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Sulfadiazine Uptake and Effects on *Salix fragilis* L. and *Zea mays* L. Plants

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Abstract Frequently, sulfonamide antibiotic agents reach arable soils via excreta of medicated livestock. In this study, accumulation and phytotoxicity indicators were analyzed to evaluate the effects of sulfonamides on plants. In a greenhouse experiment, willow (*Salix fragilis* L.) and maize (*Zea mays* L.) plants were grown for 40 days in soil spiked with 10 and 200 mg kg^{-1} sulfadiazine (SDZ). Distribution of SDZ and major metabolites among bulk and rhizosphere soil, roots, leaves, and stems was determined using accelerated solvent extraction and LC−MS/MS analysis. Accumulation of SDZ was stronger in willow. The antibiotic was mainly stored inside roots and 4-hydroxy-sulfadiazine presence increased with the administered SDZ concentration. SDZ altered root geotropism, increased the lateral root number, and affected plant water uptake. The high concentration caused serious stress in willow (e.g., reduced C/N ratio and total chlorophyll content) and the death of maize plants. Even at environmentally relevant soil concentrations (10 mg kg^{-1}), SDZ exhibited adverse effects on root growth, while at artificially high concentrations (200 mg kg^{-1}), it showed a strong potential to impair plant performance and biomass. Willow, a fast growing tree species, showed potential for possible phytoremediation purposes.

Keywords Antibiotic action · Biometrics · Rhizosphere · Root geotropism · Maize · Willow

Abbreviations

SDZ	Sulfadiazine
dm	Dry mass
fm	Fresh mass
4-OH-SDZ	4-hydroxy-sulfadiazine
5-OH-SDZ	5-hydroxy-sulfadiazine
N-Ac-SDZ	*N*-acetyl-sulfadiazine

1 Introduction

The medication of livestock with pharmaceutical antibiotics represents a normal practice in conventional

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animal production (Halling-Sørensen et al. 1998; Jjemba 2002; Jørgensen and Halling-Sørensen 2000). In particular, sulfonamides are widely used for the prevention of infectious diseases due to their broad spectrum antibacterial and anticoccidial activity (De Liguoro et al. 2007). However, following administration, up to 90 % of the parent compound is quickly excreted (Sarmah et al. 2006). As a consequence, manuring agricultural soils with excreta results in soil contamination with pharmaceutical antibiotics. In field studies, sulfonamide antibiotic agents were detected in extractable concentrations of up to 500 mg l^{-1} in pig slurry (Grote et al. 2004; Hölzel et al. 2010) and 0.5 mg kg^{-1} in field soil (Grote et al. 2004; Schmitt et al. 2005). Christian et al. (2003) and Aust et al. (2008) detected extractable sulfonamide residues in soil up to 1 year after application with manure to agricultural fields. It must be noted that the extractability of sulfonamides quickly declines due to immobilizing processes (Förster et al. 2009; Wehrhan et al. 2010). For example, already 2 days after spiking sulfadiazine (SDZ) at a concentration of 10 mg kg^{-1} to soil, <2 mg kg^{-1} remained extractable, a concentration very much resembling those reported for field soil (Grote et al. 2004; Schmitt et al. 2005). Vice versa, it must be assumed that sulfonamide concentrations extracted from field soil originated from considerably higher initial amounts. However, a decline in extractable concentration is not due to metabolization or mineralization of the parent compound, which was shown to be subordinate (Langhammer et al 1990; Sukul and Spiteller 2006). Instead, sulfonamides tend to persist for months (Aust et al. 2008; Boxall et al. 2004).

As therapeutic agents are designed to be biologically very active chemicals, once they reached the soil, their activity clearly affects soil microorganisms (Ding and He 2010; Thiele-Bruhn 2003) and could also impact vegetation (Jjemba 2002). An uptake of several antibiotics into food plants and translocation within the plant was recently reported in the literature (Dolliver et al. 2007; Ferro et al. 2010; Grote et al. 2007). The sulfonamide sulfamethazine was taken up from manure-amended soil by maize, lettuce, and other plants (Dolliver et al. 2007), and Ferro et al. (2010) reported relevant sulfadimethoxine accumulation in barley roots. Moreover, Grote et al. (2007) identified the radiolabel from ^{14}C-sulfadiazine in roots and leaves of winter wheat. However, plant uptake was small with <0.1 % of the applied amount for sulfonamides (Grote et al. 2007), which is in accordance with earlier findings from Langhammer et al. (1990). Furthermore, plant responses to the active molecules are not yet clear, and both promoting and inhibiting effects of antibiotics on plants were determined in pot experiments (Jjemba 2002; Liu et al. 2009; Migliore et al. 1995; 1996, 1998). Recent evidence that field concentrations of fluoroquinolones might negatively influence plant growth (Boxall et al. 2006) has to be corroborated in further studies for other antibiotics. However, investigations concerning plant uptake, distribution within the plant, and subsequent effects on vegetal physiology remain still limited.

Consequently, the presented study had a twofold aim: (1) to investigate the plant uptake of a sulfonamide antibiotic from soil and its possible utilization in phytoremediation and (2) to determine adverse effects on crops. To investigate this, willow (*Salix fragilis* L.) and maize (*Zea mays* L.) plants were exposed for 40 days (i.e., minimum time required to obtain enough plant material to perform the analyses described below) to SDZ, a sulfonamide antibiotic that is frequently applied in livestock husbandry to prevent and treat bacterial diseases (Boxall et al. 2004). Maize was chosen as it is an agricultural plant typically receiving high manure fertilization and respective antibiotic loads. Willow was investigated because it is a representative plant for phytoremediation purposes and short rotation plantations (Kuzovkina and Quigley 2005). Plants were grown in soil containing 10 mg SDZ kg^{-1}, corresponding to an upper concentration level that can be expected in soil, when considering the rapidly declining extractability of sulfonamides in soil (Thiele-Bruhn 2003), and 200 mg SDZ kg^{-1}, yet being an unusual high concentration, to show the potential of uptake and effects. Analyses of plant growth, physiological parameters, and the concentration of SDZ and major metabolites in plant tissues and soil sections were carried out, with particular attention to the root apparatus as it was supposed to be the main site of antibiotic accumulation and effects (Michelini et al. 2012).

