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Short term uptake and transport process for metformin in roots of *Phragmites australis* and *Typha latifolia*

H. Cui [a], B.A. Hense [b], J. Müller [b,c], P. Schröder [a,*]

[a] Helmholtz Zentrum München, German Research Center for Environmental Health, Research Unit Environmental Genomics, Ingolstaedter Landstr. 1, 85764 Neuherberg, Germany
[b] Helmholtz Zentrum München, German Research Center for Environmental Health, Institute for Computational Biology, Ingolstaedter Landstr. 1, 85764 Neuherberg, Germany
[c] Technical University of Munich, Centre for Mathematical Sciences Unit M12, Botzmannstr. 3, 85747 Garching, Germany

HIGHLIGHTS

- Metformin, carbamazepine and caffeine can be taken up by *P. australis* and *T. latifolia.*
- The uptake process of metformin was not concentration-dependent.
- The characteristic of uptake and transport of metformin is different from carbamazepine and caffeine.
- Quinidine can significantly inhibit the uptake of metformin.
- Organic cation transporters could be considered as a potential channel for metformin in roots.

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ABSTRACT

Metformin (MET) as an emerging contaminant has been detected in surface water and wastewater in numerous countries, due to insufficient retention in classical waste water treatment plants. In order to characterize the uptake of the compound during phytotreatment of waste water, a short term Pitman chamber experiment was carried out to assess the characteristics of MET uptake and transport by roots. Three different concentrations (0.5, 1.0 and 2.0 mmol L^{-1}) were applied to cattail (*Typha latifolia*) and reed (*Phragmites australis*) roots which were used to investigate the uptake mechanism because they are frequently utilized in phytoremediation. In addition, quinidine was used as an inhibitor to assess the role of organic cation transporters (OCTs) in the uptake of MET by *T. latifolia*. The transport process of MET is different from carbamazepine (CBZ) and caffeine (CFN). In both *T. latifolia* and *P. australis*, the uptake processes were independent of initial concentrations. Quinidine, a known inhibitor of organic cation transporters, can significantly affect MET uptake by *T. latifolia* roots with inhibition ratios of 70–74%. Uptake into the root could be characterized by a linear model with R^2 values in the range of 0.881–0.999. Overall, the present study provides evidence that MET is taken up by plant roots and has the potential for subsequent translocation. OCTs could be one of the important pathways for MET uptake into the plant.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) have been recognized as environmental contaminants and attracted increasing concern in the last decade (Daughton and Ternes, 1999). This is due to the fact that these compounds generally are more recalcitrant and cannot be completely removed in conventional wastewater treatment processes so that many of them are detected in the effluents of treatment plants (Ternes, 1998; Kolpin et al., 2002), from where they might enter into our fresh- and groundwater reserves. Hence, these chemicals cause a potential public health problem and an environmental safety risk, such as inhibition of growth of human embryonic cells and decrease of microbial diversity (Zhang et al., 2014). Many studies focus on their influence on aquatic ecosystems, especially on animals. However to date, knowledge about their interactions with plants, such as uptake, transport, and possible metabolism are still limited.

Metformin (MET) is an antidiabetic II drug from the biguanidine class that acts by suppressing glucose production in the liver. It is not metabolized in the human body but excreted unchanged in the urine (Scheen, 1996; Robert et al., 2003). Because of the increasing occurrence of diabetes in the ageing population, it is not surprising that a compound as stable as MET will become detectable in the

* Corresponding author at: Helmholtz Zentrum München, German Research Center for Environmental Health, Research Unit Environmental Genomics, Ingolstaedter Landstr. 1, 85764 Neuherberg, Germany.
E-mail address: peter.schroeder@helmholtz-muenchen.de (P. Schröder).

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environment. Several studies showed that the concentration of MET in wastewater treatment plant (WWTP) influents and effluents increases steadily and ranges from 20 to 129 μg L^{-1} and 2.2 to 21 μg L^{-1}, respectively (Scheurer et al., 2009; van Nuijs et al., 2010; Trautwein and Kümmerer, 2011). Some papers reported MET in surface water in the range of 60–3100 ng L^{-1} (Vulliet and Cren-Olivé, 2011; Scheurer et al., 2012). These observations are alarming, because the capacities of conventional WWTPs for its removal and degradation may be not sufficient (Oosterhuis et al., 2013).

