



UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede amministrativa UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento Territorio e Sistemi Agro-Forestali

Scuola di dottorato di ricerca in TERRITORIO AMBIENTE RISORSE E SALUTE

Indirizzo ECOLOGIA

Ciclo XXV

**SULFONAMIDE ACCUMULATION AND EFFECTS ON
HERBACEOUS AND WOODY PLANTS AND MICROORGANISMS**

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Declaration

I hereby declare that this submission, to the best of my knowledge, contains no material previously published or written by other people.

The experimental works described in this thesis are part of scientific manuscripts published, submitted or to be submitted to international peer reviewed journals. People involved within the research work are specially marked in the text.

Lucia Michelini, 11.12.2012

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Riassunto

Una delle vie principali attraverso cui i farmaci possono entrare nell'ambiente consiste nell'ampio uso che se ne fa in zootecnia. Infatti, in Europa questi principi attivi sono venduti nell'ordine di centinaia di tonnellate annue per singola nazione, per il solo utilizzo in ambito veterinario. In seguito alla somministrazione, fino al 90% della dose utilizzata di farmaco può essere escreta inalterata e, in seguito all'utilizzo del letame come ammendante organico, suolo e acque possono risultare contaminate. Il presente studio si concentra sugli effetti e sull'accumulo in piante legnose ed erbacee di sulfamidici, un gruppo di agenti antimicrobici (d'ora in poi chiamati antibiotici) frequentemente rilevati negli ecosistemi agrari, la cui persistenza rappresenta un serio rischio per gli organismi viventi ad essi connessi.

La tesi è articolata in 7 capitoli. Nella prima parte (capitolo 1) è descritta la situazione generale relativa alla presenza di antibiotici negli ambienti agrari e al loro impatto sulla crescita e lo sviluppo di organismi viventi ad essi esposti.

Successivamente, dal capitolo 2 al capitolo 6, sono presentate varie prove sperimentali, alcune effettuate in laboratorio ed altre in serra. In particolare, il capitolo 2 si occupa della risposta di piante di *Salix fragilis* L. all'antibiotico sulfadimetossina, aggiunto alla soluzione nutritiva in concentrazioni da 155 a 620 mg l⁻¹, nonché del potenziale accumulo nei tessuti vegetali. Lo studio mostra la tendenza di questa specie legnosa di assorbire e accumulare la molecola attiva a livello di apparato radicale. Il capitolo 3 ripercorre il disegno sperimentale adottato nella prova descritta nel capitolo 2, con la differenza che, in questo caso, le piante di *Salix fragilis* L. sono state esposte a dosi di sulfadimetossina che approssimano quelle registrate in alcuni ambienti agrari, ovvero da 0.01 a 10 mg l⁻¹. Lo studio ha mostrato che non appaiono effetti negativi sulla crescita delle piante di salice fino alla dose di 1 mg l⁻¹. Tuttavia, aumentando il livello del principio attivo sono state evidenziate delle importanti alterazioni sull'architettura radicale. I capitoli 4 e 5 considerano, rispettivamente, gli effetti e l'accumulo di un altro sulfamidico in piante di *Salix fragilis* L. e *Zea mays* L., coltivate in un terreno arricchito con 10 mg e 200 kg⁻¹ di sulfadiazina e il suo impatto sulle comunità microbiche e sulle attività enzimatiche associate al suolo e alla radice delle due specie vegetali. L'ultimo studio, presentato nel capitolo 6, si concentra sugli effetti indotti da circa 10 mg l⁻¹ di sulfadimetossina e sulfametazina sulla struttura e sulla funzionalità di radici di *Hordeum vulgare* L. I risultati

provano che i sulfamidici causano importanti effetti sulla morfologia dell'apparato radicale e sull'integrità delle membrane delle cellule radicali.

Concludendo, si è evidenziato (capitolo 7) che il *Salix fragilis* L. accumula e tollera meglio di *Zea mays* L. e *Hordeum vulgare* L. le molecole attive testate, mentre le specie erbacee sembrano essere più vulnerabili a questi inquinanti, di cui ne viene sconsigliato l'eventuale utilizzo nel campo del fitorimedio. Inoltre, in capitolo 7 rimarca le conseguenze negative sulla diversità funzionale e strutturale delle comunità microbiche del suolo.

Summary

One of the main routes through which pharmaceuticals may enter the environment consists in the medication of livestock. In fact, in Europe the annual national sales of active substance for veterinary consumption reach hundreds of tons. After medication, up to 90% of the administered medicine dose may be excreted unaltered and, following the use of manure as fertilizer, soils and waters are contaminated. The present work focuses on the effects and eventual accumulation on woody and herbaceous plants of sulfonamides, a group of antimicrobial agents (from now on called antibiotics) frequently detected in agricultural ecosystems, whose persistence poses a serious risk to soil and water living organisms.

The thesis consists of 7 chapters, presenting, in the first one, a general introduction on the antibiotic presence in the environment and its consequences on the growth and development of exposed living organisms.

Subsequently, from chapter 2 to chapter 6, various experimental trials are presented, some of them carried out under laboratory conditions and others in greenhouse. More specifically, chapter 2 reports the first study performed, which deals with *Salix fragilis* L. plant response and the accumulation of sulfadimethoxine antibiotic, added in the nutrient solution at doses ranging from 155 to 620 mg l⁻¹. Such a study highlights the potential of this woody species to absorb and accumulate the active molecule at the level of root apparatus. Chapter 3 retraces the experimental design of chapter 2, with the difference that *Salix fragilis* L. plants were exposed to environmental relevant sulfadimethoxine doses, from 0.01 up 10 mg l⁻¹. The trial had demonstrated that no adverse effects on the growth of willow plants appeared up to 1 mg l⁻¹ of antibiotic. Conversely, increasing levels of the antibiotic caused important alterations of the willow root architecture. Chapters 4 and 5 consider, respectively, the effects and accumulation of a different sulfonamide on *Salix fragilis* L. and *Zea mays* L. plants, grown in a soil spiked with 10 and 200 mg kg⁻¹ of sulfadiazone. Moreover, its impact on the composition of root associated soil microbial community and on the activities of selected enzymes was analyzed. The last study, presented in chapter 6, focuses on alterations induced by about 10 mg l⁻¹ of sulfadimethoxine and sulfamethazine on *Hordeum vulgare* L. root structure and function. This chapter highlights the strong effects of the antibiotics, not only on the root apparatus morphology, but also on the membrane integrity of root cells.

To conclude (chapter 7), it is highlighted that *Salix fragilis* L. seems to better accumulate and withstand the active molecules tested than *Zea mays* L. and *Hordeum vulgare* L., while the herbaceous species are more vulnerable to this kind of pollutant exposure and, therefore, not recommended for eventual remediation purposes. Furthermore, chapter 7 notes the adverse consequences on the functional and structural diversity of the soil microbial community.

CHAPTER I

General introduction

Antibiotics in the environment

Organic micropollutants represent a class of contaminants which, even if present at low concentrations, do to their relatively constant presence could turn out to be dangerous for humans and the environment. Among these compounds the following should be mentioned: pharmaceutically active compounds, personal care products, illicit drugs, endocrine disrupting compounds, persistent organic pollutants (e.g., polychlorinated biphenyls, pesticides, polycyclic aromatic hydrocarbons and dioxins) (Morosini 2009).

Organic micropollutants are increasingly identified in the environment due to the recent development of more precise analytical technologies with lower detection limits (Snyder et al. 2004). For example, a range of veterinary medicines, including hormones, antibiotics and parasiticides, have been detected in soils, surface and ground waters (Hirsch et al. 1999) and some of these substances (e.g., oxytetracyclines) may also persist in the environment for over a year (Kay et al. 2004). People may be exposed to traces of veterinary drugs present in the environment (e.g., soils, waters, sediments) via a number of routes including: the consumption of crops that have accumulated toxic substances from soils; livestock that have accumulated veterinary medicines through the food chain; fish exposed to treatments used in aquaculture; ground and surface waters containing veterinary medicines (Daughton and Ternes 1999; Boxall 2004). Consequently, once released into the environment, the potential impact of such medicines on human and animal health deserves scrupulous attention.

In the present work, among the micropollutants previously described, the focus is on antimicrobial agents (from now on called antibiotics), because of their extensive use for both human and animal therapy, and successive entrance into the environment.

Antibiotics represent a helpful tool to prevent and treat human and animal diseases. In several European countries, the overall national sales of antibiotics in 2007 were approximately 3500 tons of active substance for veterinary use and 3350 tons in terms of overall human consumption (ECDC et al. 2009). However, after administration, a significant fraction of the antibiotics and their metabolites are excreted unaltered. In fact, Winckler and Gafe (2001) reported excretion rates following the passage of sulfonamide antibiotics through the gastro-intestinal tract in the range of 40-90%. Consequently, they may move from animal and human excretions to wastewaters, where they are not always removed because of the low selectivity of the conventional treatments (Heberer 2002;

Kim et al. 2007). Moreover, following the land application of animal manure or slurry, these medicines may enter arable soils (Fig. 1.1). Normal practices of animal sludge application on soil can cause the introduction of these chemicals into the terrestrial and aquatic ecosystems (Jorgensen and Halling-Sorensen 2000).

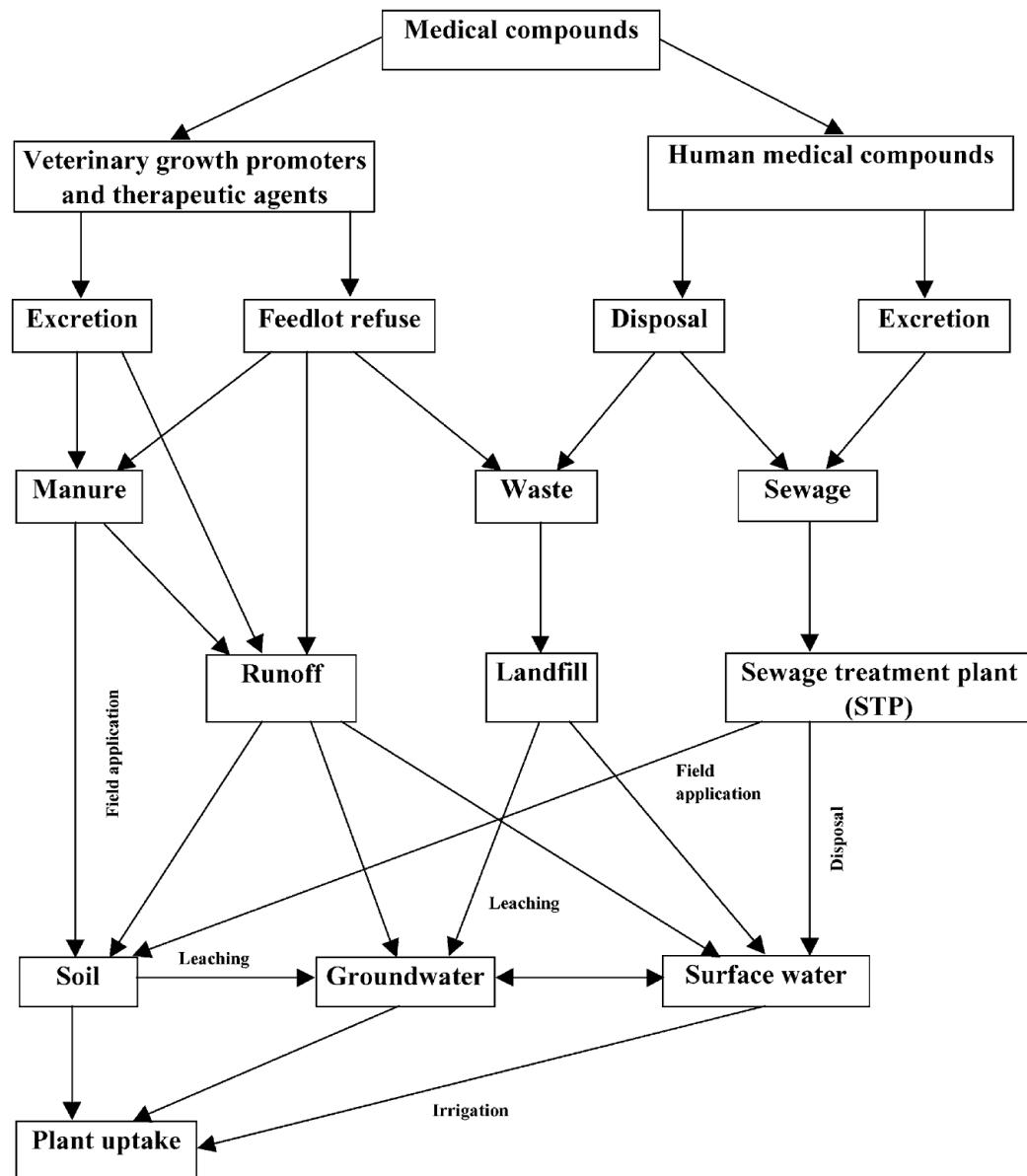


Fig. 1.1 The main distribution ways of pharmaceuticals in the environment (Jiembab 2002).