2 Materials and Methods

2.1 Experimental Design

Eighteen *S. fragilis* L. cuttings (20 cm long and 1 cm diameter), taken from a selected tree in the

experimental farm of the University of Padova at Legnaro (Italy), and 18 *Z. mays* L. seeds (cultivar PR39K13 Pioneer Hi-Bred Buxtehude) were pregrown in tap water for 10 days to allow first root and leaf development. Plants were then transferred to soil. Soil material was obtained from the Ap horizon (0–30 cm) of an Orthic Luvisol silt loam from an arable field at Jülich-Merzenhausen (Germany). The soil was not previously treated with manure and pharmaceutical antibiotics. The main soil properties are pH ($CaCl_2$) 6.3, clay 15.4 %, silt 78.2 %, sand 6.4 %, OC 2.1 %, CEC 11.4 $cmol_c\ kg^{-1}$, and maximum water holding capacity 45.8 $g g^{-1}$. The air-dried soil was sieved through a 4-mm screen, to ensure physical homogeneity. Soil was mixed with 50 $g m^{-2}$ of NPK fertilizer before planting, thus ensuring unimpeded plant nutrition but without affecting further soil properties. Kick–Brauckmann pots (25.5 cm height and 28.5 cm external diameter), made of polypropylene inert material, and ensuring the catchment of percolating water and its re-use by the plant, were filled with 7 kg soil. Each pot was split in two halves by a PE sheet and one plant per half was grown. Soil was spiked with sulfadiazine sodium salt (99.0 % minimum, CAS: 547-32-0, Sigma Aldrich, Germany) with resulting final concentrations of 0 (control), 10, and 200 mg kg^{-1}. The antibiotic was added without manure to not bias SDZ effects with those of nutrients. The experiment was conducted with six independent replicates per treatment group. In parallel, one pot per SDZ treatment was maintained without plants. Plants were cultivated in a greenhouse under natural photoperiod for 40 days (from 7 April to 16 May 2011) and at an average temperature of 25±5 °C during the day and 20±5 °C at night. Light was not artificially provided, thus depended on the meteorological conditions characterized by sunny weather during the experimental period. Pots were irrigated twice per week with the same amount of water ranging from 200 to 500 ml per pot. Soil and plant sampling was performed 40 days after the beginning of the SDZ exposure; each of the six independent replicates was treated separately. Bulk soil samples (the fraction of soil not influenced by roots) were collected and the entire root apparatus of every plant was vigorously shaken by hand in order to collect the rhizosphere soil, defined as the fraction of soil adhering to roots. Samples of roots, leaves, stems, and bulk and rhizosphere soils were stored at −20 °C prior to further analyses. Dry masses of soil samples were determined after drying at 105 °C for 24 h and at 60 °C, until complete dryness, for plant material. Root morphology of both plant species and the different treatments were documented with digital photos (SONY, Cyber-shot, DSC-S930, 10.1 megapixels).

2.2 Biometrics and Soil Moisture

Biometric measures were recorded weekly for each plant until a few days before harvest. In particular, the stem length and the total leaf number were documented for both species and, for the maize, also the length of the second to the fifth leaf. At the end of the 40-day cultivation period, root areas, root volumes, and total root lengths for both species were recorded through a scanner-based image analysis system (WinRHIZO Basic, Reg and Pro 2007a, Regent Instruments, Inc., Quebec, Canada). Additionally, the soil moisture was determined twice per week in order to get any difference in the water uptake from control and treated plants. To this intent, an ECH_2O EC-5 (Decagon Devices, Inc, Pullman, WA, USA) probe inserted at 10 cm soil depth was used together with a TDR device (INFIELD 7b, UMS, Munich, Germany).

2.3 Antibiotic Extraction Procedure

After collecting adhering rhizosphere soil and thoroughly cleaning the roots with running water (Grote et al. 2007) until they were visually free from adhering soil, root samples (0.5 g of fresh material) were sonicated in 50 ml of deionized water for 15 min to extract the fraction attached to the rhizoplane of SDZ and respective metabolites. A 1-ml aliquot of this washing solution was transferred to a 1.5-ml amber glass vial, and 10 μl of sulfamethazine (500 ng ml^{-1} in methanol), which has very similar properties to SDZ, was added as internal standard. However, correction of LC–MS data with the internal standard was not required. Subsequently, all plant tissues (i.e., willow and maize leaves, roots, and stems) were ground <0.125 mm in liquid nitrogen prior to accelerated solvent extraction (ASE 350, Dionex, Idstein, Germany) to determine the concentration of SDZ and major metabolites in the plant. A similar procedure was applied for soil. To this end, plant samples (0.5 g fresh mass) or soil (5 g field moist soil) was mixed with 1.5 or 1 g of diatomaceous earth, respectively, to prevent clogging of the extraction cells. Five (in case

of shortage in plant material) to six replicates were extracted from each sample. The solvents used for antibiotic extraction from plants were (1) methanol/deionized water 1:4 (*v*/*v*) according to Förster et al. (2008) and (2) deionized water for soil samples. These extractants proved to be most efficient in preliminary experiments. Briefly, (1) extraction yield from willow plant material using methanol/water was 1.6 times higher than that of methanol/citrate buffer pH 4.2 (3:1 *v*/*v*) and (2) recovery rate of SDZ from spiked soil samples (1 mg kg^{-1}) was 89 % (±7) using ASE water extraction compared to 75 % (±19) using ASE methanol/water extraction (unpublished data). Parameters of the applied ASE method were adjusted as follows: 9 min of preheat; two and one cycle for plants and soil, respectively; 15 min of static time; 200 °C temperature; 60 % of flush; 100 bar pressure; and 400 s of N_2 purge. A 1-ml aliquot of the extract was transferred to a 1.5-ml amber glass vial, and 10 μl of the internal standard was added in order to account for matrix effects.