The uptake of xenobiotics by plants has been reported for a number of organic contaminants including pesticides, veterinary products and especially PPCPs in the last decade (Wu et al., 2010; Herklotz et al., 2010; Gao et al., 2000; Boxall et al., 2006). Predicting the uptake of organic contaminants by plants is critical for evaluating their environmental risk and the potential effectiveness of alternative technologies like phytoremediation. However, we still know little about uptake and transport processes in wetland plants which are used in phytoremediation. Only few research papers show that MET can be taken up by crop plants and bioaccumulates in roots, leaves and seeds (Eggen et al., 2011; Eggen and Lillo, 2012).

In the context of phytoremediation, previous studies have reported the uptake of organic xenobiotics to be mediated by passive diffusion through membranes and within plant tissues (Pilon-Smits, 2005). The octanol:water partition coefficient (log Kow) has been widely used as a descriptor of xenobiotic hydrophobicity which is one of the most important properties influencing xenobiotic uptake by plants (Briggs et al., 1982; Trapp, 1994). However, recent studies indicate that some active processes might be also involved in xenobiotic uptake and translocation. Several substrate specific membrane proteins have been identified in plasma membranes where they seem to be used as transporters for water and organic solutes (Martinoia et al., 2007). Organic cation transporters (OCTs) belong to a family of proteins mediating the transport of charged organic molecules like MET across the cell membrane. They are uniporters and widely distributed in mammalian organs such as liver and kidney (Koepsell and Endou, 2004). Substrates of OCTs have diverse molecular structures, are typically hydrophilic and have low molecular mass. OCT-mediated transport is generally considered electrogenic and independent from sodium. The primary driving force is supplied simply by the electrochemical gradient of the transported compounds or the electronegative membrane potential (Klaassen and Aleksunes, 2010; Zamek-Gliszczynski et al., 2013). However, in recently published studies OCT-mediated transport was also described to occur via cation exchange (Budiman et al., 2000; Pelis et al., 2012). OCTs characterized in plants may be distributed in the vascular tissues of all organs (Küfner and Koch, 2008). It is likely that analogous transmembrane proteins can be considered as potential channels for uptake and transport of MET by plants.

The aim of this study was to (1) investigate uptake and transport of MET with different initial concentrations and plant species using Pitman chamber experiments; and (2) examine the role of OCTs during the transport process by adding quinidine as an inhibitor.

2. Materials and methods

2.1. Chemicals

Metformin HCl (97%), carbamazepine (CBZ, 99%), caffeine (CFN, 99%) and quinidine (98%) were purchased from Sigma–Aldrich (Germany), sucrose (98%) was purchased from Fluka (Germany). Table 1 shows the physicochemical properties of MET, CBZ and CFN. Acetonitrile was HPLC grade and obtained from Roth (Germany), disodium hydrogen phosphate and sodium dodecyl sulfate (SDS) were supplied from Roth (Germany). All chemicals used for nutrient solution were analytical grade.

2.2. Plant material

Typha latifolia and *Phragmites australis* plants were ordered from a local grower (Jörg Petrowsky, Eschede, Germany) and the rhizomes were thoroughly washed in tap water. Plants were grown on perlite in 5 L vessels and then transferred to a greenhouse with 12 h of light/12 h of darkness at 23/18 °C and a humidity of 65%. Nutrients were provided in a modified Hoagland nutrient solution: 2.5 mmol L^{-1} K^+, 2 mmol L^{-1} Mg^{2+}, 2 mmol L^{-1} Ca^{2+}, 2 mmol L^{-1} SO_4^{2-}, 6 mmol L^{-1} NO_3^-, 0.5 mmol L^{-1} $H_2PO_4^-$, 50 μmol L^{-1} Fe^{2+}, 50 μmol L^{-1} BO_3^{3-}, 1 μmol L^{-1} Mn^{2+}, 0.5 μmol L^{-1} Cu^{2+}, 0.5 μmol L^{-1} Zn^{2+}, 0.1 μmol L^{-1} MoO_4^{2-} and the pH was adjusted to 6.0. Plants were acclimatized to greenhouse condition more than two months before they were used in the chamber experiments.

