Uptake of oxytetracycline and its phytotoxicity to alfalfa (*Medicago sativa* L.)

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Plant uptake of antibiotic oxytetracycline is energy-dependent.

Abstract

A series of experiments were conducted in a hydroponic system to investigate the uptake of oxytetracycline (OTC) and its toxicity to alfalfa (*Medicago sativa* L.). OTC inhibited alfalfa shoot and root growth by up to 61% and 85%, respectively. The kinetics of OTC uptake could be well described by Michaelis–Menten equation with $V_{\max}$ of $2.25 \mu\text{mol g}^{-1} \text{ fresh weight h}^{-1}$, and K_m of 0.036 mM. The uptake of OTC by alfalfa was strongly inhibited by the metabolic inhibitor, 2,4-DNP (2,4-dinitrophenol), at pH 3.5 and 6.0, but not by the aquaporin competitors, glycerol and Ag^+ . OTC uptake, however, was significantly inhibited by Hg^{2+} , suggesting that the inhibition of influx was due to general cellular stress rather than the specific action of Hg^{2+} on aquaporins. Results from the present study suggested that OTC uptake into alfalfa is an energy-dependent process. © 2006 Elsevier Ltd. All rights reserved.

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1. Introduction

Veterinary pharmaceuticals are widely used for the therapy of infectious diseases of animals in intensive farming systems (Halling-Sørensen and Jørgensen, 2000; Boxall et al., 2003). They are designed to act very effectively at low doses and to be completely excreted from the body after a short time of residence. It has been reported that only a fraction of the ingested antibiotics are metabolized in the animals, hence a large percentage of the antibiotics are excreted and released into the environment, e.g. via manure, sludge and waste water used as fertilizer or irrigation water into arable soils (Jørgensen and Halling-Sørensen, 2000). Therefore, residual concentrations

of pharmaceutical antibiotics can be found in soils, surface and ground water (Hamscher et al., 2002; Kolpin et al., 2002; Simon, 2005). For example, in Denmark, concentrations of oxytetracycline ranging from 2.5 to $50 \mu\text{g g}^{-1}$ in soil and from 33 to 2000 mg g^{-1} in pig manure have been reported (Loke et al., 2002). The fate of antibiotics, including sorption and fixation, mobility and transport in soil, has been well documented (Tolls, 2001; Kulshrestha et al., 2004; Figueroa and Mackay, 2005; Figueroa et al., 2005; Gu, 2005; Jones et al., 2005), whereas knowledge of their toxicity to plants and their uptake and transport into plants is poorly understood (Boxall et al., 2003; Thiele-Bruhn, 2003). The accumulation may or not affect the growth and development of plants; however, the uptake into plants may represent an important exposure pathway of these compounds to humans and other biota. Hence, there is a potential risk that plants might transfer antibiotics from the soil into the food chain. There are some reports dealing

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with the effects of antibiotics on plant growth and development (Batchelder, 1981, 1982; Migliore et al., 1998; Bradel et al., 2000; Jjemba, 2002a,b), but information is scant on characterizing the antibiotic uptake into higher plants.

It has been shown that bacterial uptake of tetracycline, an antibiotics similar structurally to oxytetracycline, is partially energy-dependent and hence an uptake mechanism has been proposed (Nikaido and Thanassi, 1993). According to this, tetracycline traverses the outer membrane of gram-negative bacteria through the OmpF and OmpC porins channels (Schnappinger and Hillen, 1996), chelating a M^{2+} ion as cation–tetracycline complexes $[M-tc]^+$ (Fig. 1). The cationic $[M-tc]^+$ is attracted by the Donnan potential across the outer membrane, leading to accumulation in the periplasm, where the metal ion–tetracycline complex probably dissociates to liberate uncharged tetracycline, a weakly lipophilic molecule that is able to diffuse through the lipid bilayer regions of the inner (cytoplasmic) membrane. Similarly, the electroneutral, lipophilic form is assumed to be the species transferred across the cytoplasmic membrane of Gram-positive bacteria. Uptake of tetracycline is energy-dependent and driven by the ΔpH component of the proton-motive force (Chopra and Roberts, 2001).

Although there is little information available on the mechanism of plant uptake of OTC, based on the proposal for bacteria, we hypothesize that OTC uptake by alfalfa roots is an active process that requires energy. Therefore the aim of the present study was to characterize the uptake of OTC in alfalfa.

The results are likely to be important for other plants including vegetables that grow in soils contaminated with OTC.