2.4 LC–MS/MS Analysis

The concentration of SDZ and the presence of its acetyl- (N-Ac-SDZ) and hydroxy-metabolites (4-OH-SDZ, 5-OH-SDZ) in extracts from plant and soil samples were determined using a Shimadzu LC-20 HPLC (Shimadzu, Duisburg, Germany) coupled to an API 3200 LC–ESI-MS/MS (Applied Biosystems/MDS Sciex Instruments, Toronto, Canada). The HPLC consisted of two LC-20 AD pumps, an autosampler SIL-20 AC, a column oven CTO-10ASvp, and a system controller CBM-20A Lite. A Sunfire C18, 3.5 μm, 3.0×20 mm guard column and a Sunfire C18, 3.5 μm, 3.0×100 mm (Waters, Eschborn, Germany) were used for separation of SDZ and its metabolites from other matrix components. The eluent consisted of 0.1 M HCOOH in water (solvent A) and 0.1 M HCOOH in methanol (solvent B) which were delivered in a gradient program listed online in Table S1. For analysis, the API 3200 LC–MS/MS was operated in positive ionization MRM mode with a sample injection volume of 10 μl. Nitrogen was used as nebulizer gas at 413.68 kPa and as drying gas at 482.63 kPa, respectively; the latter was heated to 650 °C. Ionization voltage was set to 5.5 kV. Additional ion-dependent parameters for the specific mass transitions are listed online in Table S2. The software Analyst 1.4.2 (Applied Biosystems/MDS Sciex Instruments) was used for analysis of the data obtained. The quantification of the parent compound was done by summarizing the signal of the different mass transitions, while the ratio of two single mass transitions was used for compound identification (Antignac et al. 2003). The minimum signal-to-noise ratio for separation of a peak from baseline noise was 10. External standards containing 0, 10, 20, 50, 100, 200, 500, and 1,000 μg l^{-1} SDZ were used for the calibration curve. The metabolites 4-OH-SDZ, 5-OH-SDZ, and N-Ac-SDZ were quantified relatively to SDZ using the SDZ calibration curve. The most abundant mass transition of each metabolite was compared with the sum of SDZ transition masses. In particular, for N-Ac-SDZ, masses considered were *m*/*z* 134.2 and 198.0, while for OH-SDZ, it was *m*/*z* 155.9; the abundance of other masses was negligible. The limit of detection of the method was 5 μg l^{-1} and the limit of quantification was 10 μg l^{-1} determined using the procedure of Antignac et al. (2003). Final results are expressed in milligram per kilogram on a dry mass (dm) basis.

2.5 Bioconcentration Factor and Translocation Factor

To evaluate the ability of the two plant species to extract and accumulate SDZ in plant tissues, the bioconcentration factor (BCF, Eq. 1) was determined for roots according to Zayed et al. (1998).

$$\mathrm{BCF} = \frac{\text{Contaminant concentration in plant tissue at harvest}(\mathrm{mg\,kg^{-1}})}{\text{Initial concentration in the external growth medium}(\mathrm{mg\,kg^{-1}})} \quad (1)$$

Furthermore, to better define the active molecule fate after plant uptake, the translocation factor (Tf) was calculated using Eq. 2 in accordance to Zacchini et al. (2009). The Tf indicates the percentage of the accumulated pollutant that reaches the aerial part (leaves and stems) of the plant in relation to that remaining in roots.

$$\mathrm{Tf} = \frac{\text{Contaminant concentration in the aerial parts}(\mathrm{mg\,kg^{-1}})}{\text{Contaminant concentration in the roots}(\mathrm{mg\,kg^{-1}})} \times 100 \quad (2)$$

2.6 Element Content

Samples of leaves, roots, and stems were dried at 105 °C for 24 h, ball-milled (Retsch MM200; Retsch, Haan, Germany) until a powder-like material was reached, and transferred into tin capsules (5×9 mm; IVA Analysentechnik, Düsseldorf-Meerbusch, Germany). Total carbon and total nitrogen (percentage of dry mass) were determined after combustion using an elemental analyzer (Euro-EA 3000CNS, HEKAtech, Wegberg, Germany). Concentrations of Ca and K were determined after digesting 0.1 g of dry material per replicate at 170 °C for 6 h with 1 ml H_2O_2 30 % (Merck, Darmstadt, Germany) and 3 ml HNO_3 65 % (Carl Roth, Karlsruhe, Germany) in hermetically closed Teflon tubes. After this step, samples were purified with 125 mm diameter filters (Whatman, Dassel, Germany) and brought to 50 ml with deionized water. Ca and K contents were measured with atomic absorption spectroscopy (Agilent-Varian AA240FS, Mulgrave, Australia). Three replicates per group were carried out for these measurements and each sample was analyzed twice. Final data are expressed as gram per kilogram dm of Ca or K.

2.7 Chlorophyll Content

Chlorophyll content was evaluated in two different ways. At first, chlorophyll meter readings (SPAD-502, Minolta Camera Co. Ltd., Munich, Germany) were taken at the center of three full expanded leaves per plant at the end of the experiment. For each leaf, six independent measurements were collected, each of which was the average of five repeated measurements. In parallel to soil plant analysis development (SPAD) values, total chlorophyll content was measured according to Lichtenthaler (1987). Leaf discs (approximately 0.1–0.2 g) were cut out with a cork borer (1 cm diameter) from the youngest and fully expanded leaf. Discs were placed in glass tubes containing 5 ml methanol (MeOH; VWR, Darmstadt, Germany) and incubated at 60 °C for 30 min in the dark. After the material cooled down, absorbance of the solutions was measured with a UV/Vis spectrophotometer (UV-160a, Shimadzu, Duisburg, Germany) at 665 and 650 nm. Total chlorophyll (Total chl) concentrations (microgram per gram fm) were calculated using Eq. 3, where A665 and A650 represent the two wave lengths used in the analysis.

$$\text{Total chl} = \frac{\mathrm{MeOH(ml)} \times [(\mathrm{A665} \times 4) + (\mathrm{A650} \times 23.5)]\,(\mu\mathrm{g\,ml^{-1}})}{\text{Fresh weight(g)}} \quad (3)$$

2.8 Statistical Analysis

Open source software R (R Development Core Team 2008), with the application of "car" and "agricolae" packages, was used for statistical analyses. Significant differences ($p<0.05$) among groups were assessed by one-way analysis of variance followed by Tukey's honestly significant differences test for comparisons. Significant differences ($p<0.05$) between groups were assessed by Student's t test.

3 Results

3.1 Plant Biometrics and Soil Moisture

Willow and maize growth during the experimental time was monitored following the total number of leaves and the stem lengths per plant (Fig. 1a–d). For all the parameters analyzed, the first measuring point (0 day), immediately before the beginning of SDZ exposure, did not show statistical differences among treatment groups of the two species. In the further course of the experiment, effects due to SDZ were detected for both the number of leaves and length of stems following exposure to 200 mg kg^{-1} of SDZ. In contrast, the SDZ soil concentration of 10 mg kg^{-1} did not significantly affect the leaf numbers and the stem lengths of both plant species although in most cases there was a slight trend of smaller values for the plants growing in soil with 10 mg SDZ kg^{-1} (Fig. 1a–d). At the end of the exposure time, willow and maize plants of the control and 10 mg kg^{-1} groups reached a mean number of about 81 and 9 leaves per plant, while the stem lengths were approximately 38 and 23 cm for willow and maize, respectively (Table 1). To the opposite, 200 mg kg^{-1}