2.3. Pitman chamber experiments

The incubation chambers for present experiments had been designed during previous studies (Pitman, 1971; Schröder et al., 2007). Five roots, approx. 10 cm long, complete with root tips, were carefully cut from selected plants and inserted into the chamber immediately. Each chamber is split into three independent compartments (from left to right named A, B and C) by baffles. Vaseline was applied to all gaps in the chamber to prevent the exchange of solutes between compartments (Fig. 1). For comparison with MET, CBZ and CFN were individually spiked in compartment A at initial concentration of 0.5 mM. Experiments focusing on the transport mechanism of MET were divided into two series: First, both plant species (*T. latifolia* and *P. australis*) were used to determine transport rates; second, only *T. latifolia* was used to investigate the inhibition of OCTs. Compartment A was spiked with MET solution at three different concentrations (0.5, 1.0 and 2.0 mmol L^{-1}) in the absence of quinidine for the first series and in the presence of quinidine (0.5 mmol L^{-1}) for the second series. Compartment B and C were filled with 0.03 mol L^{-1} sucrose solution in order to provide osmotic pressure in the roots (Tazawa et al., 2001). A chamber receiving an equivalent amount of MET and sucrose solution without roots served as the control. Water samples were collected after 10, 20, 30, 45, 60, 75 and 90 min from both compartments, A and C, and stored at −20 °C for later analyses.

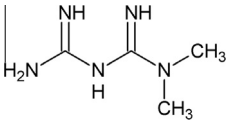
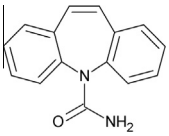
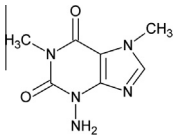
2.4. High-performance liquid chromatography (HPLC) analysis

Methods for pharmaceutical determination in water have been described previously (Kolte et al., 2005). In short, filtrated water samples (0.45 μm) were injected into the HPLC (Varian ProStar 210, Germany) by an auto sampler (Varian ProStar 410, Germany) with an injection volume of 25 μL. An XDB C18 reversed-phase column (5 μm, 4.6 × 50 mm, Agilent, Germany) was used at a column temperature of 40 °C. Detection of MET was at a wavelength of 226 nm using an UV detector (Shimadzu SPD-20, Germany). The mobile phase used was an isocratic mixture of aqueous buffer and acetonitrile (68:32, *v*/*v*) at a flow rate of 1.0 mL min^{-1}. The buffer consisted of 10 mmol L^{-1} disodium hydrogen phosphate and 10 mmol L^{-1} sodium dodecyl sulfate (SDS) in distilled water adjusted to pH 7.5 with hydrochloric acid. The mobile phase was mixed, filtered (nylon, 0.45 μm) and degassed.

2.5. Statistical analyses

Data were analyzed for normal distribution. ANOVA was used to determine significant differences between groups by comparing

Table 1
Chemical structure and physicochemical properties of metformin, carbamazepine and caffeine.

Compounds	Metformin	Carbamazepine	Caffeine
Chemical structure			
Molecular weight	129.16	236.27	194.19
pKa	12.33 and 10.27[a]	15.96[a]	−0.92[a]
Log Kow	−4.30 (pH at 7.4)[b]	2.28 (pH at 7.4)[b]	0.28 (pH at 7.4)[b]
Log Dow	−2.31[b]; −2.64[c]; −1.40[d]	2.67[b]; 2.45[c]	−0.13[b]; −0.07[c]

[a] ChemAxon.
[b] ACD/Labs.
[c] EPISuite™.
[d] ChemIDplus advanced.

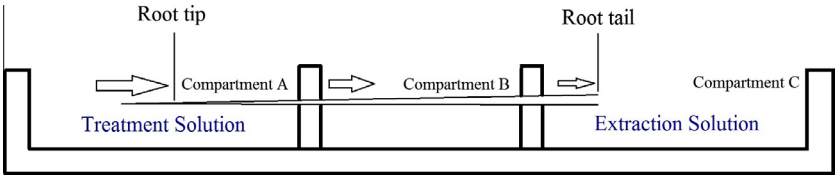


Fig. 1. Cross section of Pitman chamber. Each chamber is split into three independent compartments (from left to right named A, B and C) by baffles. Vaseline was applied to all gaps in the chamber to prevent the exchange of solutes between compartments.

the critical value of variance. These analyses were performed using SPSS v16.0 and comparisons were considered significantly different for $p < 0.05$.