Among the existing publications, Li et al. (2011) reported an alarming presence of antibiotics in vegetable farmland soils associated to livestock farms, with values around several tens of $\mu\text{g kg}^{-1}$. Moreover, Christian et al. (2003) found detectable sulfamethazine

concentrations seven months after the application of manure into German agricultural soils.

According to Bialk et al. (2005), sulfonamides that reach the soil can be covalently bound to natural organic matter through the aromatic amine group, which is a common characteristic of this class of medicines. Thorn et al. (1996; 2002) demonstrated that aromatic ammines covalently bound to fulvic acids can be displaced by ammonia in nucleophilic substitution reactions. Therefore, in accordance with these authors, an analogous release of sulfonamides from contaminated soils through ammonia-based fertilizers may be hypothesized. These studies suggest that, under field conditions, sulfonamides may be more persistent than predicted according to information obtained in laboratory studies (Bialk et al. 2005).

Not only soils, but also water bodies may be contaminated by various pharmaceuticals. For example, Watkinson et al. (2009) showed a frequent antibiotic presence up to some $\mu\text{g l}^{-1}$ in six investigated rivers in South–East Queensland, Australia. In Denmark, Holm et al. (1995) analyzed the ground water down gradient of a landfill formerly used for the disposal of waste from pharmaceutical production facilities. In this study, a large variety of sulfonamide concentrations ranging up to about 10 mg l^{-1} was found. Moreover, Hirsch et al. (1999) found concentrations of sulfamethoxazole in German sewage treatment plant effluents (STPs), and surface and ground waters up to 2, 0.48 and $0.47 \mu\text{g l}^{-1}$, respectively. Concerning the Italian situation, Zuccato et al. (2010) presented results assessing the presence and the concentration of the more consumed antibiotics for human therapy in untreated and treated wastewater of some STPs in northern Italy and in receiving rivers. Analyses of a group of antibiotics, including the most frequently prescribed penicillins, quinolones, macrolides, lincosamides, sulfonamides and glycopeptides showed that some of them are not efficiently removed and end up in the receiving water. Such analyses made it possible to estimate that since prescriptions of these substances in Italy were a total of 70 tons in 2008 (AIFA 2009), up-to 10–20% of these were discharged into the environment after consumption (Zuccato et al. 2010).

However, even if veterinary medicines are regularly monitored in food materials to ensure concentrations lower than the maximum residue limits, the magnitude of the means of contact, listed in Fig. 1.1, and the health impacts of such exposure have not been extensively quantified (Boxall et al. 2006).

Some known effects of antibiotics on plants and soil microorganisms

Metabolites of some medicines, such as chloramphenicol, sulfadiazine, estrogens, and sulfamethazine may be converted back to the parent compound once they have reached the environment (Daughton and Ternes 1999; Hirsch et al. 1999; Ingerslev and Halling-Sørensen 2000). Furthermore, when the biosolids containing the unmetabolized or partially metabolized compounds are applied to agricultural soils, the active molecule can be available for uptake by existing plants, as shown in Fig. 1.1 (Jjemba 2002).

A recent study showed that diazinon, enrofloxacin, florfenicol, and trimethoprim were taken up from manure-amended soil by carrot roots (Boxall et al. 2006) and Herklotz et al. (2010) proved that even carbamazepine, salbutamol, and sulfamethoxazole were detected in the roots and leaves of cabbage plants. The uptake of ciprofloxacin, ofloxacin, norfloxacin, sulfadimethoxine, and sulfamethoxazole from the soil into potato was also demonstrated, with higher concentrations of the pharmaceuticals found in the plant samples than in soil after 20 days of exposure (Kipper et al. 2010).

Such exposure may affect the growth and development of the plant in question, depending on the type of agent, dosage, sorption and desorption kinetics and mobility in soil (Jjemba 2002). Bioaccumulation of therapeutic agents by plants has been demonstrated (Migliore et al. 1996; 1998). However, concentrations of 300 mg sulfadimethoxine significantly depressed the growth of *Amaranthus retroflexus*, *Plantago major*, *Rumex acetosella* and *Zea mays* in vitro, as well as *Hordeum distichicum* both in vitro and in soil (Migliore et al. 1996; 1998). The reduced growth is attributed to the bioaccumulation of sulfadimethoxine in both roots and leaves. Furthermore, pinto beans (*Phaseolus vulgaris* L.) were negatively affected by oxytetracycline and chlortetracycline leading to a reduction in nodulation, fresh mass production and uptake of Ca, K, and Mg (Batchelder 1982). On the other hand, in the same study, the growth of radish and wheat was stimulated in the presence of chlortetracycline and oxytetracycline, whereas the growth of corn was unaffected by these antibiotics (Batchelder 1982).

Antibiotics usually act on particular metabolic pathways of living cells; hence, as a consequence of their presence in the environment, even non-target organisms sensitive to the mode of action of these compounds can be affected (Henschel et al. 1997). This may have negative effects on soil functions since diverse soil microbial communities are a prerequisite for ecosystem stability and services, such as the decomposition of organic compounds and nutrient cycling (Westergaard et al. 2001; Thiele-Bruhn et al. 2012). To date, a number of studies exist documenting the side effects of sulfonamides on soil

microbial communities when the antibiotics were co-applied to soil with nutrient substrates such as manure. The adverse effects of sulfonamides on microbial respiration and enzymatic activities, especially of the N-cycle (Zielezny et al. 2006; Kotzerke et al. 2008; Hammesfahr et al. 2011) and overall shifts in the functional diversity of the microbial community have been reported (Schmitt et al. 2004; Demoling et al. 2009; Liu 2012). These shifts go in line with changes in the bacteria structural community composition (Hammesfahr et al. 2008; Schauss et al. 2009; Kleineidam et al. 2010), tolerance and resistance level, respectively (Schmitt et al. 2004; Demoling et al. 2009; Heuer et al. 2011).

Sulfonamide antibiotics

The most widely used veterinary antibiotics in industrialized countries include tetracyclines, macrolides, penicillins, aminoglycosides, and also sulfonamides (Campbell 1999; Haller et al. 2002). Sulfonamides, or SAs, are synthetic medicines with a wide spectrum of activity and they act by inhibiting the growth of a large number of Gram-positive and Gram-negative bacteria. These antibiotics limit the growth of bacteria by competitively inhibiting the utilization of *p*-aminobenzoate in the biosynthesis of folic acid (Brown 1962; Stokstad and Jukes 1987). SAs belong to a large group of structurally related antibiotics and are N-substituted derivatives of sulfanilamide (Sukul 2006). They contain a 4-aminobenzene sulfonamide core and differ among one other in the N-substituent of the sulfonamide linkage (Fig. 1.2). As a consequence, such a class of antibiotics is characterized by dissimilar chemical-physical properties due to the differing side moieties (Sukul 2006).

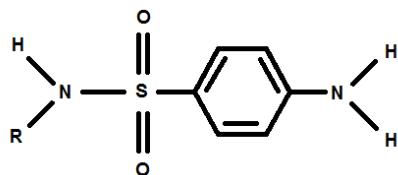


Fig. 1.2 General chemical structure of sulfonamides (Sukul 2006).

SAs are fairly water-soluble and ionize depending on the pH of the medium (Thiele-Bruhn et al. 2004). In general, SAs contain polar groups on a non-polar core, therefore showing sensitivity to bases and acids. They possess two pK_a values arising from the

protonation of the amino aromatic group at pH 2-3 and deprotonation of the sulfonamide group at pH 5-11 (Ingerslev and Halling-Sørensen 2000).

Similar to other kinds of organic contaminants, the environmental fate of antibiotics in soil is regulated by transformation, retention and transport processes (Accinelli et al. 2007). Research indicates that SAs are sorbed on soil particles with distribution constants (K_d) ranging from 0.9 to 3.5 (Thiele-Bruhn 2003; Boxall and Long 2005). Thiele-Bruhn et al. (2004) showed that soil pH and organic colloid properties are important factors in determining the strength of SAs sorption to soil.

Concerning their persistence in the environment, some authors (e.g., Richardson and Bowron 1985; Marengo et al. 1997) defined sulphamethoxine and sulphadimethoxine as persistent, suggesting that these compounds are not easily degradable.

The administration of SAs is usually performed orally (G.U. No. 82/1963, No. 98/1968 and subsequent) in the case of bacterial infections of cattle, swine and poultry, in doses ranging from 25 to 100 mg kg⁻¹ body weight over a period of 5–6 days (Forni et al. 2002). However, some SAs such as sulfadimethoxine are slowly absorbed by cattle and the non-absorbed portion (up to 90%) is eliminated unchanged in their excreta (Sarmah et al. 2006). Sulfadimethoxine concentrations were detected in fresh faeces of treated calves in the range of 300 to 900 mg kg⁻¹ (Migliore et al. 1997).

Due to SA persistence (Thiele-Bruhn et al. 2004), the application of animal sludge to soils can lead to the introduction of these chemicals into the terrestrial and aquatic environment compartments (Jorgensen and Halling-Sørensen 2000; Boxall et al. 2003; Sarmah et al. 2006). Several authors have raised concerns about the potential consequences of the environmental presence of these drugs on human and ecosystem health (e.g., Witte 1998; Bialk et al. 2005; Fatta-Kassinos et al. 2011).

Phytoremediation

Phytoremediation is a technology that utilizes plants and the associated rhizosphere microorganisms to remove, transform, or contain toxic chemicals located in soils, sediments, ground/surface waters and the atmosphere (Susarla et al. 2002). Currently, phytoremediation is used to treat many classes of contaminants including petroleum hydrocarbons, chlorinated solvents, pesticides, explosives, heavy metals, radionuclides and landfill leachates.

Numerous authors have reported the role of plants in remediating polluted soils and ground waters (Paterson et al. 1990; Shimp et al. 1993; Simonich and Hites 1995). Chang

and Corapcioglu (1998) described how plants promote the remediation of a wide range of chemicals of contaminated sites by various processes. Some of these mechanisms include:

- modifying the physical and chemical properties of contaminated soils;
- releasing root exudates, thereby increasing organic carbon;
- improving the soil aeration by releasing oxygen directly to the root zone, as well as increasing the porosity of the upper soil zones;
- intercepting and retarding the movement of chemicals;
- effecting co-metabolic microbial and plant enzymatic transformations of recalcitrant chemicals;
- reducing the vertical and lateral migration of pollutants to ground water by extracting available water and reversing the hydraulic gradient.

Many areas polluted by low concentrations of toxic compounds are suitable for phytoremediation as a long-term solution to the problem (Susarla et al. 2002). Some examples of more common phytoremediation technologies used to remove pollutant agents from waters, soils or air, are summarized in Fig. 1.3.

To use phytoremediation techniques effectively, and obtain good results, it is necessary that some conditions are respected. For example, the contaminated site should have enough space to enable vegetation growth. Moreover, time is an important factor insofar as phytoremediation represents a long-term treatment which is readily applicable provided there is no urgency to restore the contaminated area. Additionally, contaminant concentrations should not be toxic for plants (Bonomo 2005). If such assumptions are respected, phytoremediation could offer a viable solution for restore environments.

Among the advantages offered by phytoremediation, the more important are (Bonomo 2005):

- the low cost and the auto-sustainability of this technology;
- the possibility of applying it in remote areas;
- the attenuation of soil erosion, superficial water flow, infiltration and air dust;
- the application to a wide range of pollutants and their mixtures;
- the good impact on public opinion, as a “green” solution, which may also give added esthetic value to landscapes.

However, on the other hand, phytoremediation also has some limitations. The principal restriction is represented by the interaction between plant roots and pollutants, implying

root penetration in the contaminated section and soil characteristics. To derive any benefit from it, it is important that contaminated substrates are in touch with the root apparatus, which cannot always reach the target volume. Moreover, as mentioned above, phytotechnologies are relatively slow if compared with conventional active practices and are conditioned by the local climate (Bonomo 2005).

