



# Formation of biologically active benzodiazepine metabolites in *Arabidopsis thaliana* cell cultures and vegetable plants under hydroponic conditions

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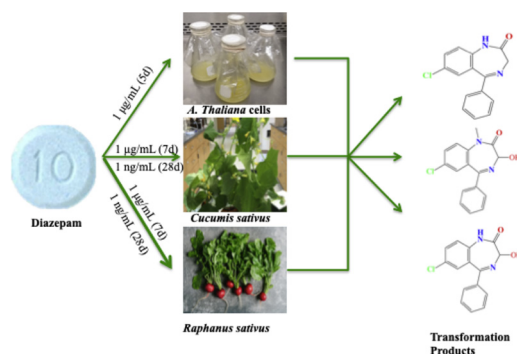
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## HIGHLIGHTS

- *Arabidopsis thaliana* cells and cucumbers and radishes can uptake and metabolize the diazepam
- Metabolism of diazepam in resulted in the formation of the bioactive metabolites
- Extensive mineralization was observed for radish seedlings
- Exposure to diazepam resulted in changes to glycosyltransferase activity

## GRAPHICAL ABSTRACT



## ARTICLE INFO

### Article history:

Received 10 September 2018

Received in revised form 18 January 2019

Accepted 18 January 2019

Available online 22 January 2019

Editor: Damia Barcelo

### Keywords:

Contaminants of emerging concern

Pharmaceuticals

Benzodiazepines

Diazepam

Metabolism in plants

## ABSTRACT

The use of recycled water for agricultural irrigation comes with the concern of exposure to crops by contaminants of emerging concerns (CECs). The concentration of CECs in plant tissues will depend on uptake, translocation and metabolism in plants. However, relatively little is known about plant metabolism of CECs, particularly under chronic exposure conditions. In this study, metabolism of the pharmaceutical diazepam was investigated in *Arabidopsis thaliana* cells and cucumber (*Cucumis sativus*) and radish (*Raphanus sativus*) seedlings grown in hydroponic solution following acute (7 d)/high concentration (1 mg L<sup>-1</sup>), and chronic (28 d)/low concentration (1 µg L<sup>-1</sup>) exposures. Liquid chromatography paired with mass spectrometry, <sup>14</sup>C tracing, and enzyme extractions, were used to characterize the metabolic phases. The three major metabolites of diazepam - nordiazepam, temazepam and oxazepam - were detected as Phase I metabolites, with the longevity corresponding to that of human metabolism. Nordiazepam was the most prevalent metabolite at the end of the 5 d incubation in *A. thaliana* cells and 7 d, 28 d seedling cultivations. At the end of 7 d cultivation, non-extractable residues (Phase III) in radish and cucumber seedlings accounted for 14% and 33% of the added <sup>14</sup>C-diazepam, respectively. By the end of 28 d incubation, the non-extractable radioactivity fraction further increased to 47% and 61%, indicating Phase III metabolism as an important destination for diazepam. Significant changes to glycosyltransferase activity were detected in both cucumber and radish seedlings exposed to diazepam. Findings of this study highlight the need to consider the formation of bioactive transformation intermediates and different phases of metabolism to achieve a comprehensive understanding of risks of CECs in agroecosystems.

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## 1. Introduction

Global climate change has resulted in shifts in precipitation patterns, causing stress on freshwater resources, especially in arid and semi-arid regions (Walther et al., 2002; Trenberth, 2011). In many of these areas, demand for water has led to increasing use of municipally treated wastewater (TWW) (Lazarova et al., 2001; Tal, 2006). Agriculture has been one of the primary targets for TWW reuse with water districts and governments promoting the adoption of recycled water for irrigation (Chen et al., 2013; Elgallal et al., 2016; Marcus et al., 2017). However, the use of TWW for irrigation may come with potential risks, as TWW is known to contain a wide variety of human pharmaceuticals (Ternes et al., 2004; Xia et al., 2005; Kolpin et al., 2002).

The use of pharmaceutical compounds has increased with population growth and economic development, resulting in over 1500 compounds currently in circulation (Guo et al., 2016). Their widespread consumption has led to their occurrence in TWW as well as in TWW-impacted surface water (Cunha et al., 2017; Xiang et al., 2018). For many of these pharmaceuticals, there is limited knowledge about their potential chronic effects in the environment (Archer et al., 2017; Bourdat-Deschamps et al., 2017). Further, many of these compounds can transform in the environment, resulting in the formation of transient or recalcitrant transformation products, many with unknown fates and effects in environmental compartments (Fu et al., 2017b).

Diazepam belongs to the class of psychoactive compounds known as benzodiazepines, one of the most prescribed classes of pharmaceuticals (Bachhuber et al., 2016). Diazepam is one of the most commonly detected pharmaceuticals in TWW, with concentration ranging from  $\text{ng L}^{-1}$  to low  $\mu\text{g L}^{-1}$  (Kosjek et al., 2012). This is likely due to its extensive use and low removal efficiency (~8%) during secondary wastewater treatment (Verlicchi et al., 2012).

In humans, diazepam is primarily metabolized via phase I oxidative metabolism by demethylation to nordiazepam (major pathway), or hydroxylation to temazepam (minor pathway), and then further oxidized to oxazepam (de Angelis et al., 1974; Wishart et al., 2018). Oxazepam undergoes phase II metabolism via rapid glucuronidation and then excretion via urine (Sonne, 1993). The three primary metabolites of diazepam are psychoactive compounds, and each is a prescribed pharmaceutical for treating psychological conditions and alcohol withdrawal symptoms (Clarke and Nicholson, 1978; Malcolm et al., 1989; Fraser et al., 2001). Both oxazepam and nordiazepam have been commonly detected in TWW, often at  $\mu\text{g L}^{-1}$  levels (Kosjek et al., 2012). However, there is little knowledge about the occurrence, formation, and fate of such metabolites outside the wastewater treatment systems (Boxall et al., 2012).

Several studies have focused on the uptake and accumulation of pharmaceuticals in agricultural plants as a result of TWW irrigation (e.g., Wu et al., 2013, 2014; Goldstein et al., 2014; Carter et al., 2014). These studies have demonstrated the capacity of higher plants to take up these compounds; however, until recently, relatively little consideration has been given to their metabolism in plants (Fu et al., 2017a, 2017b; He et al., 2017; Huber et al., 2009, 2012; Wu et al., 2015). Recent studies have shown that higher plants can metabolize xenobiotics similarly to humans with phase I modification reactions followed by phase II conjugation reactions using detoxification enzymes that function as a 'green liver' (Sandermann, 1992). In higher plants, phase I and phase II reactions are followed by a phase III sequestration, resulting in the formation of bound residues (Sandermann, 1994). Many of these studies have also highlighted a chemical-specific and species-specific nature of plant metabolism of pharmaceuticals.