3. Results and discussion

3.1. The versatility of a root chamber to study xenobiotic transport

Studying transport processes in plant tissues with Pitman chambers is a well established method. When *T. latifolia* roots were used to compare transport processes among CBZ, MET and CFN at concentration of 0.5 mM, interesting differences were observed. As is depicted in Fig. 2, CFN was taken up by the roots and transported at high rates, whereas carbamazepine and metformin had low and similar transport rates. In contrast to MET, the intercept of linear fit of CBZ and CFN are very close to zero. This indicates that differences exist between transport processes of cationic compounds like the MET and non-ionic organic chemicals. In fact, the uptake of non-ionic organic chemicals by plant roots could be just a passive, diffusive process (Collins et al., 2006). However, Calderón-Preciado et al. (2011) reported that the uptake of several personal care products, including CFN by crops was well above model predicted concentrations, indicating that other processes might play a role. Since metabolism can be excluded in the Pitman experiment, the differential behavior points to the presence of carriers with a broad substrate spectrum, allowing for the accelerated transport of distinct molecules. This is also stressed by Herklotz and co-authors (2010) who find evidence for the transfer of several pharmaceuticals across intact *Brassica* endodermis tissue which is not possible without the activity of carrier proteins.

3.2. Uptake and transport of metformin by roots of different plant species

When MET uptake was scrutinized, further evidence could be collected to confirm the observations made above. MET added to a control Pitman chamber without plant roots, did not cause any decline of MET concentration in compartment A and no diffusion

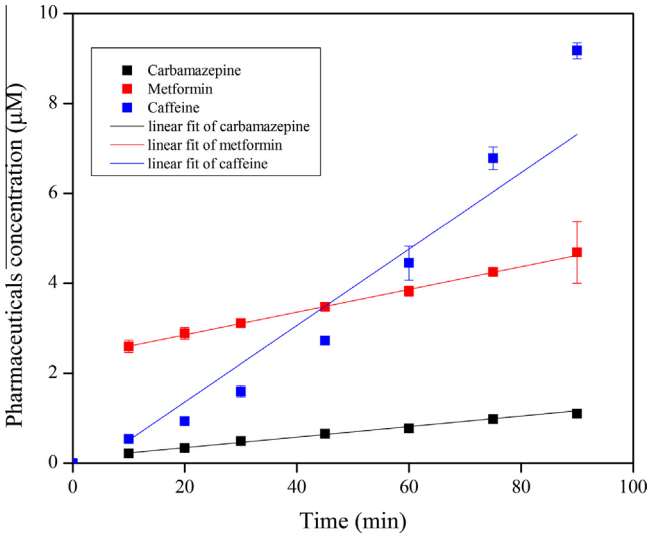


Fig. 2. Concentrations of metformin, carbamazepine and caffeine in the presence of *T. latifolia* at the initial concentration of 0.5 mmol L^{-1} in compartment C. Data points are means of independent experiments; error bar represent SD, $n = 3$.

of the compound from compartment A into B or C. On the contrary, in chambers containing plant roots, concentrations of MET steadily increased in compartment C during the experiment (Fig. 3). No contamination was observed in compartment B. After 90 min exposure of *T. latifolia* roots to concentrations of 0.5, 1.0 and 2.0 mmol L^{-1}, MET in compartment C accounted for 0.95%, 0.49% and 0.24% of the initial concentrations, respectively. Similar results were obtained when *P. australis* roots were exposed to the same experimental conditions.

Interestingly, at the end of the experiment, the concentrations of MET transported to compartment C were not significantly ($p < 0.05$) correlated to initial concentrations in compartments A in any treatment. However, the data clearly point to a saturation of the transport process system that might be carrier dependent.

