized to CBZ-2,3-quinone. This reaction can be catalyzed by a catechol oxidase, an enzyme of the polyphenol oxidase family. In plants, these enzymes are involved in oxidation of polyphenols to quinones. Their biological function remains enigmatic, but since they are induced under conditions of stress and pathogen attack, one of their functions seems to be involved in biotic and abiotic stress resistance [37]. Since there was a burst in the production of this TP during the first days only in inoculated samples (fig. 2), it is suggested that both strains trigger antioxidant responses in plant roots and therefore activate a set of responses against stress. The same reaction has been postulated to occur non-enzymatically during the oxidation of 1,2-dihydroxynaphthalene to 1,2- naphthoquinone in bacterial metabolism of naphthalene [38].

The 2,3-diol pathway exhibited a trend similar to the 10,11-diol pathway in its first steps (fig. 2). The evolution of 2,3-dihydro-2,3-dihydroxy-CBZ was comparable to those of 10,11-dihydro-10,11-dihydroxy-CBZ but approximately 30 % less. In contrast to 2,3-dihydro-2,3-dihydroxy-CBZ, 2,3-diOH-CBZ and CBZ-2,3-quinone were more significant in HRs inoculated with endophytic bacteria and specially with *R. radiobacter*. Moreover, 2,3-diOH-CBZ and CBZ-2,3-quinone were identified in negative controls inoculated with bacteria while there were negligible in autoclaved HRs (fig S1). From this it may be concluded that plants favour the first reaction involving CYP450s/peroxidases and epoxy hydrolases while bacteria prefer the 2,3-diol pathway where dehydrogenases and catechol oxidases would play an important role.

3.2.3. GSH pathway

When phase II metabolism was investigated, TPs resulting from sugars, malonic acid, amino acids or glutathione conjugation were searched for. Several TPs derived from GSH conjugates were identified (TP374, TP431 and TP560). To confirm the nature of these TPs, chemical conjugations with GSH and cysteine were performed *in vitro*. When CBZE was incubated with GSH, a molecular ion peak at m/z 560.1809 was observed. The respective ion chromatogram showed a double peak with retention time 4.99-5.04 min, corresponding to the GSH adduct (fig. S3A). Previous work had demonstrated that this double peak contains

indeed two different diastereomers formed in the absence of biological matrices [25]. Still, TP560 was found only in trace levels in HR cultures and HRs inoculated with endophytes but in greater amounts when autoclaved roots were grown with *R. radiobacter*. Identical retention and fragmentation patterns were obtained during collision experiments on the precursor ion in biological samples and in chemically synthesized GSH adducts (fig. S3B). These findings allowed us to finally identify TP560 as 10,11-dihydro-10-hydroxy-11-glutathionyl-CBZ (CBZE-GSH).

Following GSH conjugation, xenobiotic conjugates can further be degraded to the respective γ-glutamylcysteinyl- and subsequent cysteinyl-moieties by carboxypeptidases [39] or to cysteinylglycinyl- and subsequent cysteinyl- moieties by γ-glutamyl-transpeptidase (GGT), both in the vacuole [40]. Other studies have suggested a cytosolic pathway for the degradation of GSH conjugates to cysteinyl moieties, involving carboxypeptidase activity of the enzyme phytochelatin synthase (PCS) [41]. In the present study, cysteinylglycine adducts (TP431) were detected in the media of HR cultures void of or inoculated with endophytic bacteria. When autoclaved HRs were inoculated with endophytic bacteria, this metabolite was also detected suggesting that enzymes for GSH conjugation and successive degradation of CBZ are also present in bacteria (fig S1). Fragment ions with identical m/z ratios were identified when TP560 and TP431 were subjected to collision. Additionally, the retention time revealed a very similar hydrophobicity to CBZE-GSH leading us to the identification of TP431 as 10,11-dihydro-10-hydroxy-11-cysteinylglycinyl-CBZ (CBZE-cys-gly).

TP374, corresponding to the cysteine adduct was identified in all culture media. When CBZE was incubated with cysteine, a double peak at 4.88-4.92 min corresponding to a cysteine adduct was observed. Collision experiments on the precursor ion (m/z 374.1169 showed conserved fragments in biological and chemical samples (m/z = 180.0821, 210.0928, 253.0985, 313.1025, 339.0819), showing that in fact TP374 corresponds to 10,11-dihydro-10-hydroxy-11-cysteinyl-CBZ (CBZE-cys) (fig S4). TP374 was detected in autoclaved HR cultures at low levels only after 14 days of incubation suggesting chemical conjugation with free cysteine in the media (fig S1).

The γ-glutamylcysteinyl adduct was not found in the media of any of the experimental conditions. In HRs, this could be explained by a dominance of GGT activity against carboxypeptidases and phytochelatin synthase during the degradation of the GSH conjugate.

On the other hand, CBZE-cys-gly was identified in the media of HR cultures void of endophytic bacteria. Ferretti et al. demonstrated the existence of an apoplastic GGT in Barley roots, important for the recovery of extracellular GSH [43]. An apoplastic route for the degradation of GSH conjugates could explain the low content of the CBZE-cys-gly detected in growth media of HRs. In samples involving bacterial metabolism, was present at higher levels, revealing an important role of bacterial partners for the degradation of GSH conjugates. The trends of this TP and the final metabolite in the GSH degradation pathway (CBZE-cys) showed phases at different time points during the incubation where concentration decreases significantly (fig. 2). In the case of *D. nitroreducens*, CBZE-cys-gly decreases from the day 1 to the day 8 of incubation, to accumulate again up to the last day, whereas CBZE-cys decreases between day 8 and 14. In the case of *R. radiobacter*, CBZE-cys decreases from day 4 to 8, after a maximum in the fourth day, suggesting further degradation of the cysteine conjugate when plants are inoculated by endophytic bacteria. The TPs derived from this further catabolism were not identified in this study.

3.2.4. Acridine pathway

Cleavage of the carbamoyl group and rearrangement of the central ring of CBZ initiate the acridine sub-pathway. Several TPs with an acridine-related structure were found in the growth media (fig. 3). This pathway has previously been described in fungi under anoxic conditions [44] and several related TPs have been identified in soil after CBZ exposure and long aerobic incubation [30]. To our knowledge, no acridine-like metabolite has been identified in plants treated with CBZ so far. However, we can predict the order of reactions involved in this pathway from the observation of the trends and absolute amounts exhibited by each of the compounds.

The first TP of this pathway is 9-acridine carboxaldehyde which is formed from CBZ-10,11-epoxide after cleavage of the carbamoyl group and contraction of the seven-membered central ring to a six-membered ring. This compound is known to be toxic and reactive [45]. The aldehyde moiety is then cleaved to form acridine, a compound also known to be reactive and toxic in the aquatic environment [46]. Acridine can finally be oxidized to acridone (non-toxic) in two steps, with the formation of the intermediate 9-OH-acridine [47].

HR cultures showed an increase of the first three compounds accumulating at the end of the experiment. The total amount accumulated after 21 days decreased in a 10-fold manner in

each step of the pathway. These TPs were identified in the growth media of autoclaved HRs (fig S2) with trends and amounts similar to those exhibited in living roots. Acridone could barely be detected at the onset of the incubation, disappearing from the media after the fourth day. These results suggest that plants are lacking genes for the degradation of CBZ through the acridine pathway.