In this study, we examined the uptake and biotransformation of diazepam in higher plants. *Arabidopsis thaliana* cells were used for an initial kinetic evaluation and metabolic profiling (van Poecke and Dicke, 2004; Huber et al., 2009). Cucumber (*Cucumis sativus*) and radish seedlings (*Raphanus sativus*) were then used under hydroponic conditions to understand metabolism of diazepam and its effect on selected metabolic enzymes in whole plants.

## 2. Materials and methods

### 2.1. Chemicals and solvents

Analytical standards of non-labeled diazepam, nordiazepam, oxazepam, temazepam, and oxazepam-glucuronide were purchased from Sigma-Aldrich (St. Louis, MO). Diazepam-d5 was purchased from Fisher Scientific (Fair Lawn, NJ) and  $^{14}\text{C}$ -labeled diazepam (50–60  $\text{mCi/mmol}$ , N-methyl- $^{14}\text{C}$ ) was purchased from American Radiolabeled Chemicals (Saint Louis, MO). Standards were prepared in HPLC grade methanol and stored at  $-20\text{ }^\circ\text{C}$  before use. HPLC grade acetonitrile and methanol were used for extraction along with ultrapure water. Mobile phases were prepared using Optima™ grade methanol and deionized water (18 M $\Omega$ ). All solvents were purchased from Fisher (Fair Lawn, NJ).

### 2.2. Incubation experiments using *Arabidopsis thaliana* cells

PSB-D *A. thaliana* cell line (CL84840) was purchased from the Arabidopsis Biological Resource Center (ARBC) at Ohio State University (Columbus, OH) and cultured in a liquid culture suspension at  $25\text{ }^\circ\text{C}$  and 130 rpm in the dark. Cell cultures were maintained in accordance with the ARBC maintenance protocol (Gehring et al., 2018). The *A. thaliana* seed culture was produced by inoculating 7 mL of cell culture into 43 mL fresh growth media, followed by 96 h cultivation at  $25\text{ }^\circ\text{C}$  on a rotary shaker (130 rpm) in the dark. After 96 h, 3 mL of the seed culture was inoculated into 27 mL fresh growth media to create an approximate initial cell density of 3.3 g (dry weight  $\text{g L}^{-1}$ ). Flasks were spiked with 30  $\mu\text{L}$  of a stock solution of diazepam ( $1.0\text{ mg mL}^{-1}$ ) and 10  $\mu\text{L}$  of a  $^{14}\text{C}$ -diazepam stock solution ( $0.01\text{ mCi mL}^{-1}$ ) to yield an initial concentration of  $1\text{ }\mu\text{g mL}^{-1}$  and a specific radioactivity of  $7.4 \times 10^3\text{ dpm mL}^{-1}$  with an initial methanol content of 0.13% (v/v). Simultaneously, control treatments were prepared by autoclaving cell suspension flasks before chemical spiking (non-viable cell control), flasks containing diazepam without cells (medium control), and flasks containing living cells without diazepam (background control). Control treatments were used to determine adsorption, abiotic degradation, and potential toxicity to cells. Flasks were incubated for 120 h in triplicate and sacrificed at 0, 6, 12, 24, 48 and 96 h for sampling and analysis.

At each sampling time point, samples were collected and centrifuged at 13,000g for 15 min in 50 mL polypropylene tubes. The supernatant was collected and stored at  $-20\text{ }^\circ\text{C}$  until further analysis. Cells were immediately stored at  $-80\text{ }^\circ\text{C}$  and then freeze-dried for 72 h. After drying, each sample was spiked with 50  $\mu\text{L}$  of  $10\text{ mg L}^{-1}$  diazepam-d5 as a surrogate for extraction-recovery calibration and extracted using a method from Wu et al. (2012), with minor modifications. Briefly, cells were sonicated (50/60 Hz) for 20 min with 20 mL methyl tert-butyl ether and then 20 mL of acetonitrile and centrifuged at 13,000g for 15 min. The supernatants were combined and concentrated to near dryness under nitrogen at  $35\text{ }^\circ\text{C}$  and then reconstituted in 1 mL of methanol. The cells were then extracted with 20 mL acidified deionized water (pH 3) and the supernatant was combined with the methanol extract for clean-up. Prior to clean-up, 100  $\mu\text{L}$  of cell material extract and growth media were combined with 5 mL liquid scintillation cocktail I (R. J. Harvey Instruments, Tappan, NY) to measure the radioactivity in the extractable form on a Beckman LS500TD Liquid Scintillation Counter (LSC) (Fullerton, CA).

Clean-up was carried out using solid phase extraction (SPE) with 150 mg Waters Oasis® HLB cartridges that were preconditioned with 7 mL methanol and 14 mL deionized water. Samples were loaded onto cartridges and then eluted with 20 mL methanol under gravity. The eluate was dried under nitrogen and further recovered in 1.5 mL methanol:water (1:1, v/v). After re-suspension extracts were transferred to microcentrifuge tubes and centrifuged at 12,000g in a tabletop d2012 Micro-Centrifuge (SciLogex, Rocky Hill, CT). Samples were further filtered through a 0.22- $\mu\text{m}$  polytetrafluoroethylene membrane (Millipore, Carrigtwohill, Cork, Ireland) into 2 mL glass vials and stored at  $-20\text{ }^\circ\text{C}$

before analysis. Extraction of growth media was done after adjusting the solution to pH 3 using HCl, and followed by SPE with Waters HLB cartridges, as described above. The extraction recoveries for the tissues and media were  $88 \pm 7\%$  and  $80 \pm 14\%$ , respectively.

After extraction, the cell matter was air dried, and a 10-mg subsample was removed and combusted on a Biological Oxidizer OX-500 (R. J. Harvey Instruments, Tappan, NY) to determine the radioactivity in the non-extractable form. The evolved  $^{14}\text{CO}_2$  was captured in 15 mL Harvey Carbon-14 Cocktail II (R. J. Harvey Instruments, Tappan, NY) and analyzed on a LSC.

### 2.3. Whole plant hydroponic cultivation

Hydroponic cultivations were carried out using cucumber (*Cucumis sativus* cv. *sumter*) and radish (*Raphanus sativus*, cv. *cherry belle*) seedlings. Seeds were purchased from Lowes (Murrieta, CA) and germinated in a commercially labeled organic potting soil in a growth chamber (22 °C 16 h day/20 °C 8 h night cycle; a photosynthetic photon flux density of  $200 \mu\text{mol}/\text{m}^2 \text{ s}^{-1}$ , relative humidity of 75–80%). After the appearance of the first true leaf, uniform seedlings were selected, rinsed with distilled water, and individually placed in amber jars containing 900 mL hydroponic solution (Oasis® 16–4–17 hydroponic fertilizer  $4.16 \text{ g L}^{-1}$ ). After 3 d of adaption, plants were exposed to diazepam by spiking with 100  $\mu\text{L}$  of the above stock solutions to reach a nominal concentration of  $1 \text{ mg L}^{-1}$  and an initial specific radioactivity of  $2.5 \times 10^3 \text{ dpm L}^{-1}$ . The cultivation lasted for 7 d.