Although the transport of MET by roots was small in comparison to the total amount offered, our studies confirm that MET is taken up by the roots and further transported. To our knowledge, this is the first study using Pitman chambers to investigate the uptake and transport of MET by plant roots. It seems that MET can move into the symplast and is distributed inside the vascular tissue from where it has further potential to reach rhizomes, stems and leaves under the control of transporters.

Some organic xenobiotics have also been suggested to be solely adsorbed on/in plant roots (Schröder and Collins, 2002). It has been observed that root cell walls had a higher sorption capacity for PAH like phenanthrene (Chen et al., 2009). Card et al. (2012) reported the adsorption isotherms of estrogens were linear at mid to high aqueous concentrations and nonlinear at low concentrations. These investigators suspected that a low abundance high affinity sorbent existed in the roots, which could explain the observation of nonlinear adsorption at low concentrations. Other researchers found that the "non-saponifiable and non-hydrolyzable" cuticle fraction could exhibit the most nonlinear behavior (Chen et al., 2005). Although absorption seems to relate to compounds with high log Kow, the relatively high rates of disappearance of MET from the feeding compartment A could also indicate absorption and binding without further transportation.

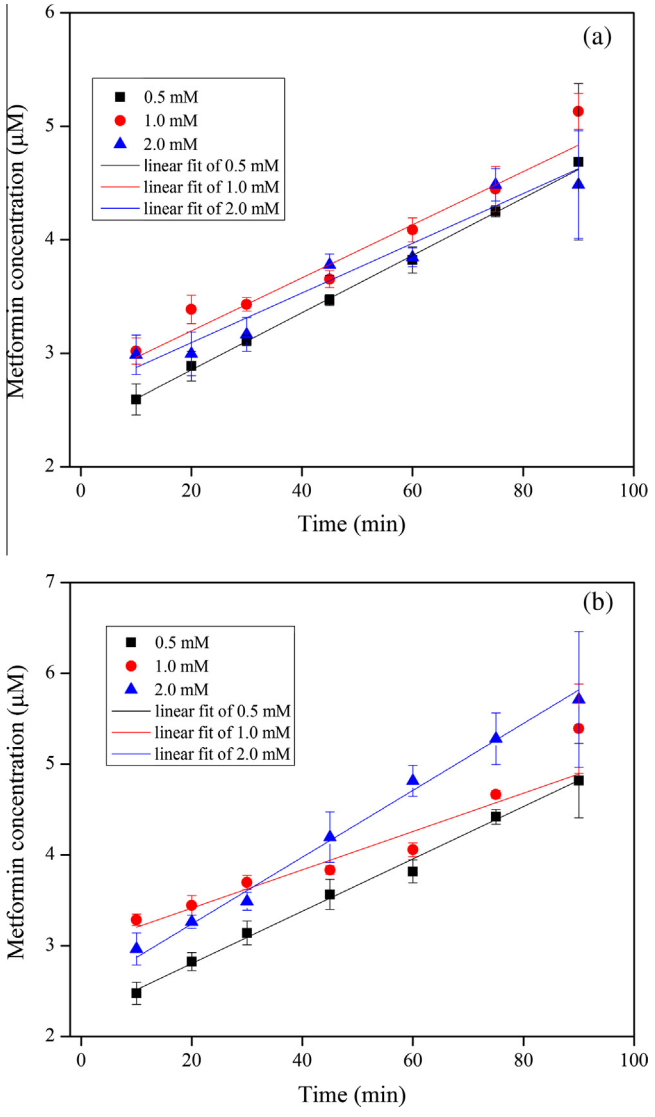


Fig. 3. Transport of metformin into compartment C in the presence of (a) *T. latifolia* and (b) *P. australis*. Data represent mean values of 3 independent biological experiments with 3 replicates ± SD.

The transport rates of MET into *T. latifolia* and *P. australis* in different initial concentrations are summarized in Table 2. For both plant root treatments, the concentrations of MET in compartment C as a function of exposure time was linear, the R^2 values in range of 0.881–0.999 for *T. latifolia* treatments and 0.942–0.993 for *P. australis* treatments (Fig. 2). Previous studies also found other pharmaceuticals such as diclofenac and CFN can be translocated from roots to shoots and the accumulation of pharmaceuticals in the shoots increased as a function of exposure time (Zhang et al., 2012; Zhang et al., 2013b). At the end of our experiments, the concentrations of MET in compartment C were approximately 0.2–1% of the initial concentration in compartment A.