The inoculation with *R. radiobacter* induced this pathway strongly, leading to a burst in the synthesis of 9-acridine carboxaldehyde, acridine and 9-OH-acridine in the first days of incubation, reaching a maximum after 4 days. Acridone accumulated in these samples from the fourth day on, but this increase does not explain the decreasing trend of the upstream TPs. Therefore, we think that acridine is further metabolized in samples containing the endophytic strain *R. radiobacter*. Similar trends were observed when HRs were inoculated with *D. nitroreducens*. At the end of the incubation, even the acridone signal decreased, revealing again the existence of further steps in the metabolic pathway of acridine-like TPs.

3.1.5. Transformation products in root extracts

The content and composition of TPs identified in the media was investigated in root extracts after 21 days of incubation (fig. 4). The amount of CBZ found in root extracts inoculated with endophytic bacteria was twice as high as in HR extracts. This result confirmed the observations performed in experiments with concentrations 10 and 25 μM (fig. 1) and can be explained by an increase in the overall CBZ metabolism causing elevated CBZ diffusion into the symplast of inoculated HRs. The distribution of TPs of the diol pathways was similar in HRs and inoculated HRs with a slight higher prevalence for the 2,3-diol pathway in roots inoculated with *R. radiobacter*. Acridine-related TPs were much higher in HRs inoculated with *D. nitroreducens* (10 fold). In absolute numbers, this was the more important metabolic sub-pathway found in root extracts. Metabolites derived from GSH conjugation and related degradation products were present in higher amounts in non-inoculated HRs. Specially, CBZE-cys was accumulated in large amounts in root tissues after 21 days of incubation.

All these observations allow to dissect the overall metabolism of CBZ in HRs and the endophytic bacteria used in this study (fig. 5). However, as indicated previously, there is evidence of further metabolism in the case of GSH and acridine pathways in inoculated HRs for which TPs downstream could not be identified. Even though the relative amounts measured in the extracts after 21 days may slightly change the final picture, the potential applications of the results remain consistent.

4. Conclusions

Application of endophytic bacteria isolated previously from *P. australis*, a plant species that has frequently proven its ability for remediation of polluted water, can improve CBZ removal, favouring distinct degradation pathways than those exclusive for plants. Our results show the potential of hairy roots as a plant model system for interaction studies between plants and bacteria and provide valuable information for applications of the endophyte-plant synergy for wastewater treatment in constructed wetlands. The dissection of the metabolic pathway of CBZ in such a system reveals differences in metabolism compared to mammals, where mainly 10,11-diol and 2,3-diol pathways lead to the excretion of glucuronide conjugates. In addition, plants and their endophytic bacteria use the acridine pathway and a pathway involving GSH conjugation. Biotechnological approaches for the targeted inoculation of plant species utilized in phytoremediation might boost the metabolic capacities of such symbiotic systems, thus improving the performance of secondary or tertiary treatment in waste water treatment.

However, the nature of the metabolic pathways established in real conditions needs to be further investigated in inoculation experiments in CWs systems. Few compounds have been studied using both, HRs and whole macrophyte plants. The metabolites identified were conserved in both systems in the case of oxybenzone (Chen et al, 2016 and 2017) and diclofenac (Huber et al, 2009). Similar results are expected for CBZ when extrapolating to whole organisms in CWs, however, the importance of each sub-pathway may be different. Plants in CWs have high transpiration rates, and hence a big amount of the parent compound is expected to be translocated to aerial parts, where further metabolism (especially phase II plant metabolism) could result in a storage of the compound and its conjugates, eventually in vacuolar compartments. In addition, endophytic bacteria inhabiting aerial plant parts could enhance breakdown of translocated compounds. From the point of view of phytoremediation, both scenarios would fulfil the main purpose, namely to remove the parent compound and its reactive metabolites from polluted water. Of course, plant biomass should be harvested and used for bioenergetic purposes, to avoid a re-entry of metabolites and parent compound into water bodies.

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Figure captions

Table 1: CBZ structure and transformation products identified in the liquid media during degradation by *A. rusticana* hairy root cultures and endophytic bacteria *R. radiobacter* and *D. nitroreducens*.

Figure 1: CBZ removal in *A. rusticana* hairy root cultures and effect of inoculation with the endophytic bacteria *R. radiobacter* and *D. nitroreducens* isolated from *P. australis*. The left panel shows the removal of CBZ after 6 days of incubation, expressed in µg of CBZ removed per g of root tissue (FW). The right panel shows the total removal of CBZ in % during the incubation time. Data are means ± SD of three replicates. The values labelled by asterisk are statistically significant (Analysis of variance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with HR groups).

Figure 2: Evolution of CBZ transformation products during degradation by *A. rusticana* hairy root cultures and effect of the inoculation with the endophytic bacteria *R. radiobacter* and *D. nitroreducens* isolated from *P. australis*: 10,11 diol, 2,3-diol and GSH subpathways. Peak area is in unit of mAU/g tissue (FW). Data are means ± SD of three replicates.

Figure 3: Evolution of CBZ transformation products belonging to the acridine pathway during degradation by *A. rusticana* hairy root cultures and effect of the inoculation with the endophytic bacteria *R. radiobacter* and *D. nitroreducens* isolated from *P. australis*. Peak area is in unit of mAU/g tissue (FW). Data are means ± SD of three replicates.

Figure 4: Composition of the different transformation products identified in root extracts during CBZ metabolism in *A. rusticana* hairy roots (HRs) and hairy roots inoculated with the endophytic bacteria *R. radiobacter* (HRs 58) and *D. nitroreducens* (HRs 55). Peak area is in unit of mAU/g tissue (FW). Data are means ± SD of three replicates.

Figure 5: proposed CBZ metabolic pathways in A. rusticana hairy roots and hairy roots inoculated with the endophytic bacteria R. radiobacter and D. nitroreducens.Figure S1: Evolution of CBZ transformation products in controls corresponding to autoclaved *A. rusticana* hairy root cultures and autoclaved hairy roots inoculated with the endophytic bacteria *R. radiobacter* and *D. nitroreducens* isolated from *P. australis*: 10,11 diol, 2,3-diol and GSH subpathways. Peak area is in unit of mAU/g tissue (FW). Data are means ± SD of three replicates.