A parallel treatment (low concentration/chronic contact) with an initial diazepam concentration at  $1 \mu\text{g L}^{-1}$  and a specific radioactivity of  $2.5 \times 10^2 \text{ dpm}$  was included to simulate more realistic exposure levels and to validate the high level treatments. The cultivation lasted for 28 d with the culture solution renewed every 3 d. Plant blanks (hydroponic solution in jars with plants but without diazepam) and treatment blanks (spiked culture solution in jars without plants) were placed alongside the treatment jars.

At the end of 7 d or 28 d cultivation, the seedlings were removed from the jars. Before sample preparation, roots were rinsed thoroughly with distilled water. Harvested plants were separated into below ground biomass (roots) and above ground biomass (shoots). Flowering buds from cucumbers were also separated to observe any potential for accumulation in fruits. Tissues were freeze-dried and then stored at  $-80 \text{ }^\circ\text{C}$  until analysis. A 0.2-g aliquot of the dried plant tissue was ground to a fine powder using a mortar and pestle. Samples were extracted and prepared as described above. Samples of hydroponic solution were filtered with  $0.7 \mu\text{M}$  GF/F filter and acidified to pH 2 with 1 M HCl, followed by SPE extraction using Oasis HLB cartridges.

### 2.4. Glycosyltransferase activity assay

To determine glycosyltransferase activity, fresh tissues of cucumber and radish plants were frozen in liquid nitrogen and homogenized with 2 mL 50 mM phosphate-buffered saline (pH 7.0) containing 1% soluble polyvinylpyrrolidone and 5 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at  $13,000g$  and  $4 \text{ }^\circ\text{C}$  for 20 min. The activity of glycosyltransferase was measured immediately by mixing 100  $\mu\text{L}$  of supernatant with 0.95 mL of the reaction mixture containing 50 mM PBS (pH 7.0), 2 mM  $\text{MgCl}_2$ , 2 mM uridine 5'-diphosphoglucose, 3.125 mM 4-nitrophenyl  $\beta$ -D-glucuronide, 3.125 salicin and 0.95 mL of 1 mM 2,4,5-trichlorophenol (TCP). The assay mixture was incubated at  $30 \text{ }^\circ\text{C}$  for 30 min, and then stopped by adding 10  $\mu\text{L}$  of phosphoric acid. After centrifugation at  $13,000g$  for 5 min, the supernatant was collected and diluted (1:4; v/v) with HPLC grade acetonitrile and 0.1% trifluoroacetic acid (TFA).

The enzyme activity was determined using an Agilent 1200 series HPLC paired with UV detector and a Thermo Scientific Acclaim™ 120 C18 5- $\mu\text{m}$  column ( $4.6 \times 250 \text{ mm}$ ). An isocratic flow was set with  $1 \text{ mL min}^{-1}$  70:30 mobile phase A (acetonitrile and 0.1% TFA) and

mobile phase B (water and 0.1% TFA) for 10 min. The TCP-glucoside was detected at 205 nm. A six-point ( $400 \mu\text{M}$ – $2000 \mu\text{M}$ ) TCP standard calibration curve was used to determine activity.

### 2.5. Structural elucidation using mass spectrometry

Instrument analysis was performed on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) combined with a Waters Micromass Triple Quadrupole (TQD) mass spectrometer equipped with electrospray ionization interface (ESI) (Waters, Milford, MA). Separation was achieved using an ACQUITY UPLC C18 column ( $2.1 \text{ mm} \times 50 \text{ mm}$ ,  $1.7 \mu\text{m}$ , Waters) at  $40 \text{ }^\circ\text{C}$ . Mobile phases A and B consisted of deionized water (18 M $\Omega$ ) and methanol, respectively, each acidified using 0.2% formic acid. The solvent gradient program, with respect to mobile phase A, was as follows: 0–0.5 min 80%; 0.5–2.5 min 45%; 2.50–3.5 min 10%; 3.5–4.0 min 80%; followed by equilibration for 1.00 min. The flow rate was  $0.4 \text{ mL min}^{-1}$ , and the injection volume was 5  $\mu\text{L}$ . Mass data were acquired using MassLynx® (Waters) multiple reactions monitoring (MRM) in the ESI positive mode. The specific instrument settings were: capillary voltage 0.69 kV, collision gas (Ar, 99.9%), dwell time 0.022 s, source temperature  $150 \text{ }^\circ\text{C}$ , desolvation temperature  $450 \text{ }^\circ\text{C}$ , desolvation gas  $900 \text{ L h}^{-1}$  and cone gas  $50 \text{ L h}^{-1}$  (Table S1).

### 2.6. Data analysis and quality control

All treatments in the *A. thaliana* cell incubation experiment were conducted in triplicate, and all hydroponic cultivations were conducted using four replicate jars containing individual plants to account for potential loss of plants. Calibration curves (ranging from 5 to  $1000 \text{ ng mL}^{-1}$ ) with standards of diazepam, diazepam-*d*5, nordiazepam, temazepam, oxazepam and oxazepam-glucuronide were used for quantification with the  $r^2$  values of at least 0.99 for all analytes. A limit of detection (LOD) of  $1 \text{ ng mL}^{-1}$  and a limit of quantification (LOQ)  $3 \text{ ng mL}^{-1}$  for diazepam and its metabolites (except oxazepam-glucuronide) were determined through preliminary experiments. For oxazepam-glucuronide the LOD was  $3 \text{ ng mL}^{-1}$  and the LOQ was  $5 \text{ ng mL}^{-1}$ . LODs and LOQs were calculated based on a signal to noise ratio of 3 and 10, respectively.

Individual peaks were detected and integrated using TargetLynx XS software from MassLynx platform (Waters). Data were analyzed with StatPlus (Walnut, CA) and graphed using Prism 6 GraphPad software (La Jolla, CA). Results were calculated as the mean  $\pm$  standard deviation (SD) ( $n = 3$ ). The Student's *t*-test was used to test significant differences in the extractable and non-extractable radioactivity and glycosyltransferase activity at  $\alpha = 0.05$ . Systematic differences in the concentration of diazepam in plant tissues were assessed using one-way ANOVA with Fisher's Least Significant Difference post-hoc ( $\alpha = 0.05$ ).