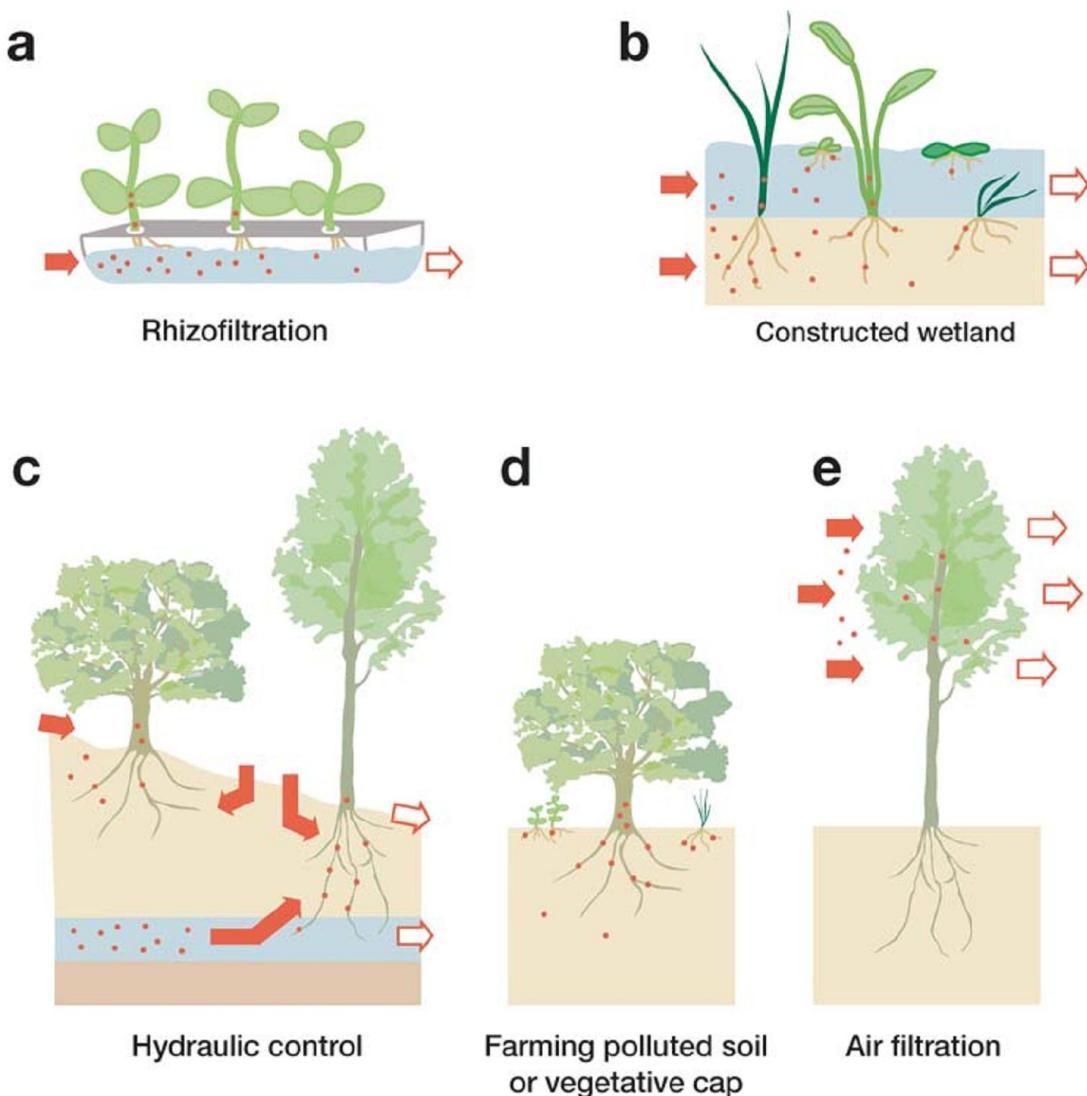


Fig. 1.3 Some examples of phytoremediation technologies used to remediate polluted waters (a, b, c), soils (b, c, d) or air (e). The red circles represent the pollutant (Pilon-Smits 2005).

Numerous authors have shed light on the emerging role of plants in the remediation of organic substances (Singh and Jain 2003; Pilon-Smits 2005; Vila et al. 2008; Kawahigashi 2009). However, at present only few studies concerning the application of plants to remediate antibiotics from soils and waters are available (e.g., Gujarathi et al. 2005; Boonsaner and Hawker 2010; Hoang et al. 2012).

Overall aims of the thesis

The main goals of this research have been:

- 1) to investigate the possible accumulation of SAs in plants and, consequently the capability of the plant to phytoremediate them. To reach this aim, various vegetal species were grown in antibiotic spiked growth media, such as nutrient solution and soil, with a view to reproducing the mechanisms that occur in the root-water and root-soil interfaces under laboratory conditions. At the end of an appropriate treatment period, long enough to collect sufficient vegetal material, the presence of the antibiotic was evaluated in root, rhizoplane, leaf and stem tissues.
- 2) In certain cases, pollutants may negatively alter the plant physiology and consequently make plants more sensitive to other biotic and abiotic stresses, thereby limiting their phytoremediation aptitude. For this reason, phytotoxicity studies are a crucial step in the assessment of the appropriateness of phytoremediation technology for specific plant species and pollutant classes. Therefore, the second aim of the overall research has been that to analyze the effects of various SAs and their concentrations on plant development and physiology. To reach this aim, studies were carried out considering, initially, antibiotic doses resembling those found in fresh liquid manure to test the potential effects, and, subsequently, environmental relevant concentrations. Furthermore, more than one active molecule within the class of SAs and various plant species were investigated. In particular, vegetal organisms used in the work have been both woody and herbaceous. The former were selected among those usually able to produce high amounts of biomass in a short time, an important characteristic in phytoremediation technology (an example is *Salix* genus). The latter, crops in particular, are chosen as important agricultural plants typically receiving elevated manure fertilization and relative antibiotic loads.
- 3) Several studies already show how sulfonamide input through liquid manure in arable soil may alter the bacterial community structure. However, they have not carefully considered the influence of vegetal organisms in the experimental design. Therefore, the last aim of the research has been to examine if an antibiotic belonging to the SA class, could alter the activity and community structure of

selected important microorganisms present in bulk soil (identified as the fraction of soil not directly investigated by roots), rhizosphere soil (the first 2-3 mm of soil attached to the root apparatus) and plant roots.

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CHAPTER II

Accumulation and effects in *Salix fragilis* L. plants exposed to sulfadimethoxine concentrations from 155 to 620 mg l⁻¹ in nutrient solution for one month

The experimental works described in this chapter are part of the following scientific manuscript: Michelini L., Meggio F., La Rocca N., Ferro S., Ghisi R. (2012). Accumulation and effects of sulfadimethoxine in *Salix fragilis* L. plants: a preliminary study to phytoremediation purposes. International Journal of Phytoremediation 14:388-402

PREFACE

Salix genus is currently under intensive scrutiny for its potential use in soil phytoremediation of organic and inorganic compounds, such as polycyclic aromatic hydrocarbons and heavy metals (Pulford et al. 2002; Cook et al. 2010; Hultgren et al. 2010).

The aim of this research has been that to investigate the possibility of using woody plants also in the phytoremediation of pharmaceuticals such as SAs. To this purpose the ability of *Salix fragilis* L. to both absorb and tolerate sulfadimethoxine, or SDM, at concentrations found in fresh calf faeces (Migliore et al. 1997) was studied. For this reason, genetically identical *Salix fragilis* L. cuttings were first grown in tap water and then in nutrient solution containing the active molecule. At fixed times, plant growth (leaf and stem length and biomass) and gas exchange (photosynthesis, stomatal conductance and transpiration rates) parameters were measured. Furthermore, at the end of the treatment period leaf fluorescence and chlorophyll, carotenoid and SDM contents were also analyzed. In a second experiment, root responses to the active molecule were investigated through scanning electron microscopy on cuttings directly grown in the presence of SDM.

MATERIALS AND METHODS

Plant growth

Cuttings (20-cm long and approximately 1-cm diameter) of *Salix fragilis* L. were taken from a selected tree in the experimental farm of the University of Padova at Legnaro (Italy) and grown in tap water. Once rooted (numerous roots per plant of about 20 cm) and after leaf out (around 20-30 leaves per stem), plants were divided into four groups each consisting of ten individuals. These groups were treated with either 0 (control), 0.5, 1 or 2 mM SDM (CAS: 122-11-2, Sigma-Aldrich, Milan, Italy), corresponding to 0, 155, 310 and 620 mg l⁻¹ respectively, dissolved in a nutrient solution (detailed below). Careful attention was given to the selection of homogenous rooted cuttings, which were then introduced into the experimental design. Each plant was grown in a 2 L pot filled with a 25 times diluted Hoagland's nutrient solution (Arnon and Hoagland 1940), pH 7.0. Plants were grown simultaneously in a growth chamber under a photon flux density (PPFD) of 400 µmol photons m⁻² s⁻¹ for 12 h per day, at a temperature of 23°C followed by a dark period when temperature was set at 20°C. Relative humidity was maintained at 60–70% for the whole period. The nutrient solution was entirely replaced twice a week to prevent

active molecule and nutrient depletion. Before replacing the growth medium, roots were rinsed with tap water to eliminate SDM traces from root surface. Each pot was equipped with an aeration system to avoid lack of oxygen in the nutrient solution.

Leaf and stem length and biomass

Biometrical parameters were measured over time from the beginning to the end of the experimental period. A selected young leaf (around 7 cm of length) of each willow cutting was measured over time to assess growth dynamics until the end of exposure. Also the length of the longest stem of each cutting was taken at different times during the experimental period. Furthermore, the total biomass of each plant was measured during the experiment.

Leaf gas exchange measurements

Single-leaf gas exchange measurements were performed using a portable open-system (infrared gas analyzer LiCor LI-6400, Li-Cor Inc. Lincoln, Nebraska, USA). Analyses were performed using the circular 2 cm² leaf cuvette equipped with the 6400-40 fluorometer as the light source. Photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) values were measured in the growth chamber, by setting the following conditions at the leaf sample: 380 $\mu\text{mol CO}_2 \text{ mol}^{-1}$, 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (PPFD), 23°C air temperature and 70% relative humidity.

At the beginning of the experiment, the first fully expanded leaf (about 12 cm long and 2 cm wide) was identified for each plant. This same leaf was then utilized in all following measurements. A first measure was taken 12 d before starting the exposure, to establish an initial time; three subsequent measurement sessions were performed 1, 11 and 25 d after the beginning of the plant treatment with SDM. Measurements were always taken the day after the nutrient solution was changed.

Chlorophyll fluorescence parameters

Chlorophyll fluorescence analyses were performed using a JUNIOR-PAM (Waltz, Effeltrich, Germany) portable fluorometer on six leaves per group at the end of the treatment period. Leaves were dark-adapted for 30 minutes at room temperature before determining minimum chlorophyll fluorescence (F_o) at a measuring light of 25 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and maximum chlorophyll fluorescence (F_m) with a saturating pulse of

10000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The maximum quantum yield of photosystem II (Fv/Fm) was calculated as (Fm–Fo)/Fm (Kitajima and Butler 1975). Leaves were then exposed to a light intensity of 190 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and the electron transport rate (ETR, $\mu\text{mol electrons m}^{-2} \text{ s}^{-1}$) was determined as $\Delta F/Fm'$ (effective photochemical quantum yield of photosystem II) \times PPFD \times 0.5 (two photons are used for exciting one electron, as it was assumed the presence of only linear electron transport, hence equal electron transport rate in photosystems I and II) and \times 0.84, which is considered the most common leaf absorbance coefficient for C₃ plants (Björkman and Demmig 1987). The light intensity of 190 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ was selected after several light curve measurements performed on both control and treated plants from 125 to 1500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. This preliminary analysis was necessary for selecting a light intensity near the saturation point that did not induce photoinhibition (data not shown). Data were analyzed using the software WinControl-3 (Waltz, Effeltrich, Germany).

Photosynthetic pigments

Each of the leaves that were used for gas exchange and fluorescence measurements was cut at the end of the exposure for photosynthetic pigment analyses. Leaf samples were treated with dimethylformamide for 48 h (1:10 = w:v) and chlorophyll *a*, chlorophyll *b* and total carotenoids were spectrophotometrically determined respectively at 664, 647 and 480 nm after dilution. The extraction coefficients and the equations reported by Wellburn (1994) were used to determine the investigated pigment contents. Final data were expressed in $\mu\text{g g}^{-1}$ of fresh mass.

Sulfonamide analysis

SDM accumulation was evaluated at the end of the experimental period. Control and treated roots were washed once with tap water buffered at a pH of 9.0 with KOH and then washed three times with deionized one to remove the SDM adsorbed by the rhizoplane. Root and leaf extraction was carried out in liquid nitrogen according to Crowdy and Jones (1956b). Plant tissues were ground in the presence of 0.5 M HCl (1:100 = w:v). A 1 h boiling step was necessary as, otherwise, SDM was detectable at very low level (data not shown). After centrifugation (14000 rpm for 10 minutes), the supernatant was analyzed spectrophotometrically (PerkinElmer, Lambda 11, UV/VIS, Wellesley, MA, USA) utilizing the reaction between sulfonamides and nitrite, which forms a diazonium salt. This, after coupling with N-(1-Naphthyl)ethylenediamine dihydrochloride produces a

colored compound ($\lambda_{\text{max}} = 540$ nm). The match of SDM in the nutrient solution and in root extracts with its corresponding standard was shown by TLC on precoated silica gel plates (E. Merck, Darmstadt, Germany) eluted with ethyl acetate:methanol:25% ammonium hydroxide, 17:6:5, v/v/v. R_f value was 0.84. Final data were expressed in $\mu\text{mol g}^{-1}$ of fresh mass.