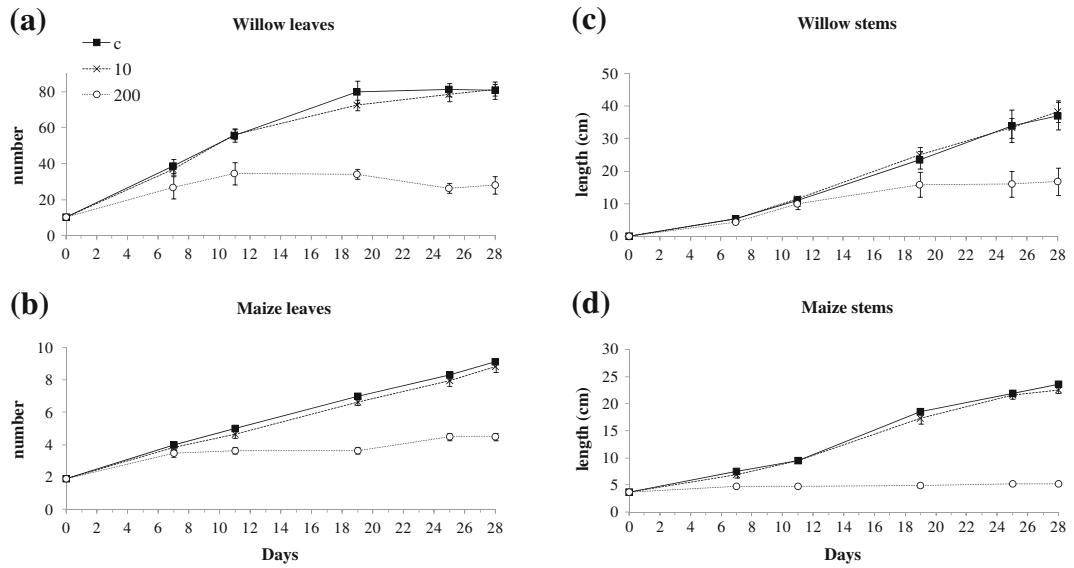

Fig. 1 Leaf number development (**a**, **b**) and stem lengths (**c**, **d**) during the experimental time in willow (**a**, **c**) and maize plants (**b**, **d**) exposed to 0 (c), 10 (10), and 200 (200) mg kg^{-1} SDZ. Values denote mean±SE. *Error bars* not shown are smaller than symbols

of SDZ caused a drastic decrease in the leaf number (28 leaves for willow and 4 leaves for maize) and in the stem length (17 cm for willow and 5 cm for maize). The length development of the second to the fifth leaf of maize plants (Fig. S1a–d) showed that plants in control soil and plants exposed to 10 mg kg^{-1} had a similar mean length development for all leaves at all measurement points, while leaves evolved within 7 days (second and third leaves), 11 days (fourth leaf), and 24 days (fifth leaf) in the treatments with 200 mg SDZ kg^{-1} exhibited a significantly ($p<0.05$) reduced leaf length. The difference to leaf length and development from maize plants of control and 10 mg SDZ kg^{-1} treatments further increased with leaf number and maize plants exposed to 200 mg SDZ kg^{-1} did not develop a fifth leaf.

Similar results were obtained for the leaf and stem mass, root areas, root volumes, and total root lengths for both species and root fresh mass in the case of maize after 40 days of exposure to SDZ (Table 1), where plant tissue development was inhibited by 200 mg SDZ kg^{-1}. Root volume and total length of maize roots and willow root area tended to be larger in the 10-mg SDZ kg^{-1} treatment compared to the control. Even more, this increase in root biometrics was significant ($p<0.05$) for the fresh mass and area of maize roots. However, for willow plants, an effect of 10 mg SDZ kg^{-1} was only found for the total root length (Table 1).

The percentage of the dry mass content was evaluated in the roots, leaves, and stems (Table 1). For both species, the dry mass content of roots and leaves was not statistically different for plants exposed to the SDZ concentrations. In contrast, a change in root structure became evident from the specific root length (SRL, root total length per unit root dry mass, in cm g^{-1} dm). This parameter increased for the spiked SDZ concentration. Willow SRL data were 2,604, 3,534, and 5,122 for control and treatments 10 and 200 mg kg^{-1}, while for maize, SRL values were 619, 675, and 1,520, respectively. The dry mass content of roots and stems of willow and maize plants exposed to 200 mg SDZ kg^{-1} was substantially altered. Dry mass content was mostly and in the case of willow stems even significantly reduced, while dry mass content of aerial parts was substantially increased to a mean of 79 % for maize plants. It must be noted that the latter dry mass data represent both leaves and stems, since the singular tissues were too small for separate sampling and analysis due to strong SDZ effects. Maize plants even wilted and died off.

With the aim to identify possible effects on the plant physiology following SDZ exposure, soil moisture was recorded and readjusted if necessary twice per week for every pot. In soil without plants, the average moisture after the first days of the experimental time remained

Table 1 Final biometrics and dry matter contents (percent) of willow and maize plants

Sample	Stem length (cm)	Leaves number	Leaves and stems	Roots				Dry mass			
			(g fm)	(g fm)	Area (cm^2)	Volume (cm^3)	Total length (cm)	Root (%)	Leaf (%)	Stem (%)	SRL ($cm\ g^{-1}$ dm)
				Willow							
Control	37.0±4.1 a	80.8±4.7 a	22.5±1.3 a	1.6±0.3	87.1±9.7 a	2.1±0.9 a	416.7±81.0 ab	9.96±1.3	20.98±0.5	25.98±0.3 a	2,604
10	38.4±3.3 a	81.2±3.2 a	22.8±1.6 a	1.6±0.3	97.1±16 a	1.3±0.3 a	600.8±77.5 a	10.38±1.1	20.77±0.7	27.08±0.4 a	3,534
200	16.9±4.3 b	28.0±4.8 b	3.2±0.8 b	0.9±0.1	40.2±4.6 b	0.5±0.1 b	256.1±22.2 b	5.57±3.2	21.63±1.5	17.23±2.8 b	5,122
				Maize							
Control	23.5±0.5 a	9.2±0.2 a	102.2±4.2 a	6.8±0.7 b	156.7±28.6 b	6.8±1.4 a	371.5±98.2 a	8.77±2.0	12.58±0.2 b	6.34±0.1 b	619
10	22.5±0.6 a	8.8±0.3 a	97.0±6.4 a	9.9±1.4 a	252.9±38.5 a	9.0±1.7 a	580.7±73.0 a	8.68±0.7	13.62±0.3 b	6.16±0.5 b	675
200	5.2±0.2 b	4.5±0.2 b	0.2±0.0 b	0.8±0.1 c	17.1±1.2 c	0.4±0.0 b	60.8±4.9 b	5.69±2.1	78.53±9 a[a]		1,520