## 3. Results and discussion

### 3.1. Kinetics of diazepam in *A. thaliana* cell cultures

Active plant metabolism of diazepam was validated using a range of controls. No diazepam was detected in the non-treated media or the cell blanks, and there was no significant degradation of diazepam in the cell-free media, suggesting no contamination or significant abiotic transformation. Moreover, no significant difference ( $p > 0.05$ ) was seen in cell mass between the chemical-free control and the treatments, indicating that diazepam did not inhibit the growth of *A. thaliana*. Furthermore, no significant amount of diazepam was adsorbed to the cell matter in the non-viable cell control. In contrast, diazepam dissipated appreciably from the media containing viable cells, with the average concentration decreasing from  $698 \pm 41.5$  to  $563 \pm 8.93 \text{ ng mL}^{-1}$  after 120 h of incubation, a decrease of nearly 20% (Fig. 1a).

Parallel with the dissipation in the medium, diazepam was detected in the *A. thaliana* cells, with the highest level appearing after 48 h and a

substantial decrease thereafter (Fig. 1b). The decrease in diazepam level in the cell fit a first-order decay model and yielded a half-life of about 68 h ( $r^2 = 0.97$ ). This half-life was in comparison to a biological half-life of 48 h in humans (Dhillion et al., 1982), indicating a moderate persistence in plant cells.

### 3.2. Metabolism of diazepam in *A. thaliana* cells

Out of the four known diazepam metabolites only nordiazepam and temazepam were detected in the *A. thaliana* cells over the 120 h incubation. Temazepam was detected first, with the highest concentration ( $645 \pm 50.4 \text{ ng g}^{-1}$ ) being observed at 12 h, which was followed by a decrease to  $58.6 \pm 17.0 \text{ ng g}^{-1}$  at the end of the 120 h cultivation. Nordiazepam gradually increased over the 120 h incubation time from  $128 \pm 61.0 \text{ ng g}^{-1}$  at 6 h to  $535 \pm 92.0 \text{ ng g}^{-1}$  at the end of incubation (Fig. 1c). These results correlated with their behavior in the human body, as nordiazepam displayed one of the most prolonged biological half-lives of the benzodiazepine family (50 to 120 h), while temazepam had a significantly shorter half-life (8 to 20 h) (Yasumori et al., 1993). The parallels observed between human and plant metabolism in this study and others (e.g., Huber et al., 2009, 2012; Fu et al., 2017a; LeFevre et al., 2016; Marsik et al., 2017) is intriguing, as it indicates that we may be able to use the knowledge of biologically active metabolites formed during human metabolism as a guide to study their formation and longevity in environmental compartments such as higher plants.

The complementary use of  $^{14}\text{C}$  labeled diazepam facilitated the determination of the fraction of diazepam and its metabolites that were incorporated into the cell matter (bound residue fraction), which could not be determined using traditional extraction and analytical methods. We observed that the radioactivity in the media decreased while the extractable and bound residue fractions increased over the 120 h incubation (Fig. 2). The extractable radioactivity in the viable cells increased to  $113 \pm 31 \text{ dpm g}^{-1}$  at 120 h (Fig. 2b). The bound residues (non-extractable radioactivity) increased steadily to a final level of  $1120 \pm 224 \text{ dpm g}^{-1}$  (Fig. 2c), indicating that *A. thaliana* cells were capable of metabolizing and then sequestering diazepam and its metabolites, likely in vacuoles and cell walls. The formation of these bound residues is commonly regarded as a detoxification pathway of xenobiotics in higher plants.

### 3.3. Uptake and translocation of diazepam in cucumber and radish seedlings

Diazepam was found in the cucumber and radish seedlings following a 7 d cultivation at the higher concentration and a 28 d of cultivation following treatment at the lower concentration (Fig. 3). After treatment with  $1 \text{ mg L}^{-1}$  with diazepam for 7 d, a significantly higher concentration of diazepam was observed in the roots as compared to the shoots in radish seedlings ( $p < 0.05$ ) whereas diazepam was more evenly distributed throughout the entire plant of cucumber seedlings (Fig. 3a). However, after the 28 d cultivation following the lower concentration treatment, this pattern appeared to be different for both plant species. In the radish plants, diazepam was more evenly distributed in the roots, but was significantly lower in the shoots ( $p < 0.01$ ). In the cucumber plants there was a significantly higher concentration in roots ( $p < 0.01$ ) and a significantly lower concentration in the shoots (Fig. 3b,  $p < 0.05$ ). These differences may be due to variations in metabolism between the two species, as well as dynamic changes as a function of contact time in both plant growth and its ability to metabolize and translocate diazepam.

The bioconcentration factors for the roots ( $\text{BCF}_R$ ) and shoots ( $\text{BCF}_S$ ) were calculated as the ratio of  $^{14}\text{C}$  activity in plant tissue ( $C_{\text{tissue}}$ ) to that in the media ( $C_{\text{media}}$ ):

$$\text{BCF} = \frac{C_{\text{tissue}}(\text{dpm/g})}{C_{\text{media}}(\text{dpm/mL})}$$