Figure1

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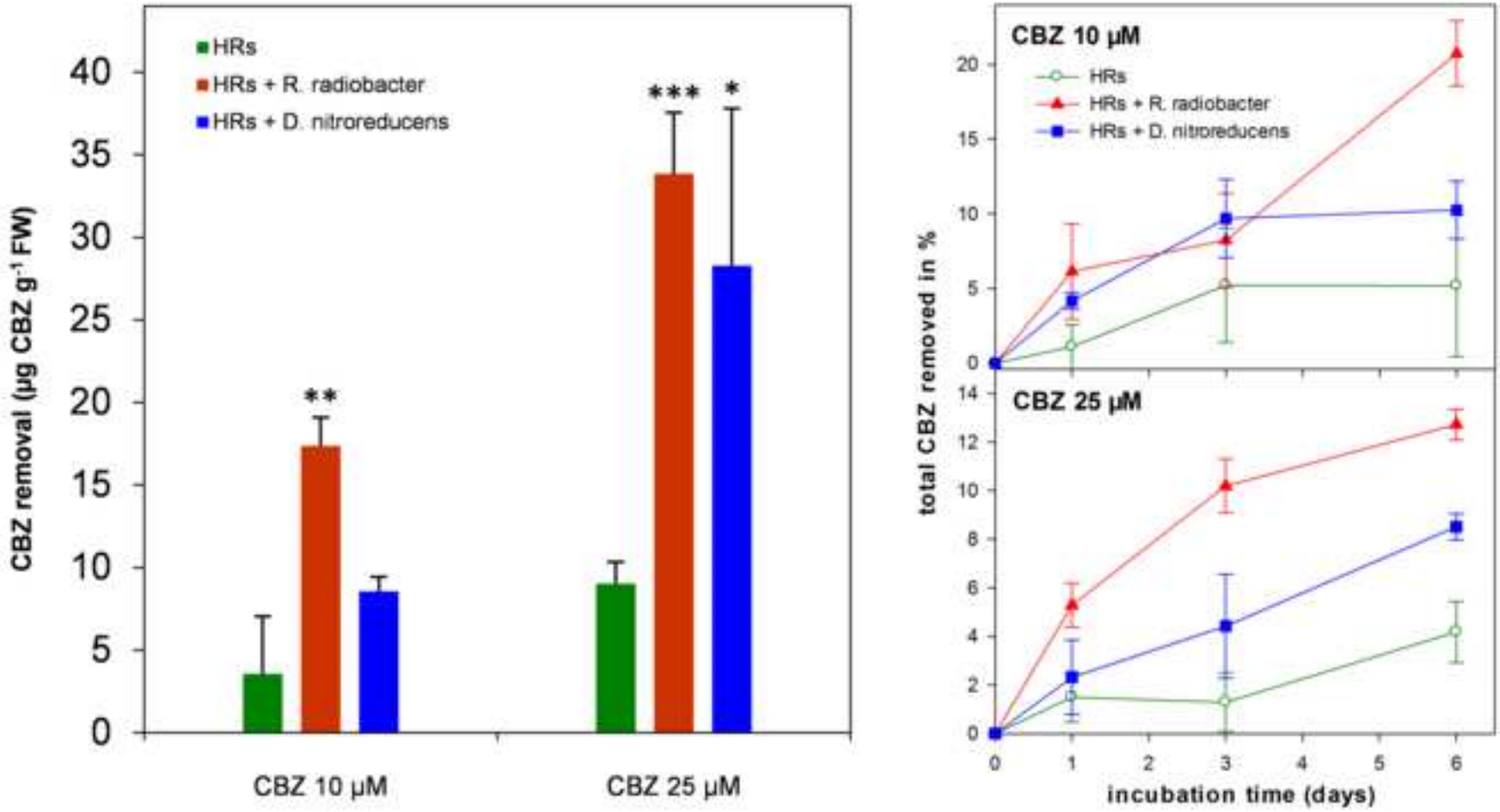

Figure2

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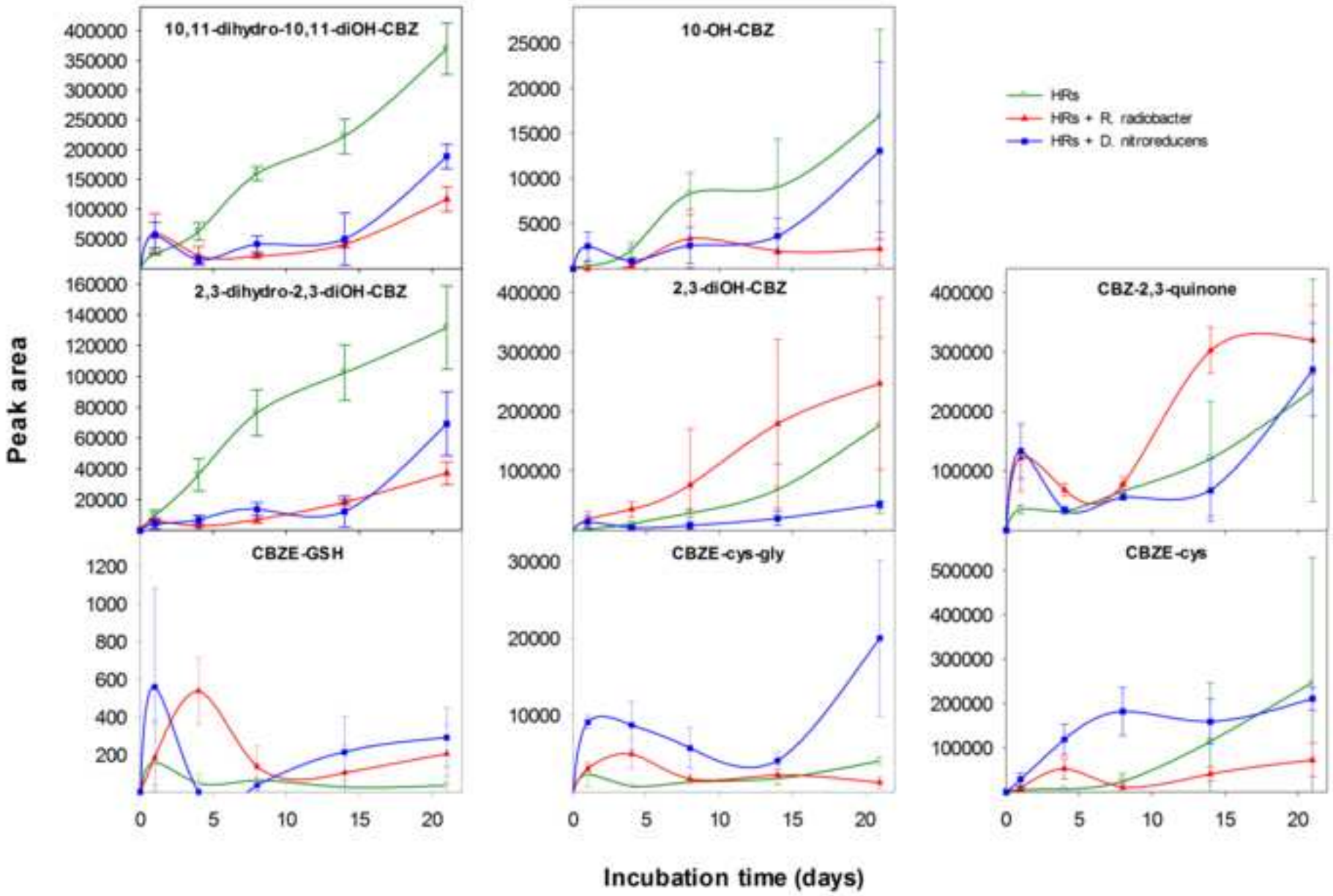