Control, 10, and 200 denote treatments 0, 10, and 200 mg SDZ kg^{-1}. Values denote mean ± SE; HSD post hoc test ($p<0.05$). Letters, when present, indicate significant difference among SDZ treatments ($p<0.05$)

fm fresh mass, *SRL* specific root length (centimeter per gram dry mass)

[a] Sum of dry mass of leaves and stems; significant difference to the respective sum of other treatments (control and SDZ 10 mg kg^{-1})

around 40–45 % (Fig. 2a), resembling the maximal water holding capacity, and with no effect of SDZ on the water content as expected. In soil without SDZ, moisture was substantially reduced in the presence of plants, due to water uptake by maize and willow, beginning from day 7 (Fig. 2b, c). Reduction in soil moisture by willow plants was similar to controls for pots with 10 mg SDZ kg^{-1} soil, while soil moisture was significantly higher in respective pots with maize. This indicated some kind of inhibition of plant functions/metabolism, which was even stronger in treatments with 200 mg SDZ kg^{-1} soil. There was no statistical difference found in comparison to bare control soils, indicating almost complete inhibition of plant water uptake.

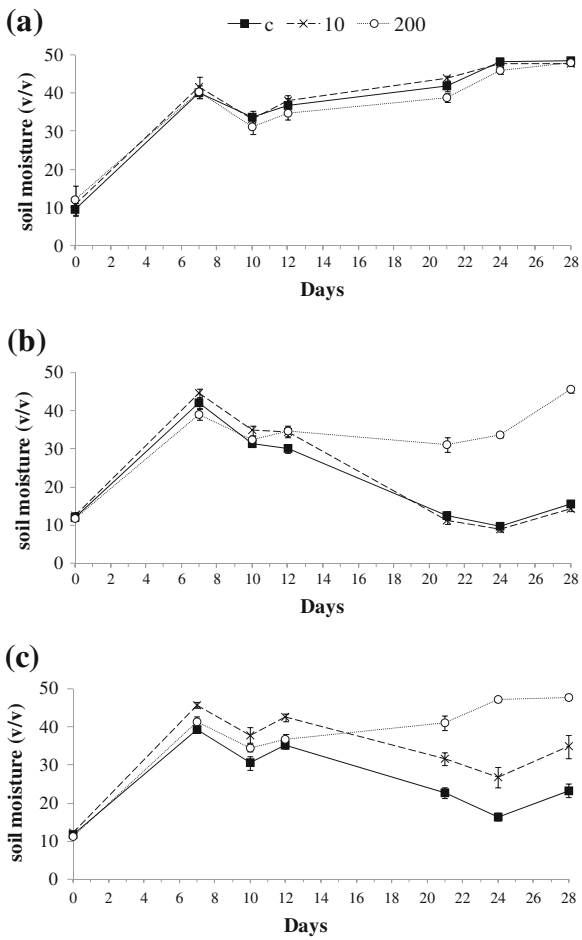

Fig. 2 Soil moisture (*v/v*) in pots without plants (**a**) and with willow (**b**) and maize plants (**c**). c, 10, and 200 denote treatments with 0, 10, and 200 mg kg^{-1} SDZ. Values are mean±SE. *Error bars* not shown are smaller than symbols

3.2 SDZ Content in Plants and Soil

Within the experimental duration of 40 days, the total extractable soil concentration of SDZ considerably decreased to a range from 1.2 to 16.5 % of the spiking level in both bulk and rhizosphere soil of treatments 10 and 200 mg kg^{-1} (Table 2). No SDZ was detected in untreated soil samples (data not shown). Differences between samples with and without plants indicated a presumable direct or indirect (i.e., through action on microbial population) plant effect on the dissipation of SDZ in or from soil at the spiking concentration of 10 mg SDZ kg^{-1} soil, with mean antibiotic bulk soil concentration of 0.12–0.13 mg kg^{-1} in planted pots and 0.34 mg kg^{-1} in pots containing only soil. Furthermore, SDZ concentrations were by a factor of 2 to 3 higher in rhizosphere soil compared to bulk soil of 10 mg kg^{-1} treatments. However, such difference was not detected at a soil spiking level of 200 mg SDZ kg^{-1}.

In plants of all control pots, no antibiotic was detected, as expected, while SDZ was taken up by plants from spiked soil and was found in several vegetal tissues (Table 2). Also SDZ was adhering to the rhizoplane as determined by ultrasound-assisted water extraction of intact roots, when plants had been exposed to 200, but not to 10 mg SDZ kg^{-1} soil. However, the majority of the active molecule was found inside roots, showing large differences between willow and maize at the lower SDZ treatment (10 mg SDZ kg^{-1} soil).

From the data, BCFs were calculated. The highest BCF of 33.3 was determined for willow plants exposed to the low SDZ concentration, while maize exhibited a BCF of 2.6. The BCFs were similar for plants treated with 200 mg kg^{-1} SDZ with mean values of 27.3 and 26.7 for willow and maize plants, respectively. The Tf data, which were calculated only for plants exposed to 200 mg kg^{-1} as for the low SDZ soil concentration the parent compound was not detected in leaves, showed higher values for maize plants (13.3) compared to willow plants (7.12).

Finally, the occurrence of two major SDZ metabolites was investigated. Specifically, the presence of OH-SDZ (mostly 4-OH-SDZ) was detected in all plant tissues (≤46 mg kg^{-1}) and soil samples (≤2.05 mg kg^{-1}) with the concentration increasing with the SDZ spiking one (Table 2). However, it was not detected in aerial parts of plants exposed to 10 mg SDZ kg^{-1} soil. The OH-SDZ was also detected in pots containing only soil spiked with SDZ. The second metabolite N-Ac-SDZ was detected only at trace levels (≤0.02 mg kg^{-1}) in a few willow leaves from pots treated with 200 mg kg^{-1} (data not shown).

3.3 Element Content in Plants

In Table 3, data are presented on the total carbon and nitrogen contents in leaves, roots, and stems collected at the end of the exposure period of 40 days. Results show that plants exposed to SDZ at 10 mg kg^{-1} had similar ability to assimilate C and N as plants grown in control soil without SDZ, since values detected for root, leaf, and stem tissues were almost equal. In contrast, in the presence of SDZ spiking concentrations of 200 mg kg^{-1}, some significant differences were determined for total C in stems (maize) and leaves (maize, willow) and total N in roots (maize, willow), stems (maize, willow), and leaves (maize) (Table 3). Differences were even more pronounced for C/N ratios and significant for all plant tissues grown at the high SDZ spiking level except for C/N ratio of the leaves of willow plants. Some alterations in the Ca and K contents were also found, in particular for the leaves of both plant species (Table 3). In fact, most leaf samples from 200 mg kg^{-1} treatments showed increased K and Ca concentrations in comparison with plants from control treatments. More evident was the effect on the ratio of K and Ca. The K/Ca ratio was higher in aerial parts especially of maize plants compared to roots. On average of all three plant tissues investigated, the ratio declined with increasing SDZ spiking concentrations to 0.9- and 0.7-fold for willow and 0.6- and 0.2-fold for maize of the control values. This clearly indicated a shift from K to Ca uptake in the plants in the presence of SDZ.