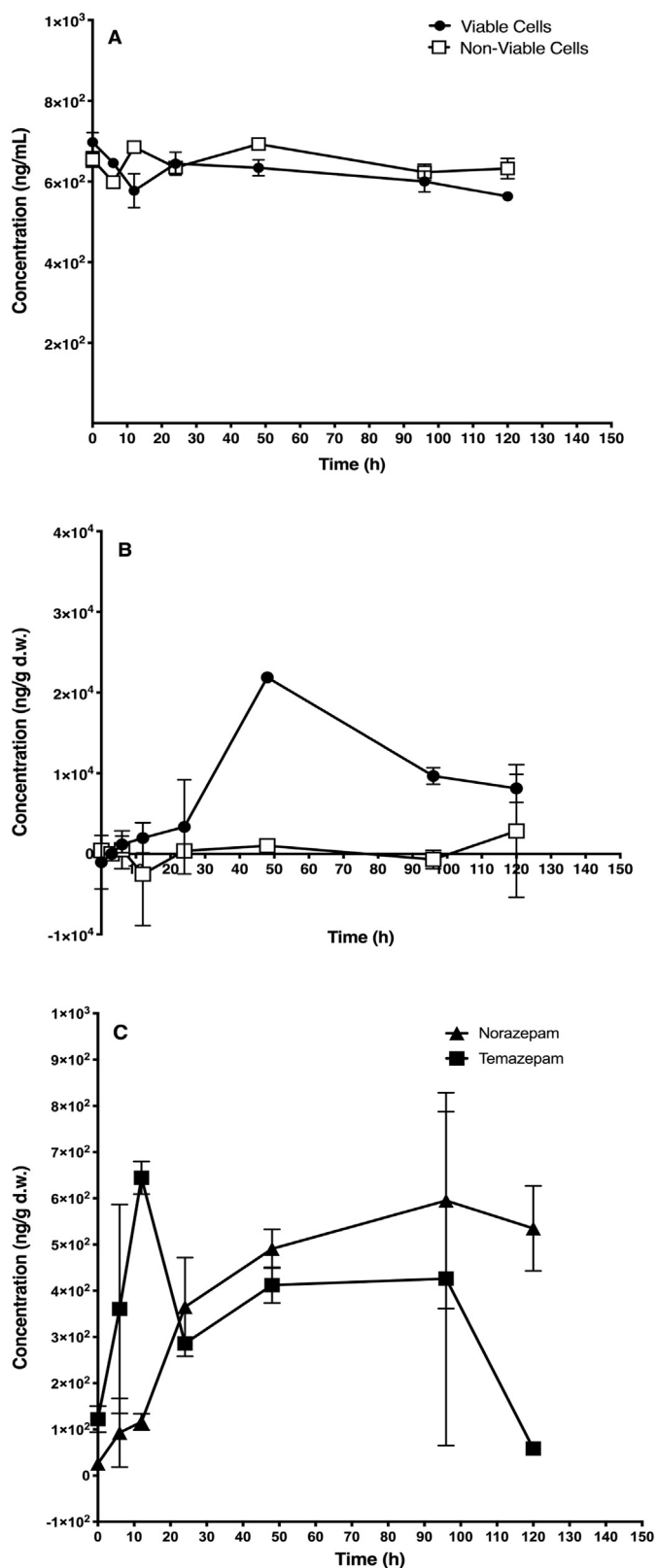


Fig. 1. Diazepam in the medium and *Arabidopsis thaliana* cells. (A) Kinetics of diazepam in cell growth media (B) kinetics of diazepam in viable and non-viable cells. (C) Kinetics of nordiazepam and temazepam in viable cells. The values are shown as mean  $\pm$  SD ( $n = 3$ ).

For the radish plants cultivated for 7 d the average  $\text{BCF}_R$  and  $\text{BCF}_S$  were 0.49 and 1.13, respectively. For the 7 d cucumber plants,  $\text{BCF}_R$  and  $\text{BCF}_S$  were 0.78 and 3.70, respectively (Table S2). The higher BCF values found for the shoots were in disagreement with Wu et al.

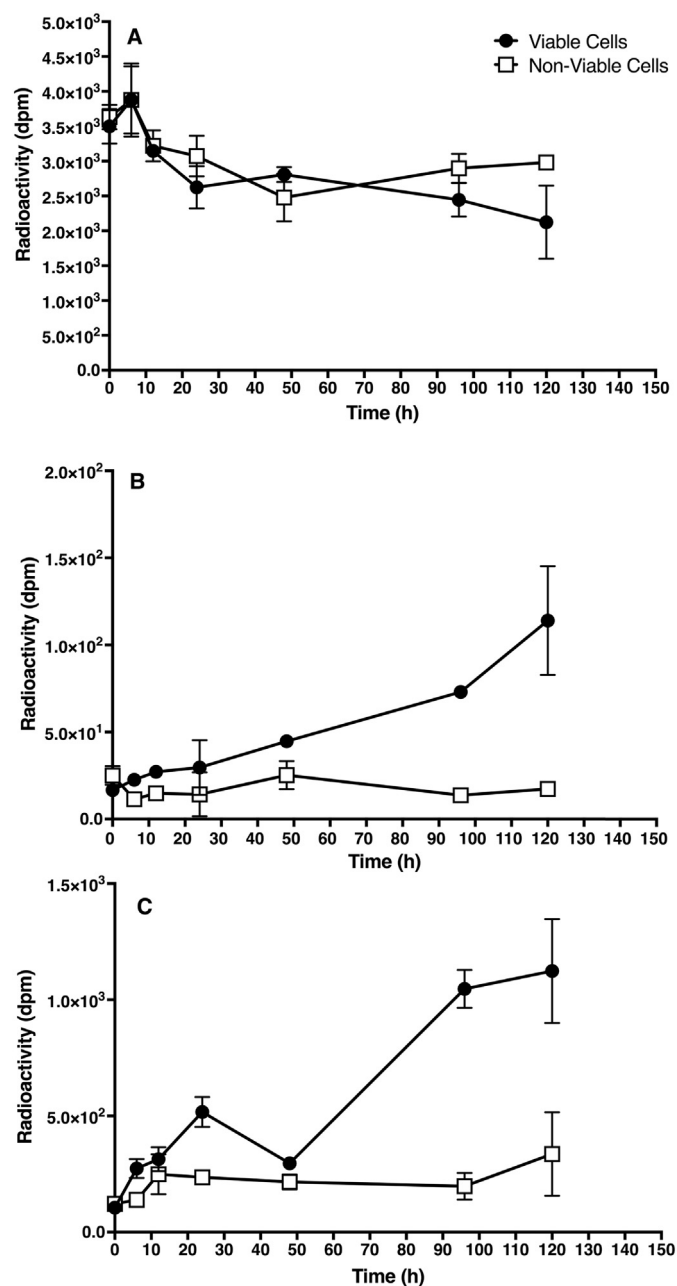


Fig. 2. Radioactivity in *Arabidopsis thaliana* cells. (A) Radioactivity in cell growth media (B) extractable radioactivity in viable and non-viable cells. (C) Non-extractable radioactivity in viable and non-viable cells. The values are shown as mean  $\pm$  SD (n = 3).

(2013). It is possible that the discrepancy between the studies was due to the use of <sup>14</sup>C-labeling in the current study, as both extractable and non-extractable parent compound and its metabolites were included in the calculation of BCFs in this study, whereas only the extractable form of the parent compound was used for calculation in the previous study. It may be argued that the use of <sup>14</sup>C accounted for both parent and metabolites in extractable and non-extractable forms, and therefore reflected the overall accumulation of diazepam in plants (Ando et al., 2015; Trapp et al., 1990; Dodgen et al., 2014; Fu et al., 2017a).