Figure3

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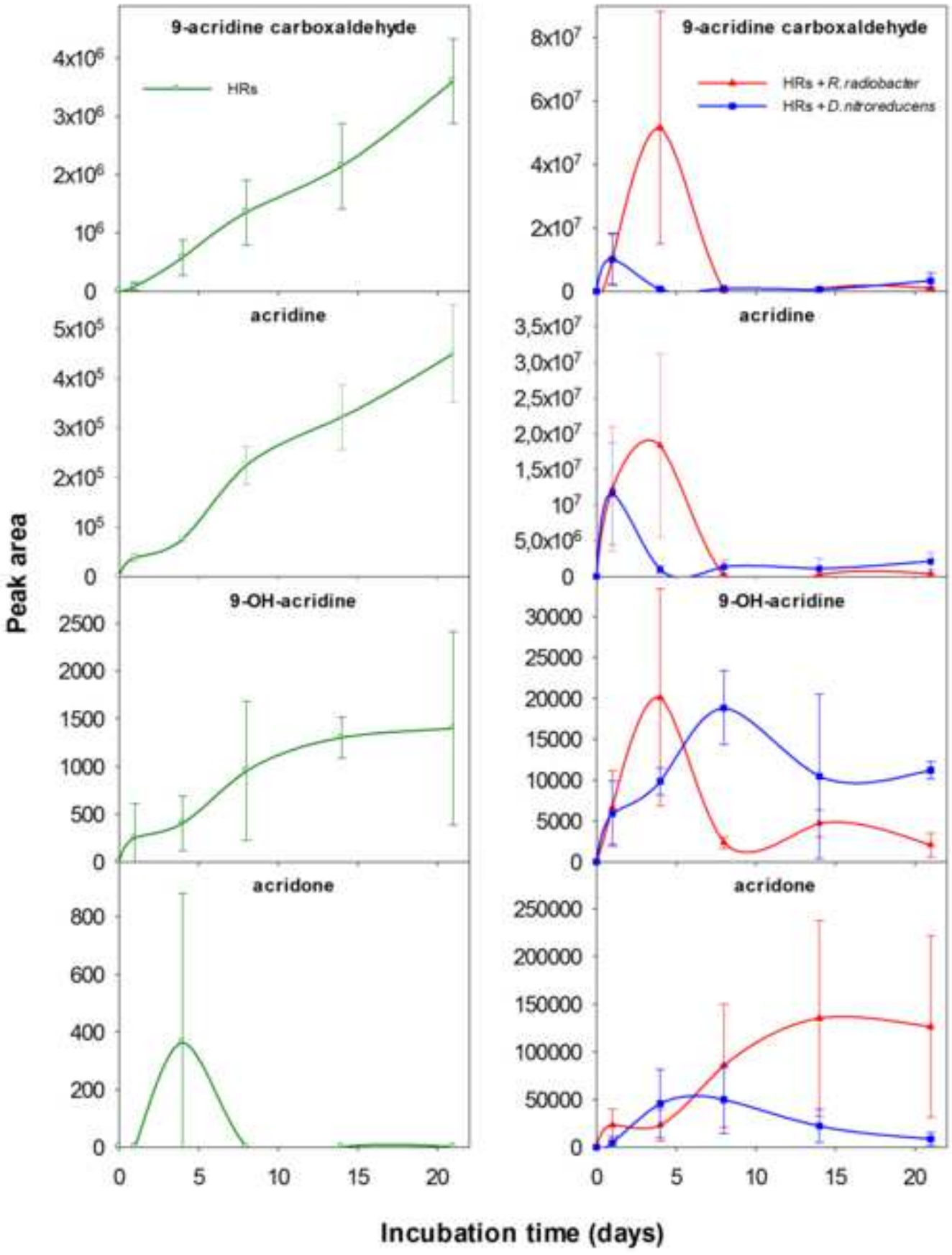

Figure4

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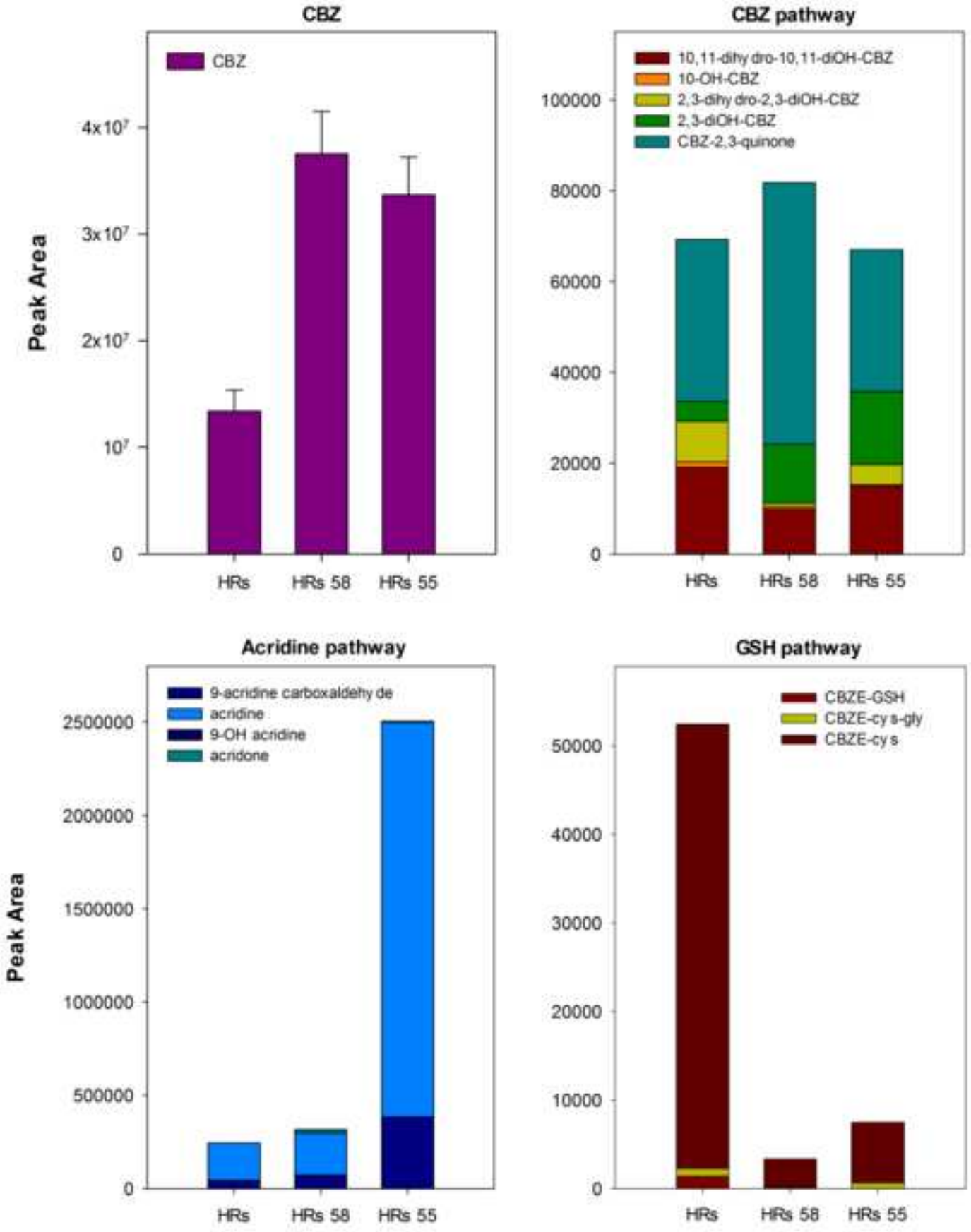