3.4 Chlorophyll Content

In this study, SPAD values and total chlorophyll content of leaves from plants grown in SDZ-contaminated soil were determined (Table 3). SPAD values did not reveal large differences between the species and the SDZ treatments, with average values around 36 for all samples. This parameter revealed a slight decrease in willow plants treated with 200 mg kg^{-1}, but the difference was not statistically significant. Instead, looking at the final contents of total chlorophylls, it appeared that plants were able to maintain normal

Table 2 Concentrations of SDZ, of two hydroxy-metabolites, and SDZ/OH-SDZ ratio in bulk and rhizosphere soil, at the rhizoplane and in plant tissues (milligram SDZ per kilogram dry mass) and resulting bioconcentration factor (BCF) and translocation factor (Tf %) after 40 days of experiment

Sample	SDZ	Soil		Rhizoplane (mg kg^{-1})	Plant			BCF	Tf (%)
	soil conc.[a]	Bulk	Rhizosphere		Roots	Stems	Leaves		
				SDZ					
Willow	10	0.12±0	0.23±0	<LOD	333±68	<LOD	<LOD	33.3	<LOD
	200	29.4±3	27.9±1	600±52	5,464±233	113±82.2	665±131	27.3	7.1
Maize	10	0.13±0	0.39±0	<LOD	26.5±10	<LOD	<LOD	2.6	<LOD
	200	33.1±4	21.1±3	699±141	5,331±210	708±184[b]		26.7	13.3
Control	10	0.34±0							
	200	22.4±2							
				OH-SDZ[c,d]					
Willow	10	0.06±0.0	0.04±0.0	<LOD	0.9±0.1	<LOD	<LOD		
	200	1.73±0.2	1.31±0.2	0.15±0.0	1.6±0.1	7.45±1.6	0.5±0.1		
Maize	10	0.02±0.0	0.03±0.0	<LOD	0.1±0.0	<LOD	<LOD		
	200	2.05±0.1	1.65±0.2	0.41±0.1	1.4±0.1	46.0±3.7[b]			
Control	10	0.07±0.0							
	200	1.71±0.1							
				SDZ:OH-SDZ					
Willow	10	6.2±0.2	6.9±0.3	<LOD	4.8±0.2	<LOD	<LOD		
	200	16.3±0.5	18.3±0.7	10.8±0.3	11.1±0.2	31.1±2.5	66.3±1.1		
Maize	10	6.5±0.2	7.4±0.3	<LOD	12.7±0.3	<LOD	<LOD		
	200	16.5±0.5	19.5±0.3	7.3±0.2	13.0±0.2	62.3±4.4[b]			
Control	10	5.1±0.2							
	200	13.1±0.4							

Values denote mean ± SE

<*LOD* below limit of detection

[a] Soil spiking concentration (milligram per kilogram)

[b] Leaves and stems together

[c] Relative quantification based on calibration for SDZ

[d] Sum of 4-OH-SDZ and 5-OH-SDZ

levels of photosynthetic pigments in both willow and maize, even in the presence of 10 mg kg^{-1} SDZ. However, a substantial reduction in chlorophyll was recorded in willow plants exposed to 200 mg SDZ kg^{-1}. Furthermore, no SPAD and total chlorophyll values of maize leaves exposed to 200 mg kg^{-1} were determined since plants suffered severely from SDZ so that not enough leaf material could be sampled for analyses at the end of the experiment. This high spiking concentration caused chlorotic and yellow areas in willow leaves (Fig. S2) and the death of maize plants.

3.5 Morphological Root Alterations

After 40 days of SDZ exposure, plants exhibited a disturbed morphology in the root system. In fact, substantial root alterations occurred in plants exposed to both 10 and 200 mg kg^{-1} of SDZ. In particular, the antibiotic promoted an abnormal root tip geotropism in maize exposed to 10 mg kg^{-1} compared to root orientation in control soil (Fig. 3a, b). Furthermore, few millimeters behind the root tips, a largely increased number of lateral roots was found for willows exposed to 200 mg kg^{-1} (Fig. 3d–f).

Table 3 Total C and N (percent), C/N ratio, amount of K and Ca (gram kilogram dry mass), K/Ca ratio, SPAD values (nondimensional), and total chlorophyll (microgram per gram fresh mass) in willow and maize tissues

		Willow			Maize		
		Control	10	200	Control	10	200
C total	Roots	39.7±0.6	38.5±0.1	38.9±0.6	36.9±1.0	36.1±0.8	37.6±0.8
	Stems	41.6±0.1	42.4±0.1	41.4±0.5	35.2±0.3 a	36.0±0.8 a	32.6±0.2 b
	Leaves	41.6±0.2 a	41.0±0.3 ab	40.5±0.4 b	39.8±0.2 ab	40.3±0.2 a	38.7±0.0 b
N total	Roots	1.86±0.1 b	1.71±0.0 b	2.83±0.0 a	0.99±0.1 b	0.91±0.1 b	2.14±0.2 a
	Stems	1.39±0.1 b	0.93±0.1 b	3.83±0.5 a	0.92±0.1 b	1.24±0.4 b	6.00±0.3 a
	Leaves	3.52±0.0	3.33±0.0	3.40±0.1	2.66±0.1 b	3.10±0.3 b	4.84±0.0 a
C:N	Roots	21.6±0.9 a	22.6±0.5 a	13.8±0.5 b	40.1±5.2 a	40.6±2.5 a	18.4±2.0 b
	Stems	30.5±1.9 b	46.7±3.4 a	11.5±1.3 c	42.4±6.0 a	27.5±7.4 a	5.5±0.3 b
	Leaves	11.8±0.2	12.3±0.2	12.0±0.5	15.0±0.3 a	13.6±1.3 a	8.0±0.0 b
K	Roots	12.1±0.2	13.6±2.9	n.a.	16.1±1.1	14.4±1.1	n.a.
	Stems	12.1±0.3	11.2±0.4	9.6±1.5	63.7±2.7 a	57.6±4.7 a	43.5±2.4 b
	Leaves	21.4±0.1 a	17.4±0.6 b	23.4±0.9 a	27.4±0.3 b	28.5±2 b	55.1±0. 7 a
Ca	Roots	4.6±0.1	5.3±0.9	n.a.	2.4±0.2	2.6±0.2	n.a.
	Stems	3.9±0.3	3.3±0.3	4.8±0.7	2.1±0.1 b	2.7±0.2 b	8.6±0.5
	Leaves	9.7±0.4 b	10.5±0.5 b	15.8±0.5 a	0.64±0.1 c	2.4±0.2 b	8.1±0.0 a
K:Ca	Roots	2.6±0.1	2.5±0.2	n.a.	6.8±0.2	5.6±0.2	n.a.
	Stems	3.2±0.1 ab	3.5±0.1	2.1±0.2 b	30.7±0.4 a	21.7±0.3 b	5.2±0.2 c
	Leaves	2.2±0.1 a	1.7±0.1 b	1.5±0.1 b	43.3±1.2 a	12.7±0.4 b	6.8±0.1 b
SPAD	Leaves	35.6±0.7	36.9±0.6	31.4±3.3	39.5±1.1	36.4±1.2	n.a.
Total chlorophyll		2,870±147 ab	3,130±160 a	2,014±482 b	1,511±26.5	1,309±145	n.a.