The translocation factor (TF) was similarly calculated using the ratio of <sup>14</sup>C activity in the shoots ( $C_{shoots}$ ) to that in the roots ( $C_{roots}$ ):

$$TF = \frac{C_{shoots}(\text{dpm/g})}{C_{roots}(\text{dpm/g})}$$

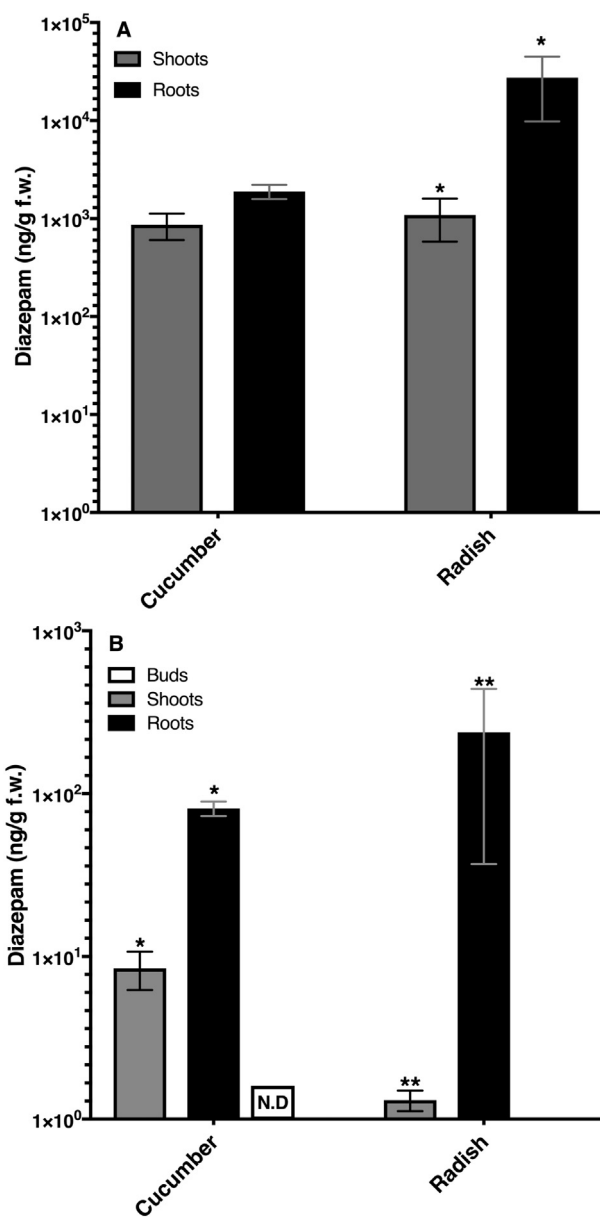


Fig. 3. Diazepam in radish and cucumber plants. (A) Concentration of diazepam in radish and cucumber seedlings cultivated for 7 d. (B) Concentration of diazepam in radish and cucumber seedlings cultivated for 28 d. The values are shown as mean  $\pm$  SD (n = 3). \*p < 0.05 \*\*p < 0.01 ANOVA (one-way).

For the 7 d cultivation experiment, TF values for radish and cucumber plants were 3.5 and 4.7, respectively. For the 28 d cultivation experiment, the TFs increased for both plants to 5.9 and 5.0, respectively. These TF values were much greater than that reported in Wu et al., 2013. However, these results were similar to the predicted values for weakly basic compounds (Table S3) under weakly basic conditions (pH 7.81) (Trapp, 2009). Again, the higher TFs observed in this study may be similarly attributed to the inclusion of the non-extractable residues and metabolites in the calculations.

#### 3.4. Metabolism of diazepam in cucumber and radish seedlings

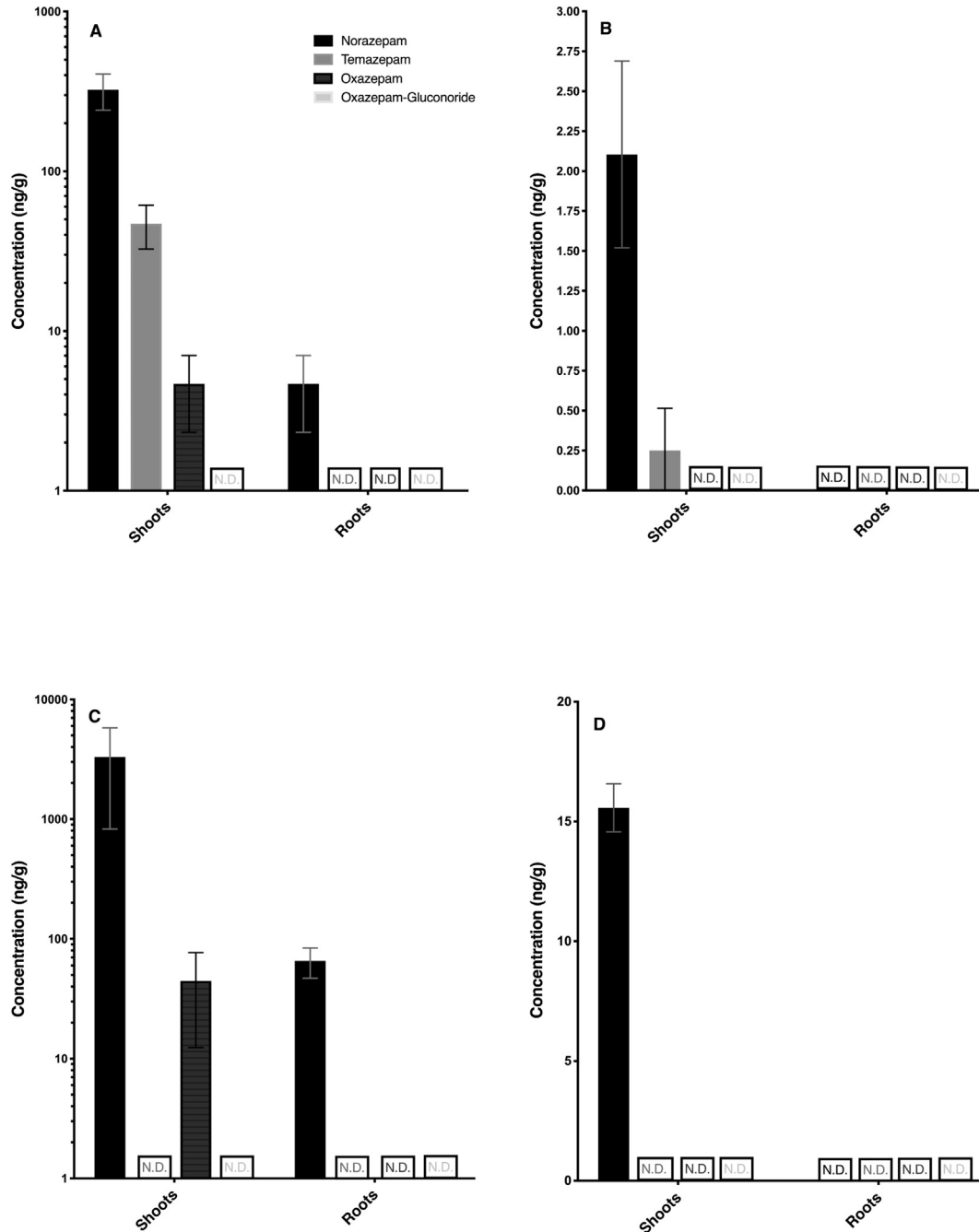
Similar metabolites to those in *A. thaliana* cells were found in seedlings grown in the nutrient solution spiked with diazepam, with nordiazepam being predominant (Fig. 4). In the 7 d and 28 d cultivation experiments, temazepam was found to be the second major metabolite in the leaves of the cucumber seedlings, and the level was higher in the