Scanning electron microscopy

New willow cuttings were grown directly in the presence of the antibiotic in order to simulate the plant growth in a SDM-polluted site and to determine if willows were able to emit roots and leaves in this condition. After one month of antibiotic exposure, samples of root tips were fixed overnight in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.9) and postfixed in 1% osmium tetroxide in a 0.1 M sodium-cacodylate buffer (pH 6.9) for 2 h. Postfixed samples were dehydrated in a graded acetone series, dried at the critical point, coated with gold and palladium and examined with a scanning electron microscope (SEM Stereoscan 250; Cambridge Instruments, Cambridge, UK) operating at 25 kV according to Rascio et al. (1991).

Statistical analysis

Open source software R (R Development Core Team, 2008), utilizing “car” and “agricolae” packages, was used for statistical analyses. Significant differences ($p \leq 0.05$) among groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences test for comparisons. When residuals were not normally distributed and homoscedasticity was not found, data were analyzed using the non-parametric Kruskal-Wallis and Duncan-Waller post-hoc tests.

RESULTS AND DISCUSSION

Biometrics

Leaf and stem length and total biomass as a function of time are shown in Fig. 2.1. Initial values of the three parameters varied very little since careful attention was paid to the selection of morphologically similar cuttings. No significant differences ($p \leq 0.05$) were detected during the SDM exposure period between controls and treatments for all observed parameters. For all treatments, leaves reached maturity level (i.e., 12 cm of length) about 11-12 d after the beginning of the experiment. Stem length and biomasses

continued to increase over the entire period of the experiment, reaching about 74 cm and 110 g respectively. In contrast to our results, some crop species (*Hordeum distichum*, *Zea mays*, *Panicum miliaceum*, *Pisum sativum* and *Phaseolus vulgaris*) and weed species (*Amaranthus retroflexus*, *Plantago major* and *Rumex acetosella*) exposed to media containing 300 mg SDM l⁻¹ (about 1 mM) showed evident decreases in leaf development and other growth parameters (Migliore et al. 1995; 1996; 1998; Sartorius et al. 2009). Therefore, it may be interpreted that woody plants such as willows are more suitable to phytoremediate high SA levels than herbaceous ones, as suggested by Zhang et al. (2001) for heavy metals.

Gas exchanges

Analyses of photosynthetic parameters, such as transpiration, stomatal conductance and CO₂ assimilation rates, can be useful tools in describing plant tolerance to pollutants, since they are measured in a nondestructive way. Moreover, the effects of pollutants on gas exchange measures become evident earlier than that on growth (Trapp et al. 2000; Pajevic et al. 2009; Pietrini et al. 2010). Numerous authors suggested that reductions in photosynthetic parameters after treatment with different organic and inorganic pollutants are appropriate indicators of phytotoxicity (Hagemeyer and Breckle 1996; Ucisik and Trapp 2006; Yu and Gu 2006; Pietrini et al. 2010). Based on these hypotheses, leaf gas exchange analyses were performed before and during the experimental period.

As shown in Fig. 2.2, transpiration, stomatal conductance and CO₂ assimilation rates can be described by a bell-shaped curve. It is well known that leaf photosynthetic activity increases during development up to maturity when it begins to decrease for senescence processes (Smart 1994; Buchanan-Wollaston 1997). Therefore, the observed decline in stomatal conductance and transpiration toward the end of the experiment can be related to the reduced exchange of CO₂ during senescence.

Before SDM exposure, all treatments, the control included, showed similar transpiration, stomatal conductance and CO₂ assimilation rates of about 3.3 mmol H₂O m⁻² s⁻¹, 0.45 mol H₂O m⁻² s⁻¹ and 11 µmol CO₂ m⁻² s⁻¹ respectively (Fig. 2.2). One day after SDM application, exposed plants exhibited lower transpiration and stomatal conductance values when compared to the control (Fig. 2.2a-b). However, at days 11 and 25 no significant differences were found between treated and control plants (about 1.3 mmol H₂O m⁻² s⁻¹ and 0.1 mol H₂O m⁻² s⁻¹, respectively, at day 25).

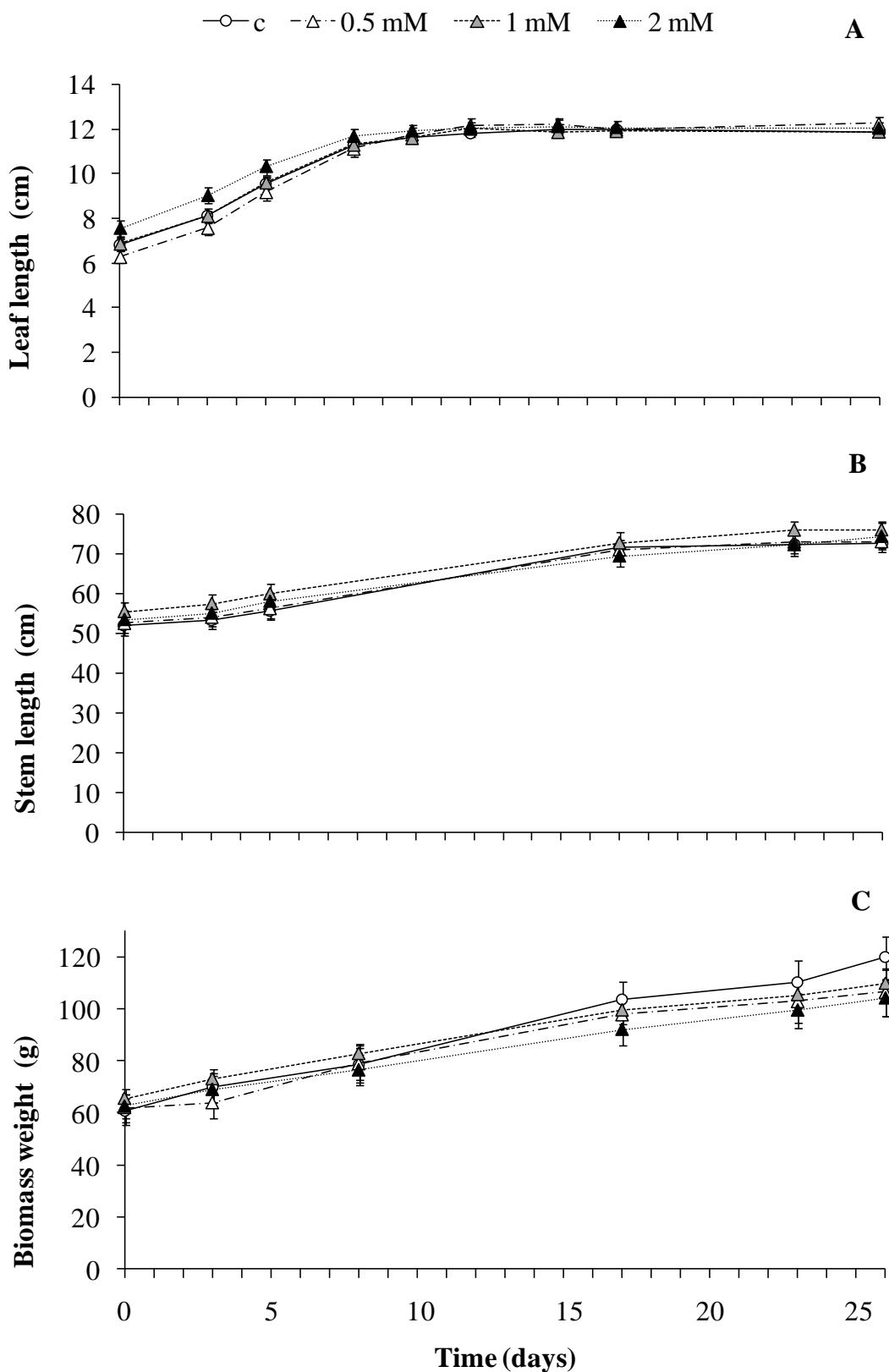


Fig. 2.1 Biometrics during the SDM exposure period. Values are means of 10 replicates \pm SE. No differences between control and treated plants were detected during the experimental period ($p \leq 0.05$).

Contrary to water exchanges, net photosynthesis was not different at day 1 between treated and control plants (about $11\text{-}12 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). However, after 11 d, exposed plants fixed $1.5\text{-}2 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ less than controls ($13.7 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$).

A further photosynthesis reduction was observed after 25 d of SDM exposure, with control plants assimilating at a rate of $8 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ and exposed plants at 7.2 , 5.4 and $4.6 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for 0.5 mM , 2 mM and 1 mM SDM, respectively (Fig. 2.2c), probably for the onset of premature senescence. Early SDM-induced senescence in *Phaseolus vulgaris* leaves was suggested also by Sartorius et al. (2009).

Plant stress due to SDM addition is indicated by transpiration and stomatal conductance decreases already at day 1. However, treated plants displayed the ability to cope with this stress since (i) transpiration and stomatal conductance did not inhibit the photosynthetic process and (ii) the longer-term measurements of the two parameters showed similar values among groups. It is likely that at day 1 photosynthates were utilized to overcome the observed water stress by supporting the leaf osmotic adjustments necessary to re-establish water transport from roots to leaves (Daie 1996). The different behavior of transpiration and photosynthetic rates (water use efficiency) after a short- (1 d) and long-term (25 d) exposure to SDM becomes more evident when transpiration is represented as a function of photosynthesis (Fig. 2.3). At days 1 and 25, the ratio between transpiration and photosynthesis was generally higher in control plants than in treated ones (Fig. 2.3, white circles). At 1 d, transpiration and photosynthesis decreased in a similar way with respect to the control indicating coupling between photosynthesis and stomatal conductance. On the contrary, data collected after 25 d revealed transpiration values around $1.3 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ for all the situations, whereas photosynthesis declined in treated plants, so indicating an uncoupling between photosynthesis and stomatal conductance. Therefore, in the short-term, stomatal limitations may be the main drivers of the slight photosynthesis reduction, whereas in the long term this parameter is likely reduced due to the inhibition of some metabolic processes (i.e., non-stomatal limitations). Flexas et al. (2002) suggested a similar explanation in response to mild and severe drought in C₃ plants.

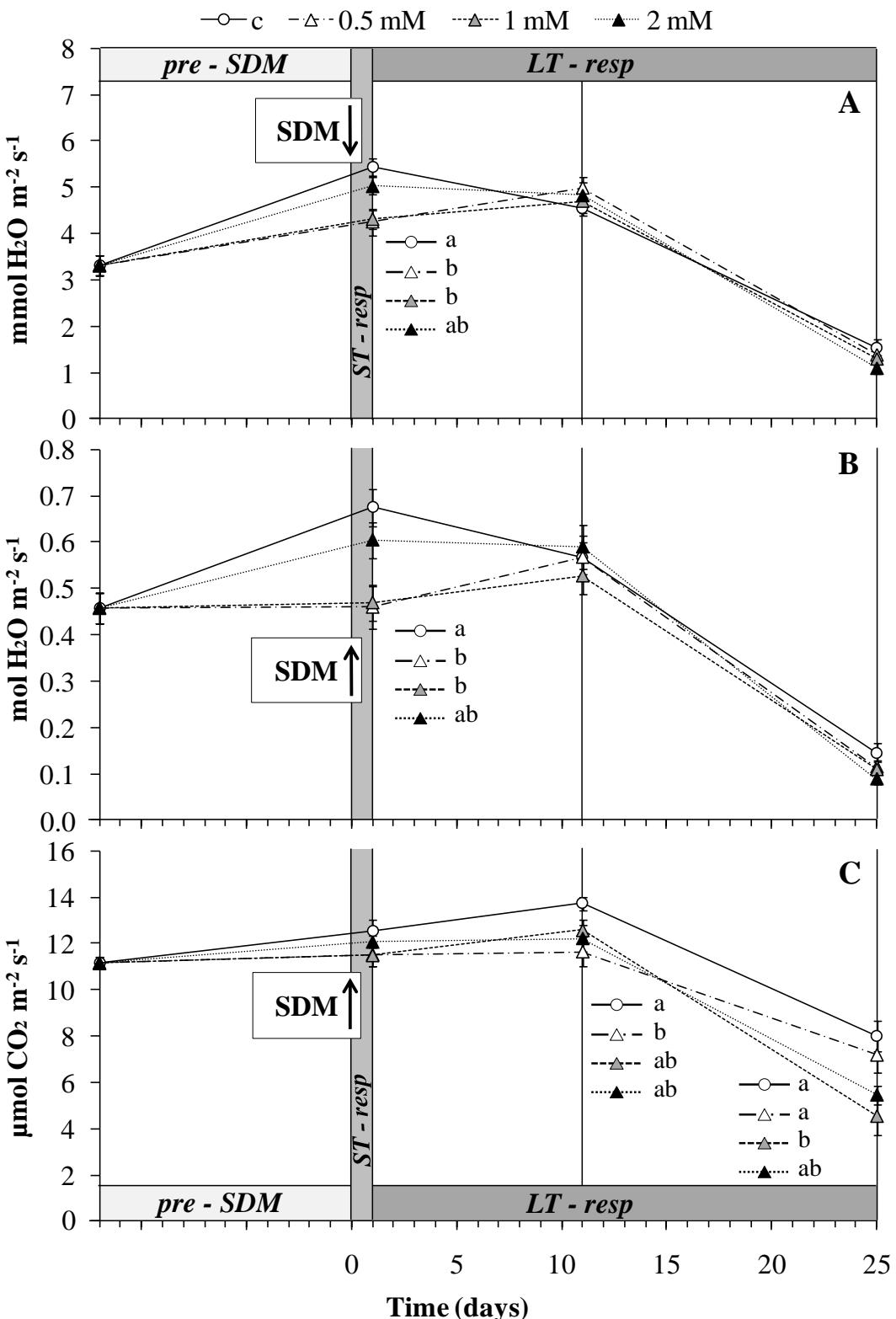


Fig. 2.2 Effect of SDM on leaf gas exchange parameters: transpiration (a), stomatal conductance (b) and net photosynthesis (c) rates measured before (pre-SDM), during and at the end of the exposure period. The arrow indicates the SDM addition. ST-resp = short term response; LT-resp = long term response. Letters, when present, indicate significant differences ($p \leq 0.05$). Values are the mean of 10 replicates \pm SE.