Figure5

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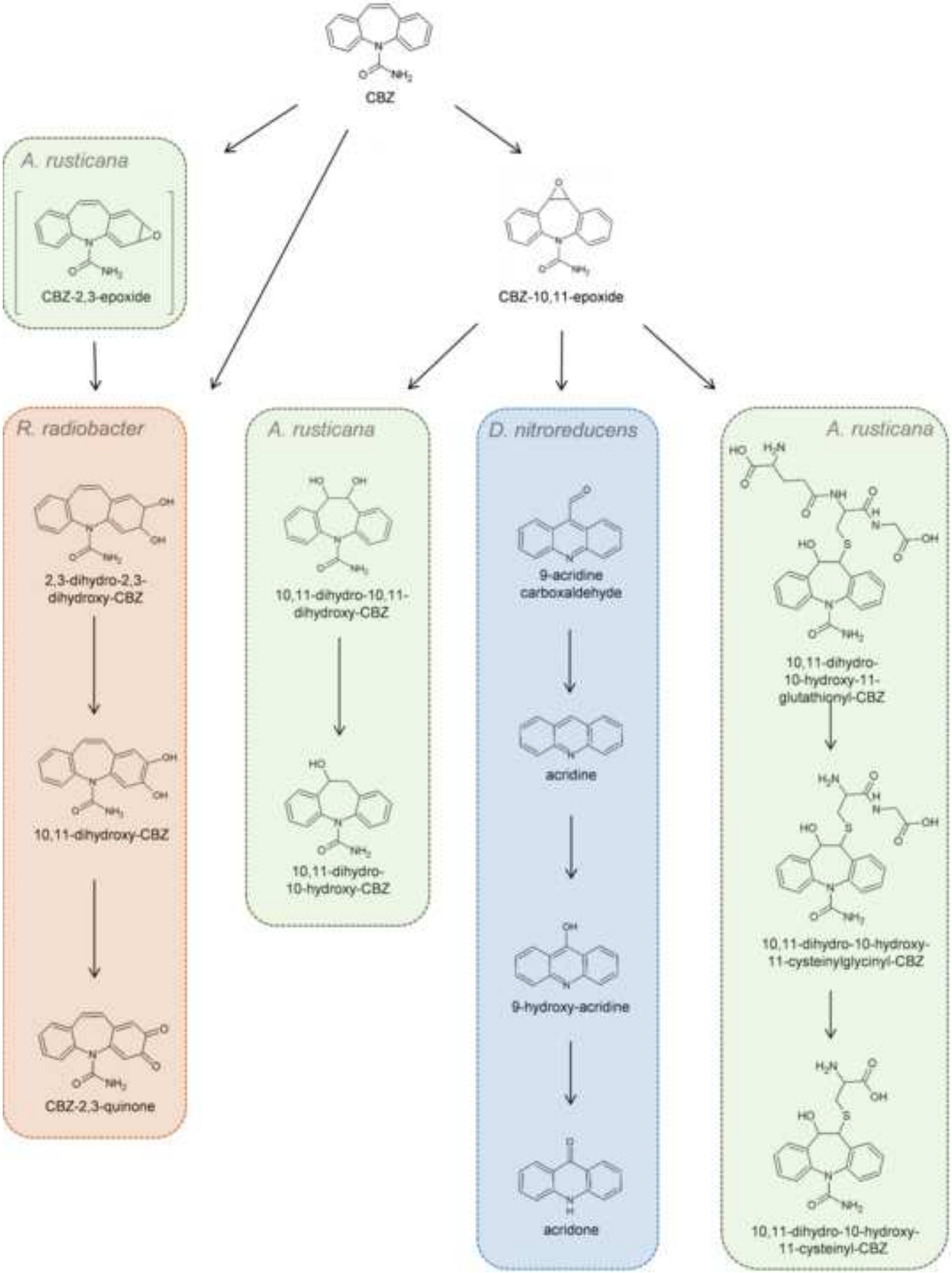
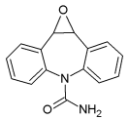
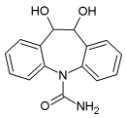
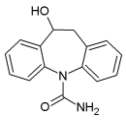
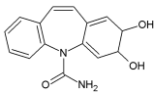
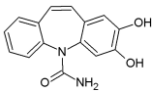
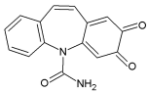
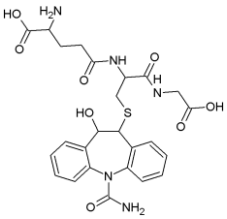
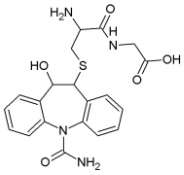
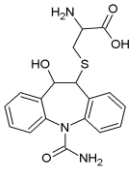
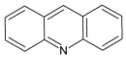
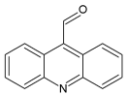
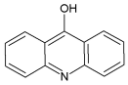
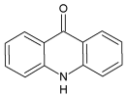


Table

Name	Exact Mass (Da)	Chemical formula	Structure	Identification	Rt (min)	log Kow
CBZ-10,11-epoxide	253.0971	C15H12N2O2		Standard	6.02	2.54
10,11-dihydro-10,11-dihydroxy-CBZ	271.1077	C15H14N2O3		Standard	5.37	1.16
10,11-dihydro-10-hydroxy-CBZ	255.1128	C15H14N2O2		Standard	5.67	2.15
2,3-dihydro-2,3-dihydroxy-CBZ	271.1077	C15H14N2O3		Proposed	5.7	0.49
2,3-dihydroxy-CBZ	269.0921	C15H12N2O3		Proposed	5.82	1.85
CBZ-2,3-quinone	267.0764	C15H10N2O3		Proposed	5.67	1.36
CBZE-GSH	560.1809	C25H29N5SO8		Proposed	5	-3.83
CBZE-cys-gly	431.1384	C20H22N4SO5		Proposed	4.93	-1.25
CBZE-cys	374.1169	C18H19N3SO4		Synthesized	4.87	-0.90
acridine	180.0808	C13H9N		Standard	4.95	3.17
9-acridine carboxaldehyde	208.0757	C14H9NO		Proposed	7.8	3.49
9-OH-acridine	196.0757	C13H9NO		Proposed	4.8	3.48
acridone	196.0757	C13H9NO		Standard	6.5	2.57

CBZ analysis:

CBZ concentration in HR nutrient solutions was determined by high performance liquid chromatography (HPLC) using a Varian ProStar HPLC system (Varian ProStar 215 solvent delivery module, autosampler Prostar 410). All samples were prepared in triplicate. Plant nutrient solution samples were filtered using 0.45 µm pore size polyvinylidene fluoride filters (Rotilabo, Carl Roth) before injection. Injection volume was 40 µL. The separation was performed on a C18 ProntoSIL Spheribond ODS 2 (5 µM, 125 x 4 mm, Bischoff) column under reversed phase conditions, applying a linear gradient of eluents (buffer A: H2O, 0.1% TFA; buffer B: acetonitrile, 0.1% TFA) and a flow rate of 1 mL/min. The gradient started with 5% B for 2.5 min, ramped up to 95% in 15 min, remained at 95% for 3.5 min and finally ramped down to 5% in 2 min. CBZ was measured at 210 nm in a photodiode array detector (Varian ProStar 335) and identified by comparison of the spectra and retention time of an authentic standard (Sigma-Aldrich). Calibration curves were constructed from a set of carbamazepine standard solutions ranging from 0.25 to 12 mg/L (1.04 to 50 µM). Chromatograms were analyzed using MS Workstation version 6.9.3 (Varian).