2. Materials and methods

2.1. Plant growth

Alfalfa (*Medicago sativa* L.) seeds were surface sterilized with 10% H_2O_2 for 10 min, rinsed with distilled water and germinated in moist filter paper for 24 h and then sown in plastic containers filled with perlite for 1 week in a growth chamber. Two seedlings of uniform size were transplanted into a PVC pot (7.5 cm in diameter, 14 cm in height) containing 550 ml of 1/3-strength aerated nutrient solution for the first week and then shifted to the half-strength nutrient solution which had the following compositions of macronutrients (in mM): KNO_3 , 1.0; $Ca(NO_3)_2 \cdot 4H_2O$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.4; KH_2PO_4 , 0.3; and of micronutrients (in μM): ferric ethylenediaminetetraacetic acid (FeEDTA), 50; $ZnSO_4 \cdot 7H_2O$, 1; $CuSO_4 \cdot 5H_2O$, 1; $MnSO_4 \cdot H_2O$, 5; H_3BO_3 , 10; $Na_2MoO_4 \cdot H_2O$, 0.5; $CoSO_4 \cdot 7H_2O$, 0.2. The initial pH of the solution was adjusted to 6.0 and the nutrient solution prepared with Millipore water (MilliQ, Billerica, MA, USA) was renewed every 3 days. Oxytetracycline was supplied as oxytetracycline hydrochloride (96% purity, Sigma–Aldrich, Inc., USA). Plants were grown in a climate chamber under controlled conditions (photoperiod 16 h light/8 h dark; temperature 25/21 °C at day/night; relative humidity 60%; light intensity $400 \mu mol m^{-2} s^{-1}$) for 3 weeks until mature roots (20–25 cm in length) were developed.

2.2. Experiment 1: Uptake and toxicity of OTC

Uniform three-week-old seedlings of alfalfa were transferred to the nutrient solutions with OTC concentration of 0, 0.0002, 0.002, 0.02, 0.10 and 0.20 mM and were allowed to grow for 10 days under controlled conditions (see above). Plants were harvested, rinsed thoroughly with running water and distilled water, and blotted dry with tissue paper. Plants were then separated into shoots and roots, and fresh weights were recorded. There were four replicates per treatment.

2.3. Experiment 2: Time-dependent uptake of OTC by alfalfa

The roots were carefully excised at the basal node to ensure that the roots were exposed to open air for a minimal period, and were used in the following experiments. Excised alfalfa roots (about 2 g fresh weight; f.wt) were incubated in distilled water at room temperature for 20 min before assay (Chen et al., 2005). Each of the samples was then quickly blotted dry with tissue paper, placed in a conical flask (200 ml) filled with 60 ml half-strength nutrient solutions (pH 6.0) containing 0.004 or 0.02 mM OTC. The incubation was kept for 0.5, 2, 4 and 8 h with continuous shaking. After the uptake period, ice-cold distilled water was used to wash the roots for 30 min to remove the surface-adsorbed OTC from alfalfa roots. The OTC concentrations in the equilibrium nutrient solutions and the washing distilled water were determined immediately by the HPLC method described below. Roots were then blotted dry with tissue paper and were immediately weighed. Sterility was maintained throughout the whole experiment. There were four replicates per treatment.

2.4. Experiment 3: Kinetics of OTC uptake

Batches of alfalfa roots (as above) were transferred to 200 ml conical flasks containing 60 ml half-strength nutrient solutions. The uptake of OTC was determined in the solutions with OTC at concentrations of 0, 0.0020, 0.0040, 0.008, 0.016, 0.032 and 0.064 mM. Other procedures were the same as described above.

Fig. 1. Uptake of tetracycline by *Escherichia coli* as described in the text. Tetracycline, protons, metal cations, and the metal-tetracycline complex are depicted as tc, H^+ , M^{2+} , and $[M-tc]^+$, respectively (Schnappinger and Hillen, 1996).

2.5. Experiment 4: OTC uptake by alfalfa roots influenced by pH

The pH value of the test solutions was buffered using 2-(*N*-morpholino) ethanesulfonic acid (MES) or 3-(*N*-morpholino) propanesulfonic acid (MOPS). Excised alfalfa roots were shaken for 1 h in the test solutions, half-strength nutrient solution with 0.02 mM OTC plus 5 mM MES (pH 5.4–6.7) or 5 mM MOPS (pH 6.5–7.9). Other procedures were the same as described above.