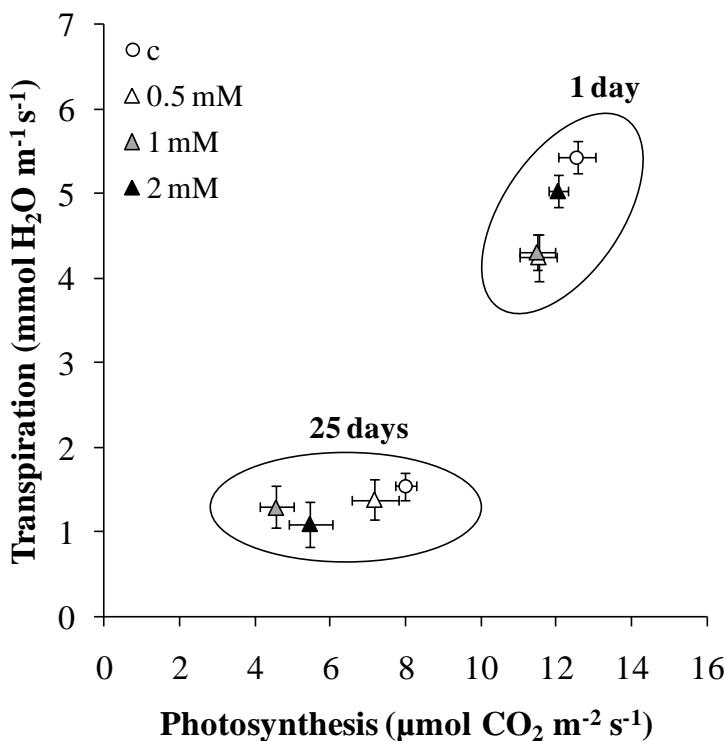


Fig. 2.3 Transpiration as a function of photosynthesis after a short- (1 d) and long- (25 d) term exposure to SDM. Values are the mean of 10 replicates \pm SE.

Fluorescence analyses

Fluorescence analysis has already proved to be a functional tool in the investigation of the effects of pollutants on plants used in phytoremediation projects (Maksymiec and Baszyński 1996). Furthermore, it gives information on the performances of the photosynthetic apparatus integrating CO_2 fixation data (Maxwell and Johnson 2000). Table 2.1 lists the fluorescence parameters surveyed in this study, thus Fv/Fm and ETR. Values of Fv/Fm indicate the potential quantum efficiency of photosystem II and are used as indicator of plant photosynthetic performance, with optimal values of about 0.83 measured for most plant species (Genty et al. 1989; Maxwell and Johnson 2000). Looking to this factor, no significant differences were found between control and SDM-treated plants, with mean values (0.82) around the optimal.

On the other hand, the electron transport rate decreased in treated plants when compared to control ones, in proportion to the administered SDM. Such different response of ETR values from Fv/Fm ones is consistent with data reported by numerous authors (Di Cagno et al. 1999; Linger et al. 2005; Pietrini et al. 2010), who indicate ETR as a better stress indicator than Fv/Fm .

Fv/Fm data show that SDM treatment does not alter the maximum quantum yield of the photosynthetic apparatus, suggesting a normal photosystem organization of willow leaves under these conditions. However, lower ETR values in exposed plants may explain the decrease in photosynthetic activity as they corroborate CO₂ fixation data (Fig. 2.2c). In fact, reductions in net photosynthesis and ETR are known to be relatively proportional (Foyer et al. 1990), supporting a close link between light-dependent and light-independent reactions of the photosynthetic process.

Chlorophyll and carotenoid contents

Chlorophyll and carotenoid contents are fundamental parameters for evaluating photosynthetic activity alterations and are often used as stress indicators in plants. These parameters are frequently used for detecting and assessing exposure of plants to environmental contaminants (Huang et al. 1997; Marwood et al. 2001; Huang et al. 2004). Therefore, to further investigate *Salix fragilis* L. aptitude to tolerate SDM, leaf pigments were also investigated.

As shown in Table 2.1, chlorophyll *a*, chlorophyll *b*, their sum, total carotenoids and the ratio total chlorophylls/carotenoids of treated plants were lower, but not statistically different, or equal in comparison to controls. Only the parameter chlorophyll *a*/chlorophyll *b* differed significantly among the treatment groups. In particular, plants exposed to 1 and 2 mM of SDM showed higher chlorophyll *a*/chlorophyll *b* ratios than control and 0.5 mM SDM-exposed ones. This behavior is in agreement with experiments performed by Huang et al. (2004), who found chlorophyll *a*/chlorophyll *b* ratio increases for plants grown in creosote-contaminated soil. In fact, some organic pollutants are known to decrease the normal total chlorophyll content. As a consequence, photochemical oxidation of the light harvesting complexes that bind chlorophyll *b* is induced (Huang et al. 1997), leading to an increase in the chlorophyll *a*/chlorophyll *b* ratio. We hypothesized a similar result due to sulfadimethoxine exposure. These data give strong support to results found on day 25 for CO₂ photosynthesis and ETR.

Sulfonamide accumulation

The application of phytoremediation technology as a “green” solution to restore polluted sites requires a knowledge of specific pollutant tolerance and accumulation aptitude by plants. Results obtained in this research showed the presence of the active compound only in root apparatus, while SDM was not detected in the epigeal part of plants. In contrast to

our findings, some authors noticed SDM presence also in the aerial apparatus of crops and weeds treated with 300 mg l⁻¹ of active product, but with higher accumulation in roots than in the foliage (Migliore et al. 1995; 1996; 1998). Antibacterial retention in roots may be due to protein binding or acetyl derivative formation, as suggested by Jones and Wignall (1955) and Crowdus and Jones (1956a) for other SAs. As frequently happens for heavy metal plant uptake (MacFarlane and Burchett 2000; Seregin et al. 2004; Snyder et al. 2004), it is possible that willow plants stabilize organic compounds such as SDM in the root cortex to limit its flow through the Caspary strip to the vascular tissues.

As shown in Table 2.1, SDM accumulation in roots was not linearly correlated with the concentration of SDM supplied to plants. Since the adopted extraction procedure can release sulfonamides from their acetyl and protein derivatives, results may indicate a non-linear formation of these products with increasing SDM concentrations.

Table 2.1 Fluorescence parameters (n = 6): Fv/Fm = maximal quantum efficiency and ETR = electron transport rate; pigment content (n = 10): chlorophyll *a* (Chl(*a*)), chlorophyll *b* (Chl(*b*)), carotenoids (Car), chlorophyll *a*/chlorophyll *b* (Chl(*a/b*)), total chlorophylls (Chl(*a+b*)) and total chlorophylls/carotenoids (Chl/Car); SDM accumulation in roots (n = 9). Photosynthetic pigment and SDM contents are expressed in fresh mass (fm).

SDM (mmol l ⁻¹)	Fv/Fm	ETR (μmol m ⁻² s ⁻¹)	Chl(<i>a</i>) (μg g ⁻¹ fm)	Chl(<i>b</i>) (μg g ⁻¹ fm)	Car (μg g ⁻¹ fm)
0	0.82 ± 0.0ns	37.9 ± 1.1a	1573 ± 95ns	493 ± 34ns	280 ± 14ns
0.5	0.82 ± 0.0ns	35.4 ± 0.9ab	1491 ± 72ns	465 ± 23ns	270 ± 12ns
1	0.81 ± 0.0ns	33.9 ± 1b	1384 ± 82ns	411 ± 27ns	254 ± 13ns
2	0.82 ± 0.0ns	33.9 ± 0.8b	1453 ± 94ns	430 ± 30ns	267 ± 16ns
SDM (mmol l ⁻¹)	Chl(<i>a/b</i>)	Chl(<i>a+b</i>) (μg g ⁻¹ fm)	Chl/Car	SDM in root (μmol g ⁻¹ fm)	
0	3.2 ± 0.0b	2066 ± 129ns	6.6 ± 0.0ns	–	
0.5	3.2 ± 0.0b	1956 ± 95ns	6.5 ± 0.0ns	14.4 ± 0.4b	
1	3.4 ± 0.0a	1795 ± 109ns	6.4 ± 0.0ns	38.3 ± 2.5b	
2	3.4 ± 0.0a	1883 ± 124ns	6.4 ± 0.0ns	88.3 ± 2.5a	

Values denote means ± SE. Letters indicate significant differences (*p* ≤ 0.05) and ns denotes no significant difference.

Root morphology alterations

At the end of the experiment, striking alterations in root morphology of all the treated plant roots were observed and further investigated in another set of cuttings through

scanning electron microscopy (SEM). SEM microscopy was performed on a group of cuttings grown in direct SDM contact. After one month exposure, there were clear alterations in the root apparatus. In fact, while control plants exhibited white, straight and tapered main roots, treated plants had yellowish-brown roots. Furthermore, for all treated plants the appearance of secondary roots 1 cm behind the root tip was clearly notable, while for control plants secondary roots were evident only at 10 cm from the tip. This phenomenon is presented in Fig. 2.4 for a control (a) and a 1 mM SDM-exposed plant (b). Alterations of root architecture were found also in common beans treated with 300 mg kg⁻¹ SDM (Sartorius et al. 2009) and spinach plants exposed to a fluoroquinolone antibiotic (Aristilde et al. 2010). According to Sartorius et al. (2009), the observed response to the antibiotic might be due to growth hormone (i.e., auxins and gibberellins) disturbance and/or to root meristematic alterations.

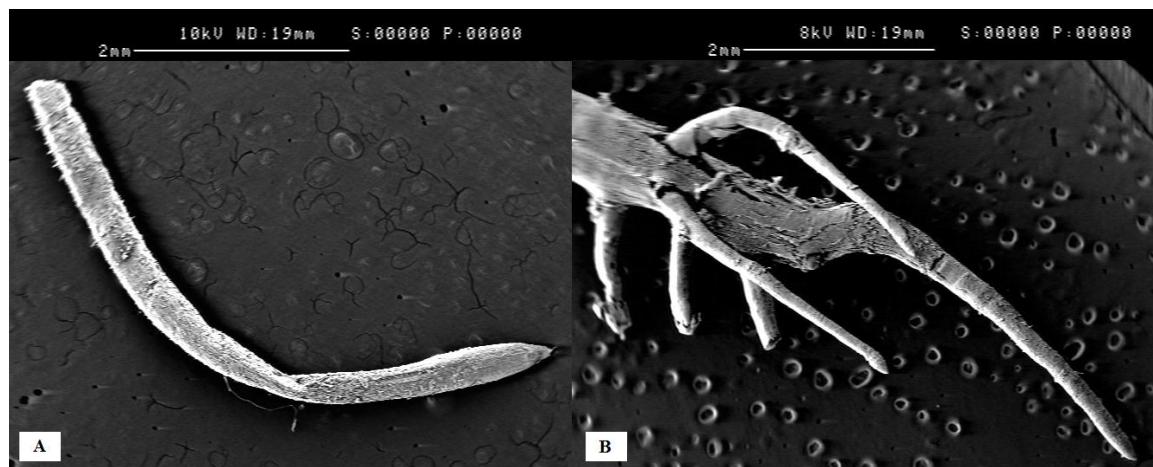


Fig. 2.4 Scanning electron microscopy of root tips of a control plant (a) compared to a plant exposed to 1 mM SDM (b).