Synthesis of GSH and CYS conjugates

The conjugates 10,11-dihydro-10-hydroxy-11-glutationyl-CBZ (CBZE-GSH) and 10,11-dihydro-10-hydroxy-11-cysteinyl-CBZ (CBZE-cys) were synthesized chemically according to [25]. Briefly, CBZE (80 µM) was incubated with GSH or cysteine (5 mM) in 0.1 M potassium phosphate buffer (pH 7.4) for 2 h at 37°C. Samples were evaporated under N_2 after mixing them with 2 volumes of acetonitrile. Residues were reconstituted in 0.1 % formic acid in ultrapure water (MS grade) and stored at -20°C before analysis. The identity of the synthesized metabolites was confirmed after MS/MS experiments based on previous results validated by NMR spectroscopy [25]. After an incubation of 2 h with GSH, three peaks were identified in the chromatogram as GSH (Rt = 2.0 min), CBZE (Rt = 6.02 min) and CBZE-GSH (Rt = 5.0 min), with a conjugation of 20% and indications of increasing rates with longer incubation. After an incubation of 2 h with cysteine, three peaks were identified in the chromatogram as cys (Rt = 0.80 min), CBZE (Rt = 6.02 min) and CBZE-cys (Rt = 4.87 min), with a conjugation success of 15% and indications of higer rates with longer incubation.

Reference

[25] H. Bu, P. Kang, A. Deese, P. Zhao, W. Pool, Human in vitro glutathionyl and protein adducts of carbamazepine-10, 11-epoxide, a stable and pharmacologically active metabolite of carbamazepine, Drug Metab. Dispos. 33 (2005) 1920–1924. doi:10.1124/dmd.105.006866.plored.

Supplementary data- Figures captions

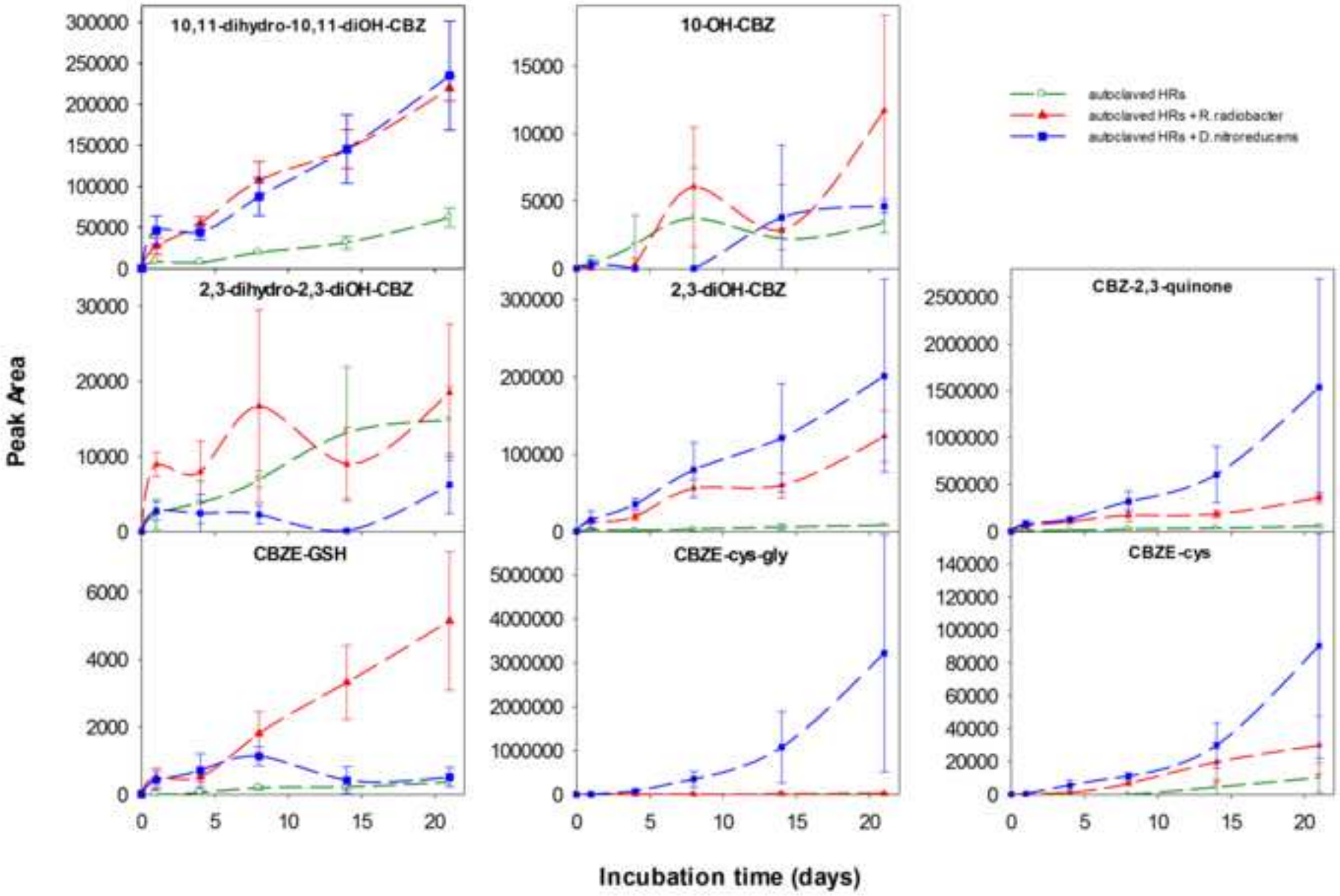
Figure S1: Evolution of CBZ transformation products in controls corresponding to autoclaved *A. rusticana* hairy root cultures and autoclaved hairy roots inoculated with the endophytic bacteria *R. radiobacter* and *D. nitroreducens* isolated from *P. australis*: 10,11 diol, 2,3-diol and GSH subpathways. Peak area is in unit of mAU/g tissue (FW). Data are means ± SD of three replicates.

Figure S2: Evolution of CBZ transformation products belonging to the acridine pathway in controls corresponding to autoclaved *A. rusticana* hairy root cultures and autoclaved hairy roots inoculated with the endophytic bacteria *R. radiobacter* and *D. nitroreducens* isolated from *P. australis*. Peak area is in unit of mAU/g tissue (FW). Data are means ± SD of three replicates.