7 d cucumber seedlings than the 28 d plants (Fig. 4a,b). Oxazepam was detected in the leaves of both plant species after the 7 d cultivation (Fig. 4a,c). The higher accumulation of diazepam and the biologically active metabolites in the leaves may have ecotoxicological ramifications; for example, many insects consume leaves, even if they are not edible tissues for humans (Pennington et al., 2017).

Our results were in agreement with recent findings in Carter et al. (2018), in which they observed the formation of nordiazepam, temazepam and oxazepam in radish and silverbeet plants exposed to diazepam and chlordiazepoxide. They similarly showed nordiazepam to be the major metabolite with oxazepam and temazepam constituting a much smaller fraction at the end of 28 d cultivation in soil. However, in that study, the authors did not track

the formation of these metabolites over time or influence of treatment concentrations.

Phase III metabolism appeared to increase from the 7 d to 28 d cultivation for both radish and cucumber seedlings (Table 1). Between the plant species, the cucumber seedlings had a greater fraction of non-extractable radioactivity in comparison to the radish seedlings (Table 1). In the 7 d cultivation experiment, the mass balances came to 99.3% for the cucumber plants but only 58.1% for the radish seedlings (Table 1). Due to the multiple water changes (Fig. S1), a complete mass balance was not attainable for the 28 d cultivation experiment. However, when a proxy mass balance (sum of roots and shoots) was calculated for both species, a similar pattern was observed. A total of 83.0% of the added  $^{14}\text{C}$  radioactivity was calculated for the cucumber



**Fig. 4.** Diazepam metabolites in radish and cucumber seedlings. (A) Concentration of diazepam metabolites in cucumber seedlings cultivated for 7 d. (B) Concentration of diazepam metabolites in cucumber seedlings cultivated for 28 d. (C) Concentration of diazepam metabolites in radish seedlings cultivated for 7 d. (D) Concentration of diazepam metabolites in radish seedlings cultivated for 28 d. The values are shown as mean  $\pm$  SD ( $n = 3$ ).

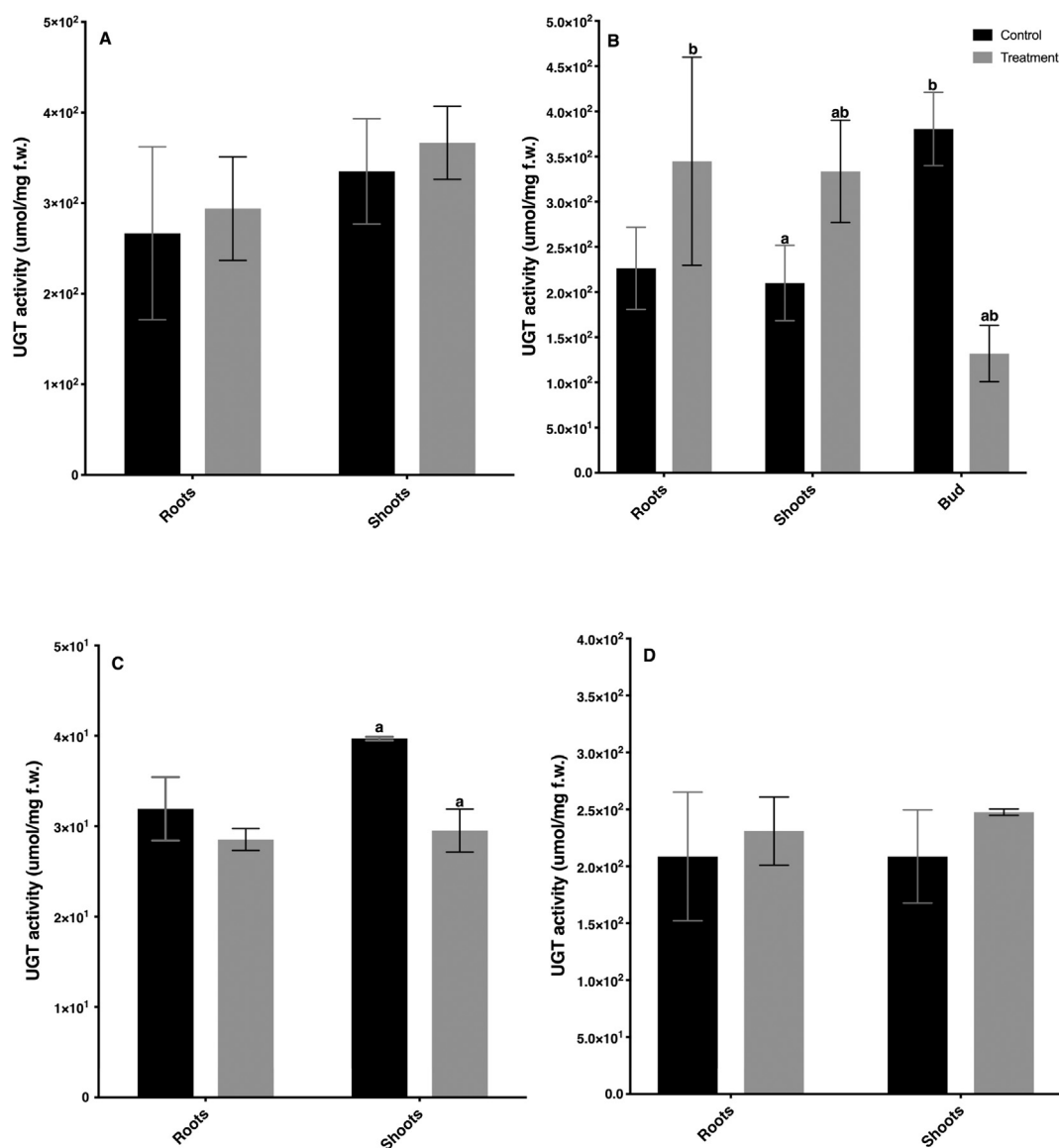
**Table 1**Extractable and non-extractable radioactivity in radish and cucumber plants cultivated for 7 d and 28 d. The values are shown as mean  $\pm$  SD (n = 3).