Achieved results allow to conclude that *Salix fragilis* L. growth and physiology are not dramatically affected by one month treatment with a sulfonamide (in nutrient solution), with higher tolerance at the concentration of 0.5 mM, higher than what is normally found in agricultural environments. Furthermore, they demonstrate that *Salix fragilis* L. is able to absorb high amounts of this kind of compound. The present study, carried out under laboratory conditions, allow to hypothesize a possible utilization of *Salix fragilis* L. for sulfonamide phytoremediation purposes.

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CHAPTER III

Accumulation and effects in *Salix fragilis* L. plants exposed to environmental relevant sulfadimethoxine concentrations from 0.01 to 10 mg l⁻¹ in nutrient solution for one month

The experimental works described in this chapter are part of the following scientific manuscript: Michelini L., Gallina G., Capolongo F. and Ghisi R. (2012). Accumulation and response of willow plants exposed to environmental relevant sulfonamide concentrations. International Journal of Phytoremediation. Submitted the 3rd of September 2012- currently under review

PREFACE

On investigating the plant accumulation and related responses to SAs, laboratory studies were performed with *Salix fragilis* L. cuttings. Trying to retrace the experimental design of a previous laboratory work (Michelini et al. 2012a), where *Salix fragilis* L. plants were exposed to sulfadimethoxine (SDM) concentrations comparable to that found in fresh faeces of treated calves (i.e., from 155 to 620 mg l⁻¹), in this work plants were exposed to the same active molecule, but at environmental relevant doses (thus from 0.01 to 10 mg l⁻¹). Cuttings were grown hydroponically to avoid potential interactions between the soil matrix and the active molecule, and willow aptitude to accumulate the sulfonamide was evaluated together with its response and acclimation to SDM, after one month of treatment.

MATERIALS AND METHODS

Plant growth

Salix fragilis L. cuttings (20-cm long and 1-cm diameter) were taken from a selected tree in the experimental farm of the University of Padova at Legnaro (Padova, Italy) and grown hydroponically similarly to a previous protocol (Michelini et al. 2012a). Briefly, after one week of growth in tap water, plants were divided into five groups, each consisting of six homogeneously rooted individuals, and treated with either 0 (control), 0.01, 0.1, 1 and 10 mg SDM l⁻¹ (CAS: 122-11-2, Sigma-Aldrich, Milan, Italy) for one month. Plants were transferred in 0.5 l pots containing a 25 times diluted Hoagland's nutrient solution (Arnon and Hoagland 1940), pH 7.0, and the active molecule. The nutrient solution was completely replaced twice a week to prevent antibiotic and nutrient depletion. For the whole period a photon flux density of 400 µmol photons m⁻² s⁻¹ for 12 h per day at 23°C was provided, followed by a dark period when temperature was set at 20°C. Relative humidity was maintained at 60–70%. Each pot was covered with an aluminum foil to protect roots from the light and equipped with an aeration system.

Sulfonamide analysis

At the end of the experimental period the SDM content in plant tissues was determined. Control and treated roots were washed once with tap water brought to pH 9.0 with KOH and then three times with deionized water to remove the SDM adsorbed by the rhizoplane (Michelini et al. 2012a). Roots and leaves were then treated as described by Crowdy and Jones (1956). Plant tissues were ground in liquid nitrogen and then treated with 0.5 M

HCl (1:10 = w:v). After 1 h boiling, extracts were centrifuged (14000 rpm for 10 minutes), the supernatants filtered and neutralized. To remove interferents a SPE cleanup procedure similar to that described by Shelver et al. (2010) was adopted. SPE STRATA-X cartridges (300 mg sorbent) were preconditioned with 5 ml 100% methanol and equilibrated with 5 ml milliQ water. Samples were then loaded and each cartridge was washed with 5 ml of milliQ water, 5 ml of 5% methanol and with 5 ml of 5% methanol/2% acetic acid. Samples were eluted with 2 ml methanol/ethyl acetate 50:50 (v:v). Eluents were dried with a stream of nitrogen (60°C for 4h) and the residues were reconstituted with the mobile phase. The HPLC apparatus consisted of a Jasco Pump PU-980 equipped with a LG 980-02 Ternary Gradient Unit, a Gastorr GT-103 degasser, a Jasco Autosampler AS 950 and a Jasco UV-975 UV/vis detector (Tokio, Japan) set at 268 nm. A Zorbax XDB C18 (Agilent Technologies, USA) column was used for analyte separation. The isocratic elution was performed using KH₂PO₄ 25 mM, brought to pH 3.0 with orthophosphoric acid (A), and acetonitrile (B), at a rate of 75:25 (v:v) for a run time of 15 min, at a flow rate of 0.5 ml min⁻¹ with a sample injection volume of 20 µl. Analyses were carried out at room temperature. The linearity of response was verified in the 0.003-30 mg l⁻¹ range.

Biometrics and microscopy observations

During and at the end of the exposure period, morphological parameters, such as leaf, stem and root lengths and areas, were recorded to visualize possible alterations induced by the SDM. To collect areas a scanner-based image analysis system was used (WINRHIZO Pro, Regent Instruments, QC, Canada). Moreover, stereoscope observations (OPTIKA SZM-2, Italy) were carried out to better investigate the morphological alterations discovered in root apexes of *Salix fragilis* L. exposed to 10 mg SDM l⁻¹.

Dry masses and carbon and nitrogen contents

Terminated the month of treatment, willow leaves and roots from each treatment group were heated at 60°C until complete dryness and then weighed. Dry samples were ground in liquid nitrogen and used to determine the carbon, nitrogen and sulfur content in leaves and roots (Vario MACRO, Macro Elemental Analyzer, Elementar Analysensysteme GmbH, Hanau, Germany). For each sample 0.05 g of plant material were mixed with 0.2 g of tungsten trioxide. Three replicates were used for each treatment group.

Indole-3-acetic acid treatment

A new set of *Salix fragilis* L. plants was used to better scrutinize the morphological alterations observed in the root apparatus after one month exposure to 10 mg SDM l⁻¹. Some growth media were supplemented with 10 mg l⁻¹ of SDM, others with 5 and 10 mg l⁻¹ of indole-3-acetic acid or IAA (CAS: 87-51-4, Sigma-Aldrich, Milan, Italy) for ten days (three plants per group). The nutrient solution and the active molecules were changed twice per week and plants were grown in the same growing condition of the previous experiment. At the end of the exposure period digital photos (SONY, Cyber-shot, DSC-S930, 10.1 megapixels) were taken as documentation of the root alterations found.

Plant response as a function of exposure time

In view to perform a valuable phytoremediation activity plants should be able to tolerate the contaminant presence for protracted periods. In order to explore the plant response to the sulfonamide according to different SDM contact times, a new trial was carried out where *Salix fragilis* L. plants were grown for 48 d in a nutrient solution containing 10 mg l⁻¹ of the antibiotic for the indicated periods: 0 (control), 28, 38 and 48 d. The hypogea area was chosen as a stress indicator and recorded (WINRHIZO Pro, Regent Instruments, QC, Canada) at days 34 and 48 from the beginning of the exposure, for all the different exposure times. Each series was composed by five willows. A representation of the experimental schema adopted in this test is given in Fig. 3.1.

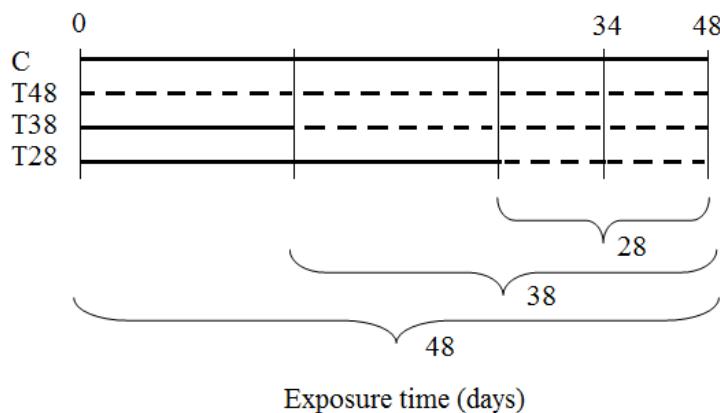


Fig. 3.1 Schematic representation of the SDM exposure conditions adopted. The black lines represent the plant cultivation in nutrient solution, while the dotted lines correspond to the three exposure periods. c: control plants; T48, T38 and T28 stand to treatment duration of 48, 38 and 28 d, respectively. Area recording was performed at days 34 and 48.

Statistical analyses

Open source software R (R Development Core Team, 2008) was used for statistical analyses. Significant differences ($p < 0.05$) among groups were assessed by one-way analysis of variance (ANOVA) followed by a LSD test for comparisons. Significant differences ($p < 0.05$) between groups were assessed by Student's t-test.

RESULTS AND DISCUSSION

Sulfonamide accumulation

Results obtained in this research showed the presence of the active compound only in the root system, while SDM was never detected in the epigeal part of plants. The accumulation data recorded of the parent compound are: 0.1, 0.3 and 14.1 mg SDM kg⁻¹ fresh mass, respectively for treatments 0.1, 1 and 10 mg l⁻¹. The presence of the active molecule was observed starting from 0.1 mg l⁻¹ and its accumulation in roots increased with the antibiotic dose, even if not linearly with the concentrations supplied to plants. It is necessary to underline that detected values were probably underestimated as derivative forms were not analyzed.

A similar preferential localization of the parent compound in roots than in shoots was observed in previous works (Michelini et al. 2012a; 2012b), where willow and maize aptitude to accumulate SAs (respectively, SDM and sulfadiazine) in a range of concentrations from 10 to 620 mg l⁻¹ was analyzed. Such greater localization in roots could be due to a possible SDM transformation (e.g., acetyl-metabolites) or to protein bindings as suggested by Crowdy and Jones (1956) and Jones and Wignall (1955) for other SAs. However, even if the active molecule was not transferred to the aerial plant, the SDM fixation to roots could be a promising mechanism for *in-situ* phytoremediation. In fact, it is well known that certain soil, sediment and ground water contaminants have a reduced mobility and bioavailability thanks to the immobilization/precipitation inside the root apparatus (e.g., in cell vacuoles to prevent contaminant transfer to the aerial part) or to the adsorption onto roots, so minimizing the pollutant migration by erosion agents and the wildlife exposure (Vangronsveld et al. 1995; Bonomo 2005; Pilon-Smits 2005).

Plant response and tolerance

Biometrics. *Salix fragilis* L. root, leaf and stem lengths and total biomasses over time are shown in Fig.3.2. No significant differences were detected at the end of the SDM exposure period between controls and treatments for leaf and stem biometrics and for

total masses (Fig. 3.2a-c). In fact, from the beginning to the end of the experiment, plants belonging to the different groups grew at similar rates. In every case, leaf and stem lengths had mean values of about 6 and 29 cm (Fig. 3.2a-b), respectively, and the total epigeal area (leaves and stems together) was about 450 cm² (Fig. 3.3a). The total biomass reached about 35 g without showing differences among groups (Fig. 3.3c). Similar results were achieved in *Salix fragilis* L. plants exposed to SDM in a range of concentrations from 155 to 620 mg l⁻¹, where no differences in the leaf and stem development were observed (Michelini et al. 2012a).

The outcomes obtained with root biometrics were different probably because of the direct contact with the antibiotic. In fact, looking at Fig. 3.2d, which shows the root lengths over time, it appears that after about 18 d of treatment plants started to develop differently depending on the antibiotic dose. In particular, root growth in the less SDM concentrated solution had an increased elongation in comparison to control and treated plants ($p < 0.05$). This evidence was highlighted also in the total root area analysis. In fact, the antibiotic stimulated the total root area development at 0.01 mg l⁻¹ and inhibited it at the higher concentrations, respectively 1 and 10 mg l⁻¹ (Fig. 3.3b). This particular behavior, consisting in a hormetic response characterized by an inverse U-shaped curve, was clearly evident also looking at the hypogea weights (Table 3.1). This root hormetic trend was found to influence the total biomass as well, since leaf, stem and cutting masses remained similar among all the treatments (Table 3.1). An analogous hormetic reaction, but only at a shoot level, was recently observed by Migliore et al. (2010b) in *Lythrum salicaria* L. plants exposed for one month to 0.005, 0.05, 0.5, 5, 50 mg l⁻¹ of SDM. Not only SAs, but also other kind of antibiotics tended to stimulate the plant development, such as carbenicillin towards strawberry roots (Qin et al. 2011) and tetracyclines towards maize plants, in both laboratory and field tests (Migliore et al. 2010a).

According to Calabrese (2005), hormesis is a very common phenomenon occurring in many organisms and with numerous chemically agents. This kind of overcompensatory response could represent an adaptive reaction which involves an additional activity of the plant (e.g., increased allocation of resources) to cope with the stress situation (Calabrese 2005). However, information concerning possible toxic implications for plants following hormetic strategies is still lacking.