In the present study, the concentrations of MET in compartment A decreased in correspondence to the uptake over time. A maximum of 14.4% MET disappeared from compartment A upon treatment of 2.0 mM for both *T. latifolia* and *P. australis* experiments, and 8.9% (*P. australis*) for the initial concentration of 0.5 mM (Table 2), respectively. A linear relationship between eliminated concentration and exposure time was found in the studies. Statistical analysis showed there are no significant differences ($p < 0.05$) among the treatments with different initial concentration. The R^2 values of different initial concentrations of MET were in the range of 0.503–0.872 for *T. latifolia* treatment and 0.944–0.984 for *P. australis* treatment. Similar results were also reported for other pharmaceuticals (e.g. CBZ, diclofenac and clofibric acid, Zhang et al., 2013a).

We hypothesize that the uptake process is separated in two steps: (1) MET will enter the root tissue by passive diffusion through the apoplastic pathway, where it may also accumulate to a certain percentage; (2) MET is transported across the endodermis into the vascular bundles by active transport via a symplastic pathway. The concentrations in compartment C characterize only the second part of this process while the decrease of concentrations in compartment A characterizes both, including the possible accumulation inside the root tissue and adsorption on the surface of the roots without further movement.

3.3. Uptake and transport of metformin by T. latifolia in the presence of an inhibitor

Quinidine is an inhibitor of MET uptake by OCTs in mammals. We show here that this substance can significantly affect the transport process of MET in *T. latifolia* roots. After 90 min exposure to MET in the presence of the inhibitor, its concentrations in the 0.5, 1.0 and 2.0 mmol L^{-1} treatment increased in compartment C to 1.233, 1.327 and 1.346 μmol L^{-1}, respectively (Fig. 4). The concentration of MET in compartment C was 73.7%, 74.1% and 70.0% lower in the presence of quinidine compared to the absence of quinidine, respectively. This implies that in the presence of quinidine, MET transport is at least in part outcompeted. Still, the residual transport of MET in the roots is concentration dependent. Clearly, the lowest fluxes towards the root are observed at 0.5 mmol L^{-1} MET, where quinidine is present at equimolar concentration. It seems that the quinidine inhibitor, which acts competitively on the transporter protein, is expelled from the binding sites in a typical concentration dependent manner. However, the transport process could not be completely inhibited when higher concentrations of quinidine were added.

Previous studies showed that Arabidopsis OCTs are expressed in vascular tissues of roots (Lelandais-Brière et al., 2007). Subcellular localization also verified OCTs were present in the plasma membrane and tonoplast (Küfner and Koch, 2008). Thus, OCTs can be considered as an important pathway for MET taken up by plant roots. In the present study, we observed a fast transport process

Table 2
The metformin concentrations in compartment C, transport rates and metformin eliminated concentration in compartment A.

	T. latifolia			*P. australis*			*T. latifolia* with quinidine		
	0.5 (mmol L^{-1})	1.0 (mmol L^{-1})	2.0 (mmol L^{-1})	0.5 (mmol L^{-1})	1.0 (mmol L^{-1})	2.0 (mmol L^{-1})	0.5 (mmol L^{-1})	1.0 (mmol L^{-1})	2.0 (mmol L^{-1})
MET concentrations in compartment C (μmol L^{-1})	4.69 ± 0.69	5.13 ± 0.16	4.49 ± 0.47	4.82 ± 0.41	5.39 ± 0.49	5.71 ± 0.75	1.23 ± 0.05	1.33 ± 0.04	1.35 ± 0.05
Transport rates (μmol L^{-1} min^{-1})	0.0250	0.0230	0.0220	0.0290	0.0210	0.0370	0.0023	0.0027	0.0029
MET eliminated concentration in compartment A (μmol L^{-1})	60.3 ± 7.8	50.0 ± 5.0	87.8 ± 48.5	44.5 ± 9.5	36.0 ± 2.2	44.8 ± 4.3	11.8 ± 2.1	14.2 ± 7.6	19.8 ± 4.5