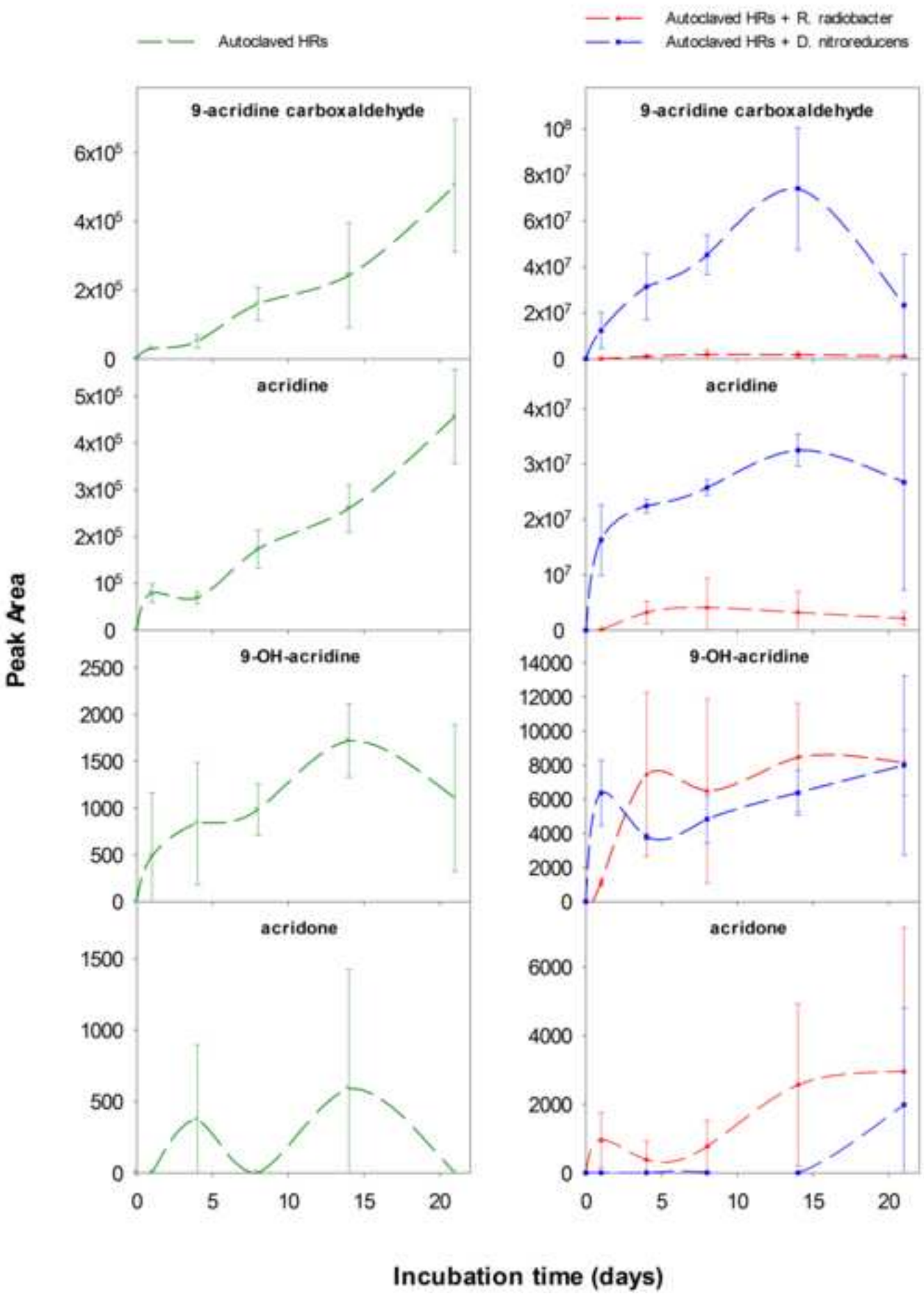
Figure S3: Chromatograms showing the elution and fragmentation pattern of the GSH conjugate formed with carbamazepine-10,11-epoxide after chemical synthesis (A) and in biological samples (B).

Figure S4: Chromatograms showing the elution and fragmentation pattern of the cysteine conjugate formed with carbamazepine-10,11-epoxide after chemical synthesis (A) and in biological samples (B).