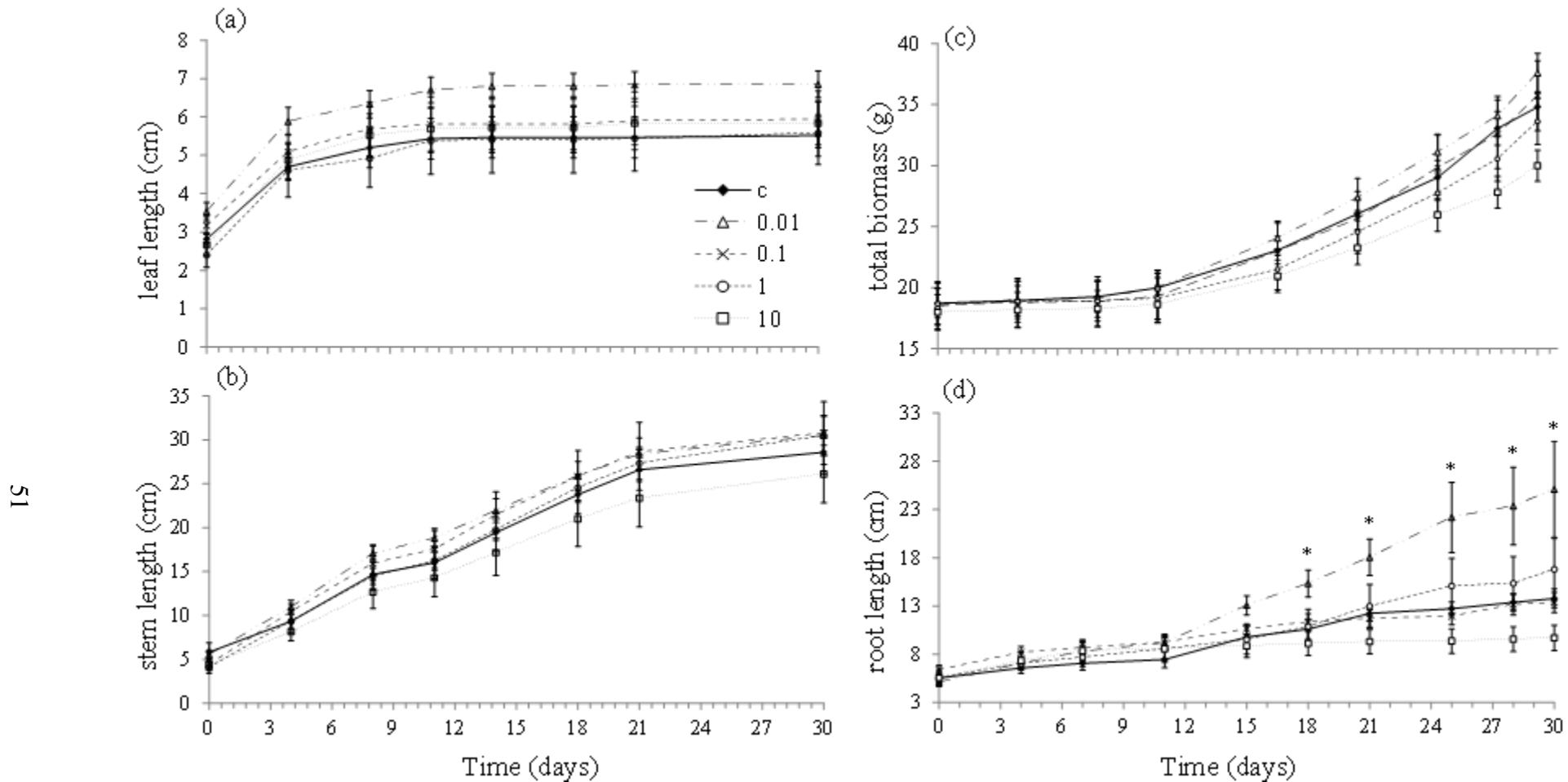


Fig. 3.2 Leaf length (a), stem length (b), total biomass (c) and root length (d) during the exposure period. c: control plants; 0.01: 0.01 mg SDM L⁻¹ treatment; 0.1: 0.1 mg SDM L⁻¹ treatment; 1: 1 mg SDM L⁻¹ treatment; 10: 10 mg SDM L⁻¹ treatment. Asterisks identify significant differences among groups.

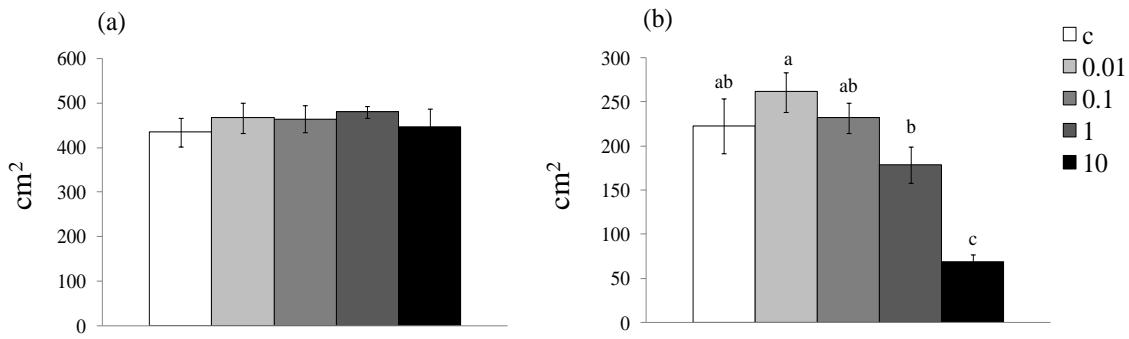


Fig. 3.3 Areas of stems and leaves (a) and roots (b). c: control plants; 0.01: 0.01 mg SDM l^{-1} treatment; 0.1: 0.1 mg SDM l^{-1} treatment; 1: 1 mg SDM l^{-1} treatment; 10: 10 mg SDM l^{-1} treatment. Values denote means \pm SE ($n = 6$). Where present, letters indicate significant differences among groups ($p < 0.05$).

Dry matter and nutrient content. The dry matter content in plants (i.e., the dry to wet weight ratio) was evaluated in order to define if the SDM exposure affected the water relationships in willows (Table 3.1). This value was found to be about 8% and 31%, respectively, for roots and leaves of controls and plants treated from 0.01 to 1 mg SDM l^{-1} . A remarkable increase in the dry mass percentage ($p < 0.05$) was found in leaves and roots coming from plants treated with 10 mg SDM l^{-1} (respectively, 12% and 39%). Similar results were obtained with barley exposed to 11.5 mg SDM l^{-1} (Ferro et al. 2010) and *Lythrum salicaria* L. plantlets treated with 50 mg SDM l^{-1} (Migliore et al. 2010b). An increased dry weight was also found in rice plants exposed to organics as phenanthrene and pyrene (Li et al. 2008). According to the last authors, it is possible that plants try to limit the contaminant uptake by reducing the water flow from the growth medium to the roots, thereby protecting the photosynthetic apparatus. Huang et al. (2004) analyzed the tolerance of three plant species to creosote in soil and noticed that the more sensitive species showed a lowest root dry to fresh weight ratio in contrast to the more tolerant one. In accordance, dry matter data observed in the present work might indicate that the pharmaceutical dose of 1 mg l^{-1} could be assumed as the maximum SDM toleration threshold of *Salix fragilis* L.

To investigate the plant ability to assimilate nutrients during the month of treatment with SDM, the total amounts of carbon and nitrogen in both roots and leaves were evaluated (Table 3.1). Data concerning the carbon content showed that only plants growing with 10 mg SDM l^{-1} exhibited a slight alteration (+ 3.2 and + 3.4% for roots and leaves, respectively) of this parameter, whereas all the other treatment groups were largely unaffected. These findings are in agreement with a previous work (Michelini et al.

2012a), where no remarkable alterations in the CO₂ assimilation rate were observed in willow plants treated with up to 155 mg SDM l⁻¹. Looking to the nitrogen content, percentages were found to be similar in all the groups for both roots and green tissues. Consequently, such results suggest that the assimilation and/or allocation of carbon and nitrogen in *Salix fragilis* L. are not deeply affected by antibiotic levels similar to those tested in the present trial; the plant can still produce biomass useful for energetic purposes and contribute to the control of water eutrophication.

Table 3.1 Total, hypogea, stem-leaf and cutting final fresh masses, dry masses, carbon and nitrogen contents in root and leaf tissues. Letters indicate significant differences among groups ($p < 0.05$) and ns denotes no significant difference. fm: fresh mass.

SDM	Total mass		Hypogea mass		Stem-Leaf mass		Cutting mass	
	mg l ⁻¹	g (fm)	g (fm)	g (fm)	g (fm)	g (fm)	g (fm)	g (fm)
0	34.8 ± 1.2 ab	9.2 ± 1.5 b	9.7 ± 0.3 ns	17.0 ± 1.1 ns				
0.01	37.6 ± 1.6 a	13.1 ± 1.7 a	10.2 ± 0.7 ns	16.5 ± 1.1 ns				
0.1	35.7 ± 2.9 a	10.4 ± 1.1 ab	8.8 ± 0.8 ns	16.5 ± 2.0 ns				
1	33.6 ± 1.9 ab	9.5 ± 1.3 ab	8.8 ± 0.6 ns	16.7 ± 1.4 ns				
10	30.0 ± 1.3 b	2.7 ± 0.3 c	9.2 ± 0.7 ns	17.0 ± 1.3 ns				

SDM	Roots		Leaves		Roots		Leaves	
	mg l ⁻¹	% dry mass	% dry mass	% C	% N	% C	% N	
0	7.9 ± 0.3 b	29.9 ± 2.3 b	45.6 ± 0.4 b	1.9 ± 0.2 ns	46.0 ± 0.2 b	2.1 ± 0.1 ns		
0.01	8.1 ± 1.2 b	31.2 ± 0.8 b	45.4 ± 0.1 b	1.8 ± 0.0 ns	46.1 ± 0.3 b	1.7 ± 0.2 ns		
0.1	7.3 ± 0.6 b	32.9 ± 1.0 b	46.2 ± 0.3 ab	1.7 ± 0.0 ns	45.9 ± 0.2 b	1.7 ± 0.0 ns		
1	7.8 ± 0.4 b	31.4 ± 0.5 b	45.8 ± 0.2 b	2.0 ± 0.1 ns	46.2 ± 0.2 b	1.8 ± 0.0 ns		
10	12.3 ± 2.3 a	38.6 ± 1.8 a	47.2 ± 0.2 a	2.2 ± 0.1 ns	47.6 ± 0.3 a	1.6 ± 0.1 ns		

Root architecture and growth hormone assay. After the one month of SDM exposure, important morphological alterations in the root system were observed at the antibiotic concentration of 10 mg l⁻¹. In particular, this treatment promoted an abnormal emission of numerous lateral roots, which were never found in control willows (Fig. 3.4). Similar alterations were previously found in other *Salix fragilis* L. plants grown for about one

month in nutrient solution and soil, respectively spiked with 155 mg SDM l^{-1} and 200 mg kg^{-1} of sulfadiazine (Michelini et al. 2012a; 2012b). Moreover, root number increase was noticed following treatment of strawberry plants with equal concentrations of carbenicillin and cefotaxime antibiotics from 100 to 250 mg l^{-1} (Qin et al. 2011). According to Potters et al. (2009), several abiotic stressors, as heavy metals, paraquat, hydrogen peroxide or low phosphate in the rhizosphere, can induce lateral root development through alterations of the normal homeostasis of reactive oxygen species, antioxidants, auxin and ethylene. Sartorius et al. (2009) as well, suggested hormonal disturbances to explain alterations of bean root architecture due to SDM treatment.

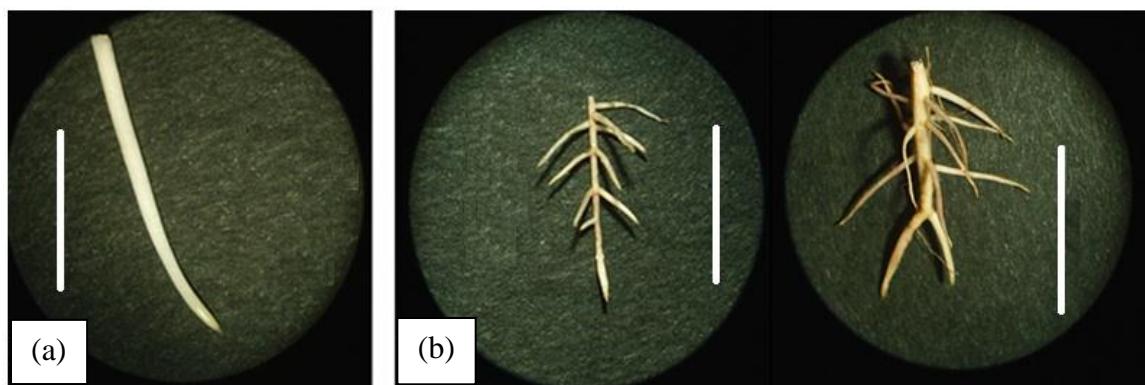


Fig. 3.4 Stereoscope images of a control (a) and of two roots treated with 10 mg SDM l^{-1} (b). The white lines correspond to 1 cm.