2.6. Experiment 5: OTC uptake influenced by metabolic and aquaporin inhibitors

OTC uptake was investigated using the procedure described elsewhere (Meharg and Jardine, 2003; Liang et al., 2005). The metabolic inhibitor, 2,4-DNP, was added at a concentration of 1 mM to suppress the uptake of OTC. For the treatments with aquaporin inhibitors, either HgCl₂ (0.01 and 0.1 mM Hg²⁺) or AgNO₃ (0.5 and 5 μM Ag⁺) or glycerol (1 and 10 mM) was used. Test solutions had 0.02 mM OTC or a range of concentration of inhibitors plus 0.02 mM OTC. The 3-week-old alfalfa roots were transferred to the test solutions and shaken for 1 h. The OTC concentrations in the equilibrium test solutions and the washing distilled water were determined immediately. There were four replicates per treatment.

2.7. Experiment 6: OTC uptake by excised roots influenced by 2,4-DNP at two pH values

Initial observation showed that the addition of 2,4-DNP decreased the pH value of nutrient solution. In order to exclude the possible inhibitory effects of pH, we characterized OTC uptake by alfalfa roots and by alfalfa plants as influenced by 2,4-DNP at two pH values. There were four treatments with four replicates each: i.e. Control (nutrient solution pH 6.0), MES (pH 6.0), 2,4-DNP (pH 3.5), and 2,4-DNP plus MES (pH 6.0). All test solutions contained 0.02 mM OTC. Excised alfalfa roots were excised and placed in a 200 ml conical flask containing 60 ml test solutions described above. Other procedures were the same as described above.

2.8. Experiment 7: OTC uptake by intact plants influenced by 2,4-DNP at two pH values

The experiment consisted of four treatments with four replicates: i.e. control (nutrient solution, pH 6.0), 0.02 mM OTC, 0.02 mM OTC plus 1 mM 2,4-DNP (pH 3.5) and 0.02 mM OTC plus 1 mM 2,4-DNP (pH 6.0).

Uniform 3-week-old seedlings were transferred to distilled water for 30 min and blotted dry with tissue paper. Two plants were transferred to an aluminum-foil wrapped brown flask containing 300 ml test solutions described

above after the light period had already started for 2 h. After 24 h, the concentration of OTC in the uptake solution was determined by HPLC and the water loss from transpiration was also recorded by weighing.

2.9. Determination of OTC by the HPLC method

Acetonitrile and methanol were of HPLC grade, and ammonium acetate, formic acid, sodium hydroxide were of analytical reagent grade. Water was prepared in-house by a Milli-Q system (Millipore, Eschborn, Germany). Stock solutions of OTC standard prepared by dissolving 1 mg of OTC in 1 ml of methanol were stored at –80 °C and were stable for at least 2 months. Working solutions were prepared freshly for the assay of OTC.

OTC was determined following the method of Hamscher et al. (2002). The concentration in equilibrium solution was measured by the reversed-phase HPLC method. The HPLC system was a gradient system consisting of a Waters 515 pump, a Waters 2996 photodiode array detector acquiring spectra at 355 nm with a resolution of 1.2 nm and a data acquisition rate of 1 spectrum/4 s and a Sunfire C18 column (5 μm, 4.6 × 150 mm, Waters Corp., USA) operated at 25 °C. The mobile phase consisted of 0.5% formic acid in water with 1 mM ammonium acetate (solvent A, pH 2.5), and acetonitrile (solvent B). The flow was of 1 ml/min. The gradient run was 0–50% B in 9 min and then held at 50% B for 1 min. After each run, the column was rinsed for 3 min with 99% acetonitrile and re-equilibrated for 12 min with solvent A. The injection volume was 20 μl.

Calibration curves constructed for OTC ranged from 0.001 to 40 mM for OTC concentration-dependent experiment with $r^2 = 0.9999$ and from 0.002 to 0.040 for other experiments with $r^2 = 0.9995$. Qualification was obtained by comparing the peak areas of the sample, which was assessed using the Millenniumatography manager V2.10 software, with that of the external calibration curves.

2.10. Statistical analysis

Analysis of variance on plant shoot and root biomass and concentrations of OTC was performed with SPSS 11.0 (SPSS Inc., Chicago, MI, USA).

3. Results

3.1. Uptake and toxicity of OTC

Both shoot and root biomass of alfalfa decreased sharply with increasing concentrations of OTC in the growth solution (Fig. 2). OTC had no effect on alfalfa shoot biomass at OTC concentration of 0.002 mM, but decreased the root biomass significantly ($p < 0.05$) when the OTC concentration was

Fig. 2. Effects of oxytetracycline on (A) shoot and (B) root fresh weights in Experiment 1. Each point is the average of four replicates, and bars represent $\pm$ SE of the mean.

above 0.002 mM. In addition, the ratio of shoot/root increased significantly at lower OTC concentrations, suggesting that the roots are more sensitive to OTC than shoots. OTC decreased shoot and root fresh weight by a maximum of 61% and 85%, respectively (Fig. 2). The leaves became weakly green at 0.02 mM, and the shoots wilted at higher concentrations (0.10 and 0.20 mM).