Control, 10, and 200 correspond to soils treated with 0, 10, and 200 mg SDZ kg^{-1}. Values denote mean±SE; HSD post hoc test ($p<0.05$). Letters, when present, indicate significant difference among SDZ treatments ($p<0.05$)

n.a. not analyzed due to shortage in sample material resulting from strongly inhibited plant development

4 Discussion

4.1 SDZ in Soil

Soil spiking concentrations of SDZ strongly declined within 40 days and the soil extractable SDZ diminished to concentrations that are frequently detected in arable soils (e.g., Hamscher et al. 2003; Höper et al. 2002). The resulting formation of non-extractable residues was previously reported (Kreuzig and Höltge 2005) and was possibly linked to chemical incorporation into humic substances through covalent cross-coupling mediated by soil oxidoreductases (Bialk et al. 2005; Schwarz et al. 2010). Even more, sorption and diffusion processes most likely contributed to the sequestration of SDZ (Förster et al. 2008) that, with a $pK_{a,1}$ of 6.5±0.30 (Sukul and Spiteller 2006), predominantly occurred as neutral (55 %) and acidic species (44 %). Thus, polar bonds as well as hydrophobic interactions with soil organic matter and mineral surfaces will have been the reason for the sorption and observed sorption nonlinearity of SDZ (Chiou et al. 2000; Thiele-Bruhn et al. 2004). Taking into consideration the total mass balance of SDZ recovered in the whole plant biomass grown in a single pot, it was calculated that within 40 days, willow removed 0.16 % of the total amount of SDZ spiked to soil at the low level and 1.35 % of SDZ at the high spiking level, while uptake by maize equaled 0.003 and 0.04 %, respectively. These findings closely matched data from Dolliver et al. (2007), who found sulfamethazine accumulation in maize, lettuce, and potatoes being less than 0.1 % of the initial amount applied to soil. The mild solvent extractable fraction of SDZ

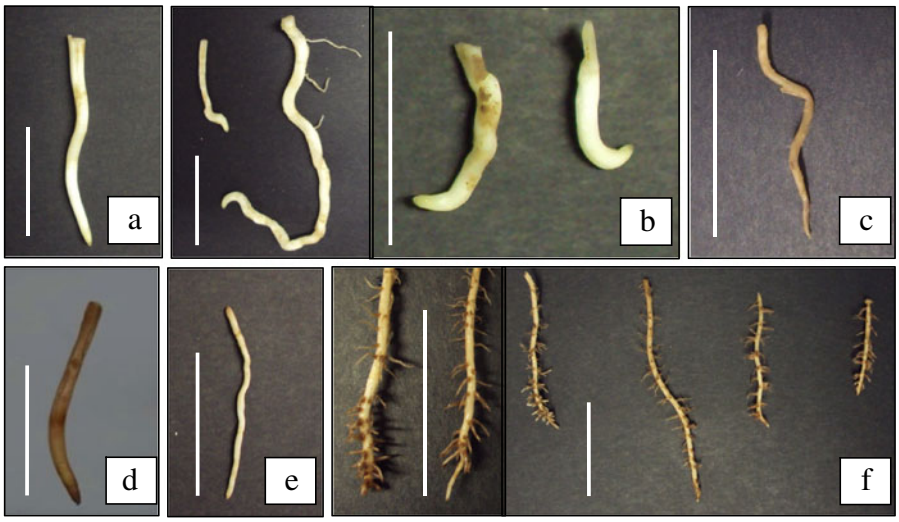

Fig. 3 Root tips from *Zea mays* L. (**a**–**c**) and *Salix fragilis* L. (**d**–**f**) plants exposed to soil concentrations of 0 (**a**, **d**), 10 (**b**, **e**), and 200 (**c**, **f**) mg kg^{-1} SDZ. *White lines* correspond to 2 cm

from soil planted with willow equaled 1.7 % of the low and 14.2 % of the high spiking concentration, while for maize, values were 2.5 and 13.4 %. These results highlight that more than 85 % of the applied SDZ was incorporated into the soil matrix. It was previously shown that plants may affect the non-extractable fraction of xenobiotics by enhancing the transformation and bound residue formation (Pilon-Smits 2005).

Based on similar findings, the application of phytoremediation to tetracyclines and sulfonamides was recently proposed (Boonsaner and Hawker 2010; Ferro et al. 2010). However, from our findings, SDZ total uptake was low, which was probably aggravated by the young plant age and a relatively low plant number per soil volume in the pot experiment. Furthermore, in our study, the SDZ concentration in planted pots with 10 mg SDZ kg^{-1} was higher in the rhizosphere soil compared to bulk soil, probably owing to passive transport with water moving towards roots. Similar contaminant migration to plant rhizosphere was reported, e.g., for polycyclic aromatic hydrocarbons (Gerhardt et al. 2009).

4.2 SDZ in Plants

In plant samples, the antibiotic was much more abundant inside roots than at the rhizoplane level. Accordingly, Ferro et al. (2010) showed that root cell wall preparations of barley sorbed much less sulfadimethoxine and sulfamethazine than the fresh roots. The determined SDZ concentrations in plant parts were clearly higher, especially at a soil spiking concentration of 200 mg kg^{-1} (up to 5,464 mg kg^{-1} dm for roots and up to 708 mg kg^{-1} dm for leaves), compared to sulfamethazine concentrations of 0.1 to 1.2 mg kg^{-1} dm in maize, lettuce, and potato after 45 days of exposure to 2.5 mg sulfamethazine kg^{-1} soil (Dolliver et al. 2007). As for soil, also for plant samples, the extracted SDZ was not linearly related with the spiking concentration, which is in agreement with previous findings (Michelini et al. 2012). In fact, root concentrations and BCF values of the 200-mg-kg^{-1} treatment clearly highlighted that the maximum uptake of SDZ in willow and maize was reached, probably because of the high stress and hampered water uptake experienced by the plants.