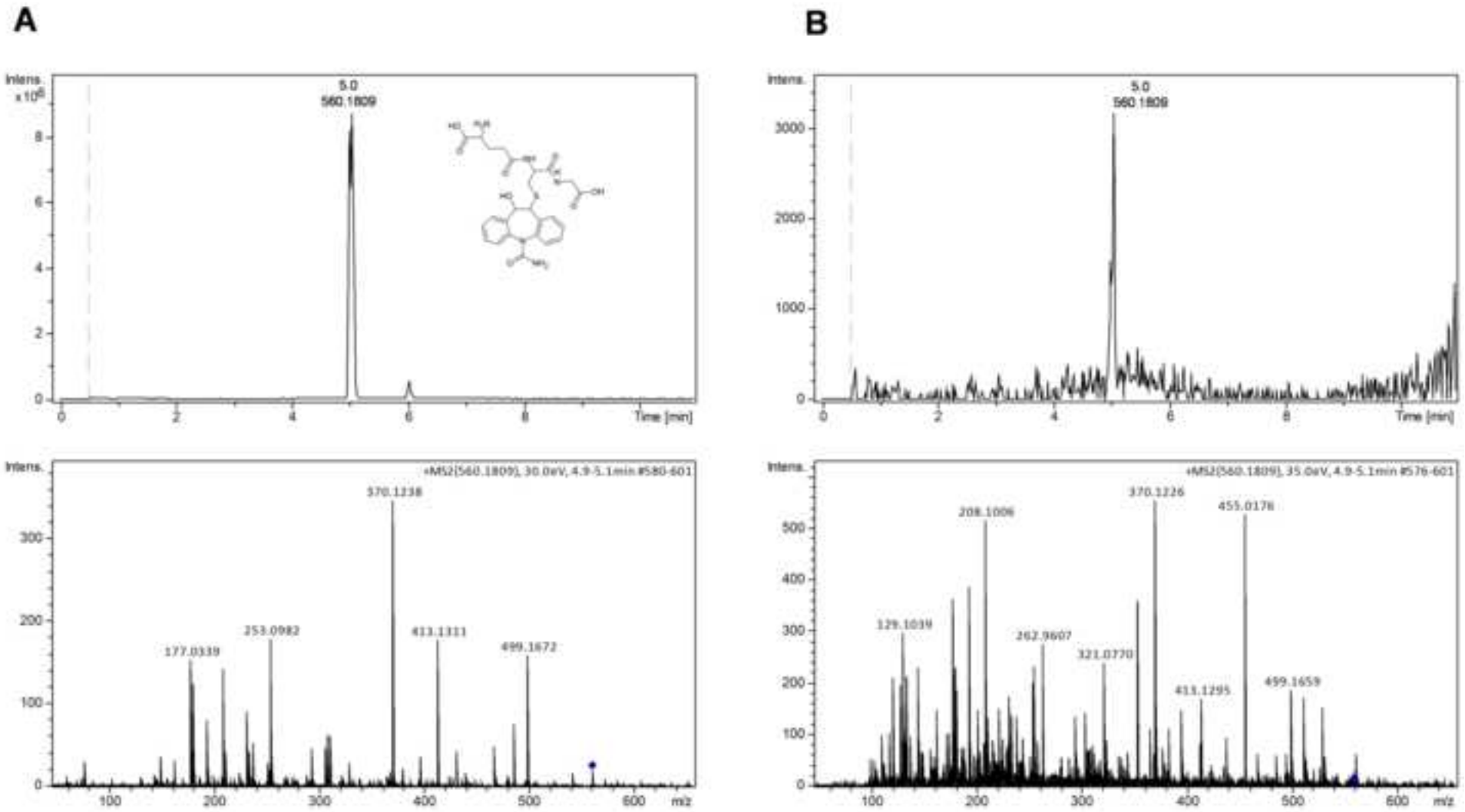
Supplementary Fig.1



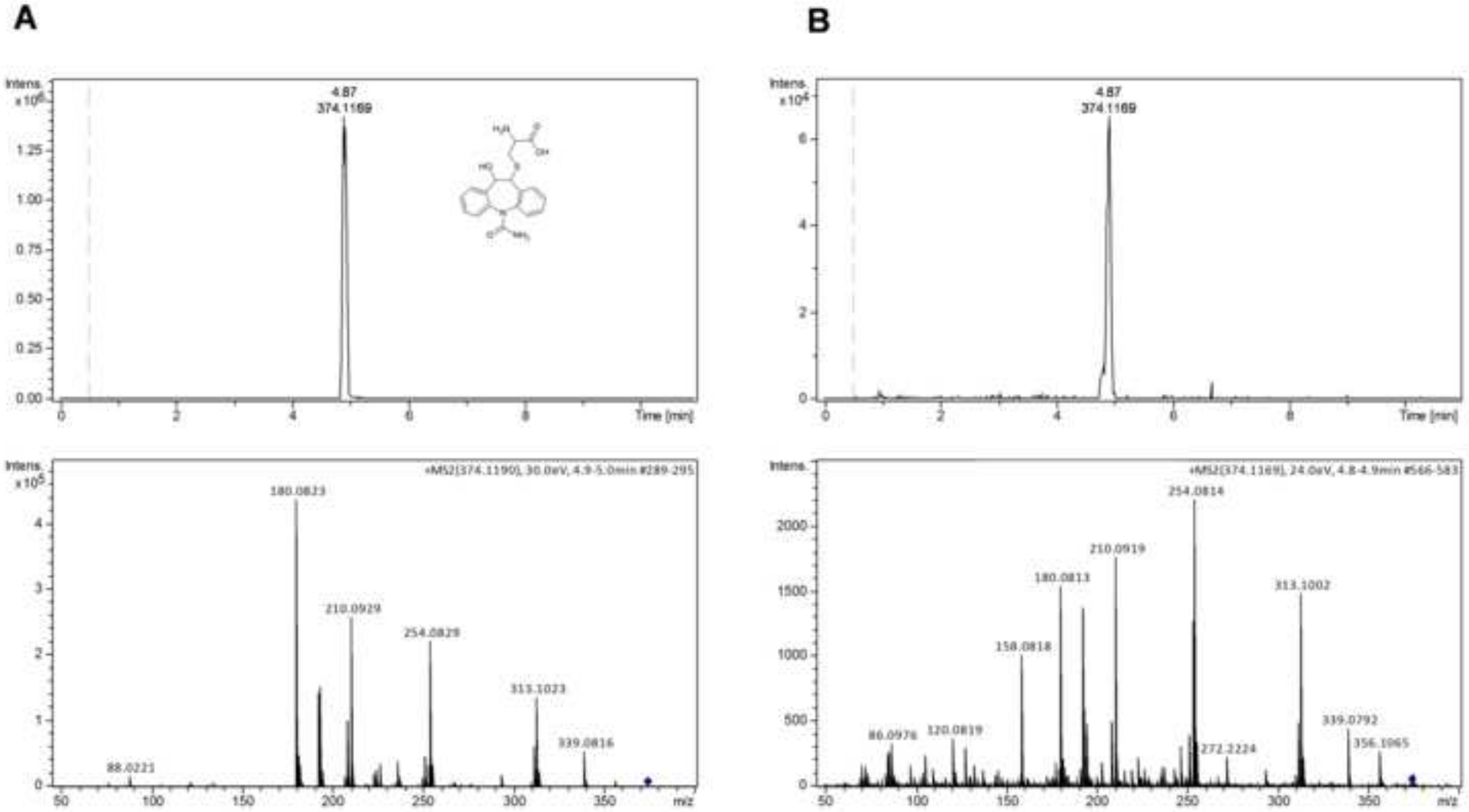
Supplementary Fig.2



Supplementary Fig.3



Supplementary Fig.4



*Abstract

Abstract:

Carbamazepine (CBZ) is a pharmaceutical frequently categorized as a recalcitrant pollutant in the aquatic environment. Endophytic bacteria previously isolated from reed plants have shown the ability to promote growth of their host and to contribute to CBZ metabolism.

In this work, a horseradish (*Armoracia rusticana*) hairy root (HR) culture has been used as a plant model to study the interactions between roots and endophytic bacteria in response to CBZ exposure. HRs could remove up to 5% of the initial CBZ concentration when they were grown in spiked Murashige and Skoog (MS) medium. Higher removal rates were observed when HRs were inoculated with the endophytic bacteria *Rhizobium radiobacter* (21%) and *Diaphorobacter nitroreducens* (10%). Transformation products resulting from CBZ degradation were identified using liquid chromatography−ultra high-resolution quadrupole time of flight mass spectrometry (LC-UHR-QTOF-MS). CBZ metabolism could be divided in four pathways. Metabolites involving GSH conjugation and 2,3-dihydroxylation, as well as acridine related compounds are described in plants for the first time.

This study presents strong evidence that xenobiotic metabolism and degradation pathways in plants can be modulated by the interaction with their endophytic community. Hence it points to plausible applications for the elimination of recalcitrant compounds such as CBZ from wastewater in CWs.

***Novelty Statement (maximum limit:100 words)**

Statement of novelty

This manuscript covers the metabolism of a widely used pharmaceutical in hairy root cultures. Recently, carbamazepine attracted attention due to its persistence in water. Few studies show its degradation in mammals, uptake in plants and partial metabolism. Evidence for alternative methods like phytoremediation is urgently required. We demonstrate for the first time that plants spiked with endophytes degrade carbamazepine in four parallel pathways. No comparable literature has been published, to our best knowledge. The scientific impact will be extremely high and meet the interest of a broad readership.

Highlights

- Hairy root cultures are a suitable tool to study plant microbe interactions in response to xenobiotic stress
- *Rhizobium radiobacter* and *Chriseobacterium nitroreducens*, endophytes in *P. australis*, improve CBZ removal
- CBZ metabolism undergoes four different pathways in hairy root cultures
- A CBZ-GSH conjugate and its degradations products were identified for the first time in horseradish hairy roots
- Specific metabolic pathways are activated when HRs are inoculated with endophytic bacteria