3.2. Time-dependent and kinetics of OTC uptake

The results of time-dependent uptake of OTC at external concentrations of 0.004 and 0.02 mM (Experiment 2) showed that the net uptake increased linearly within 4 h, after which OTC uptake started to slow down (Fig. 3). Kinetics of OTC uptake (Experiment 3) by alfalfa roots was adequately described by the Michaelis-Menten function (Fig. 4, $p < 0.0001$). The calculated $V_{\max}$ was $2.25 \mu\text{mol g}^{-1} \text{ f.w. h}^{-1}$, and K_m was 0.036 mM.

3.3. OTC uptake influenced by pH, metabolic and aquaporin inhibitors

OTC uptake was the lowest at pH 5.0, and the highest at pH 7.0 (Fig. 5). Treatment with 1 mM 2,4-DNP decreased OTC uptake by alfalfa roots by 74.8% (Table 1). OTC uptake at external concentration of 0.02 mM was decreased by 42.4 and 46.1% when the plants were treated with 0.01 and 0.1 mM Hg^{2+} (Table 1). Silver had no effect on OTC uptake by alfalfa roots at 0.5 or 5 mM. Glycerol at either 1 or 10 mM had no effects on OTC uptake (Table 1).

3.4. Inhibitory effects of 2,4-DNP on OTC uptake at two pH values

The uptake of OTC by alfalfa roots was inhibited significantly by 2,4-DNP at two pH values (pH 6.0 and 3.5) (Fig. 6). Furthermore, OTC uptake by intact alfalfa plants was also inhibited notably by 2,4-DNP at pH 3.5 within a 24-h period, although low pH value (3.5) reduced OTC uptake by alfalfa plants compared with the control (pH 6.0)

Fig. 4. Concentration-dependent kinetics of OTC uptake by alfalfa (*Medicago sativa*) roots in Experiment 3. Each point is the average of four replicates and bars represent $\pm$ SE of the mean.

(Fig. 7). Fig. 7 also shows that OTC had no effect on transpirational water loss compared with the control, but 2,4-DNP significantly inhibited transpiration.

4. Discussion

OTC had significant inhibitory effect on alfalfa growth at concentrations higher than 0.02 mM. Root growth was more sensitive to OTC than shoot, which is in agreement with the findings of Migliore et al. (2003), who showed that plant roots are the main sites of antimicrobial drug accumulation. Alfalfa leaves turned light green or even yellow with increasing OTC doses. The phytotoxicity might be caused by the inhibition of translational activity of chloroplast and chloroplast (p)ppGpp synthase activity by tetracycline, which is structurally similar to OTC (Bradel et al., 2000; Kasai et al., 2004).

Dissolved species of OTC may have net positive charges (pH < 3.6), neutral (3.6 < pH < 7.5) and negative (pH > 7.5) (Fig. 8) because OTC is a weak polyprotic acid. Our data showed that OTC uptake by alfalfa was pH-dependent with

Fig. 3. Time-dependent uptake of OTC at external concentrations of (A) 0.004 mM and (B) 0.02 mM by alfalfa (*Medicago sativa*) roots in Experiment 2. Each point is the average of four replicates and bars represent $\pm$ SE of the mean.

Fig. 5. Effects of solution pH on OTC uptake by alfalfa roots in Experiment 4. Each point is the average of four replicates and bars represent $\pm$ SE of the mean.

Fig. 6. Effects of 2,4-DNP at pH 3.5 and 6.0 on OTC uptake in Experiment 6. Bars represent $\pm$ SE of the mean.

the lowest uptake at pH 5.0, suggesting that OTC molecules could be easily taken up by alfalfa roots at higher pH. This pH-dependent uptake of OTC was observed not only by alfalfa roots but also by intact alfalfa plants (Fig. 7). Thus, soil solution pH is an important factor influencing the bioavailability of OTC in soil (Kulshrestha et al., 2004; Figueroa and Mackay, 2005; Figueroa et al., 2005; Gu, 2005; Jones et al., 2005) and the transport from soil to plant.