Only in willow and maize plants exposed to 200 mg kg^{-1} SDZ was transported to the leaves, corresponding to the decreased translocation of sulfadimethoxine from roots to shoots of crops (*Panicum miliaceum* L., *Pisum sativum* L., *Z. mays* L., and *Hordeum distichum* L.) and weeds (*Amaranthus retroflexus* L., *Plantago major* L., and *Rumex acetosella* L.) (Migliore et al. 1995, 1996, 1998). The antibiotic movement was probably driven by diffusion and/or advection with the transpiration stream, the main processes of the passive uptake of organic pollutants such as chlortetracycline (Kumar 2005; Pilon-Smits 2005; Trapp et al. 1990). Therefore, the low SDZ concentration in leaves could have been due to an inhibited transpiration, which was reflected by the soil moisture data recorded. Vice versa, decreases in transpiration might have been related to leaf damages induced by SDZ (Fig. S2).

It is assumed that after plant uptake, toxic contaminants, in this case SDZ, are subsequently sequestered in places where they could do the least damage to essential cellular processes (Pilon-Smits 2005), such as vacuole or cell wall (Burken 2003; Li et al. 1997). However, investigating this was beyond the scope of this study. Detoxification of organic contaminants in plants is mostly driven by cytochrome P-450 enzymes (Barret 1995), which frequently catalyze transformation reactions, as hydroxylation (Trapp and Karlson 2001). In our study, OH-SDZ was the most prominent metabolite in both plants and soil, which exhibits a strongly reduced antibiotic potential (Hammesfahr et al. 2008). The OH-metabolites were found in both planted and unplanted soils, confirming that abiotic and/or biotic degradation processes in soil contribute to SDZ metabolism (Schwarz et al. 2010). However, the ratio of SDZ/OH-SDZ differed between soil and plant and was mostly lower in roots but higher in aerial plant parts compared to soil (Table 2). Hence, from the data, it remains unclear whether OH-SDZ in plants originated from plant metabolism or root uptake from soil.

4.3 Effects on Plants

Biometric analyses evidenced that SDZ has the potential to adversely affect *S. fragilis* L. and *Z. mays* L. plants even within a short exposure period. This potential was clearly observed at a spiking concentration of 200 mg SDZ kg^{-1} soil, which is highly above what can be typically expected in agricultural soil though (e.g., Aust et al. 2008; Christian et al. 2003; Hamscher et al. 2002). However, even at an environmentally relevant soil concentration of 10 mg kg^{-1} SDZ led to alterations in root morphology. Correspondingly, sulfadimethoxine had similar effects on *S. fragilis* L. roots (Michelini et al. 2012). According to Sartorius et al. (2009), a growth regulator disturbance could be the reason of the abnormal root geotropism and leaf pigmentation noticed. In fact, sulfonamide antibiotics inhibit the synthesis of folic acid (Stokstad and Jukes 1987; Thiele-Bruhn 2003), a phytohormone precursor. If this pathway is hampered by the drug, abnormal cell division and differentiation can occur (Boonsirichai et al. 2002; Migliore et al. 1995). Since the architecture of a root system determines its exploration of the soil (Lynch 1995), the modified root morphology (i.e., weight and area reduction), combined with an indicated reduced transpiration, adversely affected the plant water uptake. The drought stress was obviously reflected by substantially increased dry matter contents of plant tissues. In agreement with these results, Sartorius et al. (2009) found evident decreases in leaf and root length development when plants were grown in liquid medium containing 300 mg l^{-1} of sulfadimethoxine. Also, Mikes and Trapp (2010) noticed decreased transpiration of *Salix viminalis* L. exposed for a few days to trimethoprim at 100 mg l^{-1}. Contrary, the 10-mg-kg^{-1} concentration tested in our study did not reduce the plant development, while, in some cases, it even enhanced root growth. A similar hormetic answer was described for the aerial parts of *Lythrum salicaria* L. treated with sulfadimethoxine nominal concentrations in a range between 0.005 and 50 mg $l-^{1}$ (Migliore et al. 2010).

The high SDZ concentration caused serious disequilibria in the nutrient contents. The C/N ratio was lower in both roots and stems of the two species exposed to 200 mg SDZ kg^{-1}. This is at least partly explained by SDZ effects on photosynthesis that were evidenced by a reduced biomass production, while N uptake appeared to be unaffected. Normally, N uptake of juvenile plants starts before C assimilation begins. Assimilated C then dilutes the N concentration to normal C/N ratios (Marschner 2012) which was not the case in the presence of SDZ. It is suggested that the decreased water uptake caused the particularly concentrated nutrient content in willow and maize leaves treated with 200 mg SDZ kg^{-1}. Even more, the K/Ca ratio clearly showed that with more SDZ in soil, relatively more Ca was taken up by the plants. Although the bulk of the K and Ca uptake is notoriously passive (Schachtman and Schroeder 1994; Taiz and Zeiger 2009), K is also absorbed through the ionophoric protein systems (Pressman et al. 1967). As numerous single carbon transfer reactions are altered following interference of folic acid synthesis, it is plausible that formation and/or regulation of these lipid-soluble membrane molecules resulted in being disturbed. Consequently, lack of K probably compromised plant nutrition, growth, tropism, enzyme homeostasis, and osmoregulation (Schachtman and Schroeder 1994; Taiz and Zeiger 2009).

5 Conclusions

This study focused on some of the ecological consequences of antibiotic contaminated waste application on agricultural lands. The overall physiological parameters tested in this study, i.e., water uptake, nutrient accumulation, and photosynthetic pigments, clearly showed the potential of the sulfonamide antibiotic SDZ to adversely affect the growth and yield of important agricultural crops such as maize. In particular, the lower concentration tested may be expected in arable soils as the upper level of sulfonamide contamination. Additionally, it must be considered that more than one pharmaceutical antibiotic is often used for livestock at a time and thus may end up in soil. On the other hand, willow, which is like other fast growing tree species preferred for phytoremediation purposes, proved to withstand and take up higher SDZ concentrations. Also in view of possible phytoextraction and/or phytodegradation aims, toxic effects of SDZ to vegetal organisms deserve further investigation, certainly with longer term works, considering the peculiar interactions between the soil matrix and the tested antibiotic.

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