Radioactivity	Media	Shoots	Roots	Buds
7 d extractable radish	30.8% $\pm$ 3.8	12.9% $\pm$ 10 <sup>a</sup>	9.35% $\pm$ 3.6	N.A.
7 d non-extractable radish	N.A.	2.0% $\pm$ 0.4 <sup>a</sup>	2.95% $\pm$ 1.4	N.A.
28 d extractable radish	N.A.	2.23% $\pm$ 1.0 <sup>b</sup>	11.6% $\pm$ 1.0 <sup>b</sup>	N.A.
28 d non-extractable radish	N.A.	37.3% $\pm$ 6.4	10.4% $\pm$ 4.0	N.A.
7 d extractable cucumber	21.9% $\pm$ 12	34.0% $\pm$ 7.5 <sup>a,b</sup>	10.0% $\pm$ 5.1 <sup>b</sup>	N.A.
7 d non-extractable cucumber	N.A.	28.6% $\pm$ 1.09 <sup>a,b</sup>	4.65% $\pm$ 2.3 <sup>b</sup>	N.A.
28 d extractable cucumber	N.A.	13.3% $\pm$ 10 <sup>b</sup>	10.3% $\pm$ 4.8 <sup>b</sup>	0.96% $\pm$ 0.4 <sup>b</sup>
28 d non-extractable cucumber	N.A.	30.3% $\pm$ 8.4 <sup>b</sup>	13.5% $\pm$ 9.5 <sup>b</sup>	14.5% $\pm$ 2.5 <sup>b</sup>

<sup>a</sup> Significant differences ( $\alpha = 0.05$ , student *t*-test) relative to the inter-plant tissues.<sup>b</sup> Significant differences ( $\alpha = 0.05$ , student *t*-test) relative to intra-plant tissues.

treatments while the fraction was 61.3% for the radish plants. This could be due to increased mineralization in the growth media and respiration of <sup>14</sup>CO<sub>2</sub> through plant in the radish cultures. As mineralization is viewed as the final stage of detoxification (Dodgen et al., 2014), it is likely that the radish plant was more efficient in their ability to detoxify

diazepam than cucumber plants. The Brassicaceae family, which includes the common radish, has been shown to be effective for phytoremediation due to their possession of genes that increase tolerance to stressors (Peer et al., 2006) and activation of enzymes capable of extensive biotransformations (Piotrowska-Długosz, 2017).



**Fig. 5.** Glycosyltransferase activity in radish and cucumber seedlings. (A) Glycosyltransferase activity in cucumber seedlings cultivated for 7 d. (B) Glycosyltransferase activity in cucumber seedlings cultivated for 28 d. (C) Glycosyltransferase activity in radish seedlings cultivated for 7 d. (D) Glycosyltransferase activity in radish seedlings cultivated for 28 d. The values are shown as mean  $\pm$  SD (n = 3). (a) Significant differences ( $\alpha = 0.05$ , student *t*-test) relative to the intra-plant tissues. (b) Significant differences ( $\alpha = 0.05$ , student *t*-test) relative to inter-plant tissues.

### 3.5. Diazepam-induced changes to glycosyltransferase activity

The activity of glycosyltransferase was measured in the control seedlings as well as seedlings exposed to diazepam for the 7 d and 28 d cultivation experiments (Fig. 5). Glycosyltransferase catalyzes the transfer of sugars, such as glucuronic acid, to many types of acceptor molecules, including xenobiotics (Gachon et al., 2005). The conjugation of glucuronic acid with oxazepam is the major detoxification pathway of diazepam in humans (Sonne, 1993).

No detectable level of oxazepam-glucuronide was observed in radish or cucumber seedlings for either the 7 d or 28 d cultivation. However, there was a significant difference in the glycosyltransferase activity in radish seedlings treated with diazepam for 7 d and 28 d, although a distinct pattern in the changes of the enzyme activity was absent (Fig. 5). For the 7 d cultivation experiment, a significant decrease in glycosyltransferase activity was observed in the shoots of radish seedlings when compared to the control (Fig. 5c,  $p < 0.01$ ). In contrast, no significant change in glycosyltransferase activity was observed in the shoots of cucumber seedlings when exposed to diazepam (Fig. 5a). In the 28 d cultivation experiment, only the cucumber seedlings exhibited significant differences in the enzyme activity, with an increase in activity detected in the shoots ( $p < 0.01$ ) and a decrease in the cucumber buds (Fig. 5b,  $p < 0.01$ ).

Even though we did not detect oxazepam-glucuronide in the exposed plants, changes in the glycosyltransferase activity indicated that conjugation might have occurred with the parent and its metabolites, including those not examined in this study, or at levels below our detection capability. In addition, it may be postulated that rapid phase III metabolism may have limited the accumulation of such conjugates in the plant tissues, making the conjugates transient metabolites. In previous studies, glycosyltransferase was observed to catalyze the detoxification of ibuprofen in *Phragmites australis* during a 21 d exposure (He et al., 2017). Further, the formation of a glucose conjugate has been considered to be a major detoxification pathway for several environmental contaminants (e.g., Ando et al., 2015; Fu et al., 2017a, 2017b). These studies together suggest the importance of phase II metabolism in the metabolic fate of pharmaceuticals in higher plants.

## 4. Conclusions

Agricultural irrigation using TWW may pose unintended risks, such as exposure to pharmaceuticals by humans through dietary intakes and unexpected ecological consequences following consumption of pharmaceuticals by terrestrial organisms such as insects. In this study, the pharmaceutical diazepam was found to be taken up and metabolized by *Arabidopsis thaliana* cells, as well as cucumber and radish seedlings. The uptake resulted in extensive phase I metabolism, as demonstrated by the formation of the three major diazepam metabolites, nordiazepam, temazepam and oxazepam. However, the three plant species displayed notable metabolic differences. Further, exposure to diazepam resulted in alteration of glycosyltransferase that is involved in phase II metabolism. The observed interaction between a plant's ability to accumulate and metabolize diazepam and diazepam's apparent ability to affect a plant's detoxification enzymes warrants further consideration, as it may be indicative of other previously unobserved metabolites. Results from this study present new insights on plants' ability to take up and metabolize pharmaceuticals, including their ability to create other biologically active compounds. This and other studies highlight the need to consider plant metabolism and bioactivation in multiple crops and under realistic environmental conditions when assessing the safety of TWW reuse for agricultural irrigation.

## Acknowledgements

The authors would like to thank the U.S. Environmental Protection Agency STAR program (Award No: R835829) and the National Science

Foundation—the Water SENSE Integrative Graduate Education Research Training program (No. DGE-1144635) for funding support.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2019.01.259>.

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