Time-dependent uptake of OTC by alfalfa showed that there was an equilibrium status of OTC uptake after 4 h (Fig. 3). Thus, follow-up short-term uptake experiments were performed within one hour, because OTC uptake within the 4-h-uptake period increased linearly ($r^2 = 0.994$ for 0.004 mM and 0.999 for 0.02 mM) with time. The kinetics of OTC uptake into alfalfa roots was well fitted to the Michaelis–Menten equation. Further experiments in the present study showed that OTC uptake by alfalfa roots and plants was inhibited significantly by the metabolic inhibitor 2,4-DNP. It is well documented that 2,4-dinitrophenol (2,4-DNP) is regarded as an uncoupler of oxidative (and to a lesser extent, photosynthetic), phosphorylation, and of proton-coupled fluxes at the plasma membrane and endomembrane via the dissipation of trans-membrane electrochemical gradients of protons (Liang et al., 2005). The significant inhibition of OTC uptake by the uncoupler DNP and the good description of OTC uptake by Michaelis–Menten model (Figs. 4–7 and Table 1), suggested that the transport of OTC into alfalfa roots was an energy-dependent process, which requires selective binding sites.

To investigate whether OTC uptake by plant could be facilitated by water channels, we used Hg^{2+} , Ag^+ and glycerol to inhibit or compete for the aquaporins in alfalfa roots. Aquaporins are sensitive to Hg^{2+} , which inhibits water via covalent

modification of cysteine residues within the water pore and in other regions of the protein causing either blockage or conformational changes leading to the inhibition of water transport (Barone et al., 1998). OTC uptake by alfalfa roots was decreased when exposed to 0.01 and 0.1 mM Hg^{2+} compared with the control (Table 1). However, the reduction in OTC uptake after the addition of Hg^{2+} could be due to general disruption of plasma membrane biochemistry caused by the highly toxic Hg^{2+} ion (Dordas and Brown, 2001; Martre et al., 2001; Du et al., 2005), rather than specific inhibition of aquaporins (Meharg and Jardine, 2003). To further elucidate the possible role of aquaporins in OTC uptake, we used the other competitors, such as glycerol and Ag^+ . Glycerol is widely used to assay aquaporins activity (Biela et al., 1999), and has a low phytotoxicity. Silver is another more potent inhibitor of aquaporins than the commonly used mercury (Niemietz and Tyerman, 2002). It is likely that silver reacts with the sulfhydryl group of a cysteine in the vicinity of the conserved NPA motif and thus effectively blocks the constriction region of the water channel. Our findings showed that glycerol and Ag^+ did not compete with OTC for uptake and transport into alfalfa roots, suggesting that OTC does not enter into the root through water channels. The glycerol concentration of 10 μM used in the present study was high enough to inhibit arsenite uptake by rice within 20 min, which is sufficient for the inhibition of aquaporins (Meharg and Jardine, 2003), and the 5 μM of Ag^+ was also suitable (Niemietz and Tyerman, 2002). Therefore, our data convincingly demonstrated that OTC uptake was aquaporins-independent.

The adverse effects observed on alfalfa and other plants have important implications in agricultural systems where livestock

Table 1

OTC uptake as influenced by metabolic inhibitors (2,4-DNP) and aquaporin inhibitors (Hg^{2+} , Ag^+ and glycerol)

	Control	2,4-DNP (mM)	Hg^{2+} (mM)	Ag^+ (μM)	Glycerol (mM)
Concentration		0.001	0.01	0.1	0.5
OTC influx (nmol ⁻¹ f.wt h ⁻¹)	138.7 $\pm$ 3.9	35.0 $\pm$ 2.1	79.9 $\pm$ 4.1	74.7 $\pm$ 8.8	129.8 $\pm$ 9.7

Data are expressed as mean $\pm$ SE.

Fig. 7. OTC and water uptake by intact alfalfa plants grown with OTC at concentration of 0.02 mM for 24 h in Experiment 7. Bars represent $\pm$ SE of the mean.

manure, wastewater or treated sewage sludge which might contain some of the commonly used antibiotic agents are applied to agricultural fields (Kümmerer, 2001). Therapeutic agents might convey antibiotics from the soil into the food chain of human beings due to the uptake and bioaccumulation of these agents by plants grown in the antibiotic-contaminated soils.

In conclusion, antibiotics are potentially phytotoxic to and are accumulated by higher plants, including vegetables and crops. Results from the present study demonstrate that plant uptake of OTC is energy dependent, and aquaporins in root cell membranes may not contribute to the uptake of OTC. The accumulation of antibiotics such as OTC in plants may pose health risks to animals and humans. Therefore, further research activities should focus on investigating the mechanism for uptake of other veterinary antibiotics into plant roots, their root to shoot translocation, and their accumulation in plants, especially in vegetables.

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Antibiotic	pKa1	pKa2	pKa3
OTC	3.57	7.49	9.88

Fig. 8. OTC chemistry and solution speciation shown with environmentally relevant pH value (Tavares and McGuffin, 1994).

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