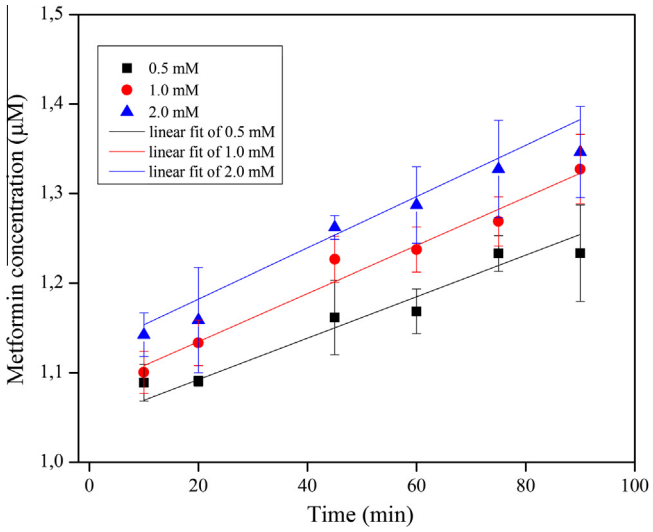


Fig. 4. Transport of metformin into compartment C in the presence of quinidine. Data points depict mean values of independent biological experiments with 3 replicates ± SD.

between 10 min and 90 min, with a positive correlation between initial concentration and transport rate in the presence of quinidine. However, this phenomenon disappeared in the absence of quinidine. This indicated that the uptake and transport of MET by plant roots is a complex process involving parallel activity of several mechanisms. Amino acids can be taken up directly by the roots and many amino acid transporters in plant have been identified (Fischer et al., 1998; Tegeder, 2012). As metformin resembles the molecular structure of arginine, it may be considered a potential substrate for the high affinity arginine transporters. Detaille et al. (2002) reported asymmetrical dimethylarginine, a cationic amino acid transporter inhibitor which can markedly reduce the uptake of MET by *Xenopus* oocytes.

Some authors quote that the movement of PPCPs into plants is a physical process such as passive diffusion (Pilon-Smits, 2005). A passive diffusion driven transport mechanism should be significantly dose-dependent, which was not observed in the present studies. Therefore, the mechanisms for uptake and transport of MET by plants points into a different direction. In addition, it is generally known that many non-selective cation channels (NSCCs) are extensively distributed in the plant cell membrane (Hedrich and Schröder, 1989) that could assist transport processes of xenobiotics to various extents.

In analogy with the absorption process in human liver our research indicates that OCTs may be considered as potential ports for symplastic transport of MET. OCT1, OCT2 and OCT3 are essential for the hepatic uptake of MET (Wang et al., 2002; Higgins et al., 2012; Nies et al., 2011) and other substrates for OCTs were found such as choline and carnitine (Sweet et al., 2001; Lelandais-Brière et al., 2007; Küfner and Koch, 2008). However, OCTs in plant have not been studied in depth as yet (Omote et al., 2006). Therefore, our use of quinidine as OCT inhibitor in the present study delivers first insight in the function of OCTs in plants during an uptake and transport process for cationic xenobiotics. Further studies are required to investigate the transport mechanisms in depth.

4. Conclusions

We demonstrated that MET can be taken up by plant roots and has the potential for subsequent translocation. No significantly difference has been found between *T. latifolia* and *P. australis* for the transport rate. Interestingly, the transport of MET was not correlated to the initial treatment concentrations. Quinidine can significantly inhibit the transport of MET by *T. latifolia* roots, with an inhibition ratios of 70–74%. The transport process of organic cationic compounds like MET is different from non-ionic organic chemicals, and OCTs may be considered as a potential conduit for cationic xenobiotics such as MET. Our studies indicate that plants can take up charged pollutant molecules and even translocate them to their arial parts, which might easily be harvested in a phytoremediation approach, in order to reduce the pollutant load in effluents towards surface waters. Of course, further research is required to explore the role, capacity and inducibility of OCTs and other membrane proteins for pollutant transport, and to screen species used in phytoremediation for such transporters.

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