In order to corroborate the hypothesis of a growth hormone implication in the alterations observed, new *Salix fragilis* L. cuttings were treated with IAA (from 5 to 10 mg l^{-1}), as it is known that auxin represents an important regulator of the lateral root development (Rashotte et al. 2001; Jeong et al. 2007; Baque et al. 2010). Root architecture induced by IAA was compared to that caused by a 10 mg SDM l^{-1} treatment after 10 d of exposure. From this test it was observed that the exogenously applied IAA induced a morphological disturbance of the hypogea organs comparable to that stimulated by the antibiotic (Fig. 3.5a-d). Hence, these phenotypic responses support the hypothesis that SDM induces a strong hormonal disequilibrium.

However, it is important to underline that despite the abnormal root phenotype described above, plants were able to accumulate both SDM and nutrients from the growth solution.

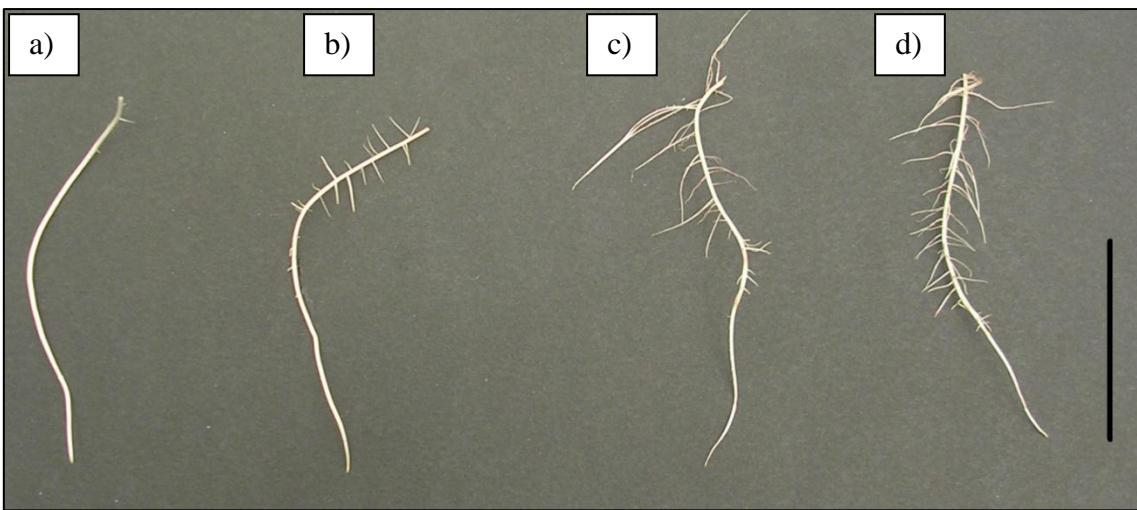


Fig. 3.5 Primary roots of a control plant (a), a plant treated with 10 mg SDM l^{-1} (b), a plant treated with 5 mg IAA l^{-1} (c) and 10 mg IAA l^{-1} (d). The black line corresponds to 5 cm.

SDM acclimation as a function of the exposure period. In order to find out if the contact with the sulfonamide induced some acclimation mechanisms in *Salix fragilis* L. species, new cuttings were grown for a period of 48 d, during which they were exposed to 10 mg SDM l^{-1} for different times, respectively, 0 (control), 28, 38 and 48 d (experimental scheme described in Fig. 3.1). The root area was chosen as a non-destructive and sensible indicator of plant response to SDM. In Fig. 3.6 the percentage increases of this parameter between values measured on the 34th and 48th d are presented. From these data it appears that the control plants exhibited the absolute highest growth rate with a mean increase of 48%. However, in treated plants growth increased with time of exposure to SDM. In fact, plants treated for the whole period (i.e., 48 d) presented an average root area increase of 32%, which was higher than that of the other two groups (22% and 8% for plants exposed for 38 and 28 d, respectively). This evidence clearly highlights that *Salix fragilis* L. plants develop a higher SDM tolerance during the time of exposure, which is a good characteristic for plants designed to phytoremediate toxic molecules in long-term field projects. Plants were found to acclimatize also towards environmental O₃ in long-term growth conditions (Bussotti et al. 2007). In our case, it is possible to hypothesize that plants develop some defense mechanisms with time, such as antibiotic compartmentalization (e.g., intracellular sequestration) and transformation in a less toxic derivative, which allowed *Salix fragilis* L. to grow similarly to control plants.

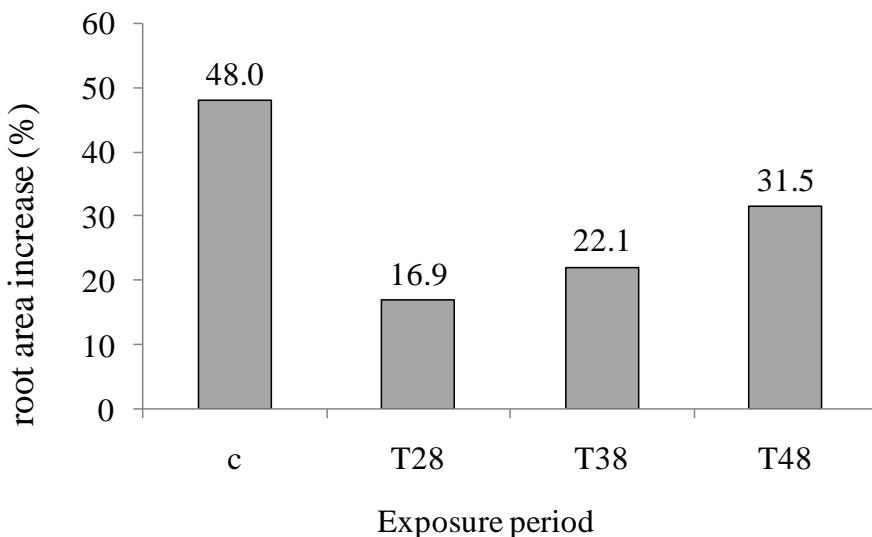


Fig. 3.6 Root area increase (%) among values measured on 34th and 48th d. c, T28, T38 and T48 correspond, respectively, to controls and plants exposed for 28, 38 and 48 d.

This study highlights the aptitude of *Salix fragilis* L. to accumulate SDM in root tissues from the dose of 0.1 mg l⁻¹. Furthermore, results obtained after exposing cuttings to 10 mg SDM l⁻¹ for different periods suggest that willow tolerance to SDM increases with the exposure duration, probably because of the onset of some acclimation mechanisms. These findings enable to speculate about the possible future use of willow also for long-term remediation projects. As some stress symptoms on the root development were visible after treatment at the highest dose, *Salix fragilis* L. seems to tolerate well the antibiotic presence up to 1 mg SDM l⁻¹. Furthermore, considering the dominant presence of SDM in the hypogea apparatus, the use of willow plants for *in-situ* application could limit the interaction with soil living organisms, thanks to the ability of these plants to stabilize the active molecule.

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CHAPTER IV

Accumulation and effects in *Salix fragilis* L. and *Zea mays* L. plants grown in soil spiked with sulfadiazine concentrations of 10 and 200 mg kg⁻¹ for one month

The experimental works described in this chapter are part of the following scientific manuscript: Michelini L., Reichel R., Werner W., Ghisi R., Thiele-Bruhn S. (2012). Sulfadiazine uptake and effects on *Salix fragilis* L. and *Zea mays* L. plants. Water Air and Soil Pollution 223: 5243-5257

PREFACE

Once in the soil, the extractability of sulfonamides quickly declines due to immobilizing processes (Förster et al. 2009; Wehrhan et al. 2010). For example, already 2 d after spiking SDZ at a concentration of 10 mg kg^{-1} to soil, $< 2 \text{ mg kg}^{-1}$ 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 (Boxall et al. 2004; Aust et al. 2008).

As therapeutic agents are designed to be biologically very active chemicals, once reached the soil their activity clearly affects soil microorganisms (Thiele-Bruhn 2003; Ding and He 2010) and could also impact vegetation (Jjemba 2002). An uptake of several antibiotics into food plants and translocation within the plant was recently reported in literature (Dolliver et al. 2007; Ferro et al. 2010). 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.

At present, investigations concerning plant uptake, distribution within the plant and subsequent effects on vegetal physiology remain still limited. For this reason, the presented study had a twofold aim, (i) to investigate the plant uptake of a sulfonamide antibiotic from soil and its possible utilization in phytoremediation and (ii) to determine adverse effects on crops. To investigate this, willow (*Salix fragilis* L.) and maize (*Zea mays* L.) plants were exposed for 40 d to sulfadiazine (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 $10 \text{ mg SDZ kg}^{-1}$, 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 $200 \text{ mg SDZ kg}^{-1}$, 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).

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

MATERIALS AND METHODS

Experimental design

Eighteen *Salix 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 eighteen *Zea mays* L. seeds (cultivar PR39K13 Pioneer Hi-Bred Buxtehude) were pre-grown in tap water for 10 d 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 $\text{cmol}_c \text{ kg}^{-1}$; 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 d (from 7 April to 16 May 2011) and at an average temperature of $25\pm5^\circ\text{C}$ during the day and

20 ± 5 °C in the 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 d 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 24h and at 60 °C, until complete dryness, for plant material. Root morphology of both plant species and the different treatments was documented with digital photos (SONY, Cyber-shot, DSC-S930, 10.1 megapixels).

Biometrics and soil moisture

Biometric measures were recorded weekly for each plant until 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 fifth leaf. At the end of the 40 d 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 ECH₂O EC-5 (Decagon Devices, Inc, Pullman, Washington, USA) probe inserted at 10 cm soil depth was used together with a TDR device (INFIELD 7b, UMS, Munich, Germany).

Antibiotic extraction procedure

After collecting adhering rhizosphere soil and thoroughly cleaning the roots with running water 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, were 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) were mixed with 1.5 g 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 (i) from plants were methanol/deionized water 1:4 (v/v) according to Förster et al. (2008) and (ii) deionized water for soil samples. These extractants proved to be most efficient in preliminary experiments. Briefly, (i) 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 (ii) 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 cycles for plants and soil, respectively; 15 min of static time; 200 °C temperature; 60% of flush; 100 bar pressure and 400 sec of N₂-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 were added in order to account for matrix effects.

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, CA). The HPLC consisted of two LC-20AD pumps, an autosampler SIL-20AC, 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 0.1M HCOOH in water (solvent A) and 0.1M HCOOH in methanol (solvent B) which were delivered in a gradient program listed online in Table S1 (Supplementary material). 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 (Supplementary material). The software Analyst 1.4.2 (Applied Biosystems/MDS Sciex Instruments, Toronto, Canada) 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 1000 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 (LOD) of the method was 5 µg l⁻¹ and the limit of quantification (LOQ) was 10 µg l⁻¹ determined using the procedure of Antignac et al. (2003). Final results are expressed in mg kg⁻¹ on a dry mass (dm) basis.

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).

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

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.

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

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, HEKAttech, 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 H₂O₂ 30% (Merck, Darmstadt, Germany) and 3 ml HNO₃ 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 g kg⁻¹ dm of Ca or K.

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 SPAD (Soil Plant Analysis Development) 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 (µg g⁻¹ fm) were calculated

using Eq. 3, where A665 and A650 represent the two wave lengths used in the analysis.

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

Statistical analyses

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 (ANOVA) followed by Tukey's Honestly Significant Differences test for comparisons. Significant differences ($p<0.05$) between groups were assessed by Student's t-test.

RESULTS

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. 4.1a-d). For all the parameters analyzed the first measuring point (0 d), 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 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 $10 \text{ mg SDZ kg}^{-1}$ (Fig. 4.1a-d). At the end of the exposure time willow and maize plants of 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 4.1). To the opposite, 200 mg kg^{-1} 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 fifth leaf of maize plants (Supplementary material, 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 d (2nd and 3rd leaves), 11 d (4th leaf) and 24 d (5th leaf) in the treatments with $200 \text{ mg SDZ kg}^{-1}$ 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 masses, root areas, root volumes and total root lengths for both species and root fresh mass in the case of maize after 40 d of exposure to SDZ (Table 4.1), 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 4.1).

The percentage of the dry mass content was evaluated in roots, leaves and stems (Table 4.1). For both the 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 2604, 3534 and 5122 for control and treatments 10 and 200 mg kg⁻¹, while for maize SRL values were 619, 675 and 1520, 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 around 40-45% (Fig. 4.2a), 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. 4.2b-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, no statistical difference was found to bare control soils, indicating almost complete inhibition of plant water uptake.

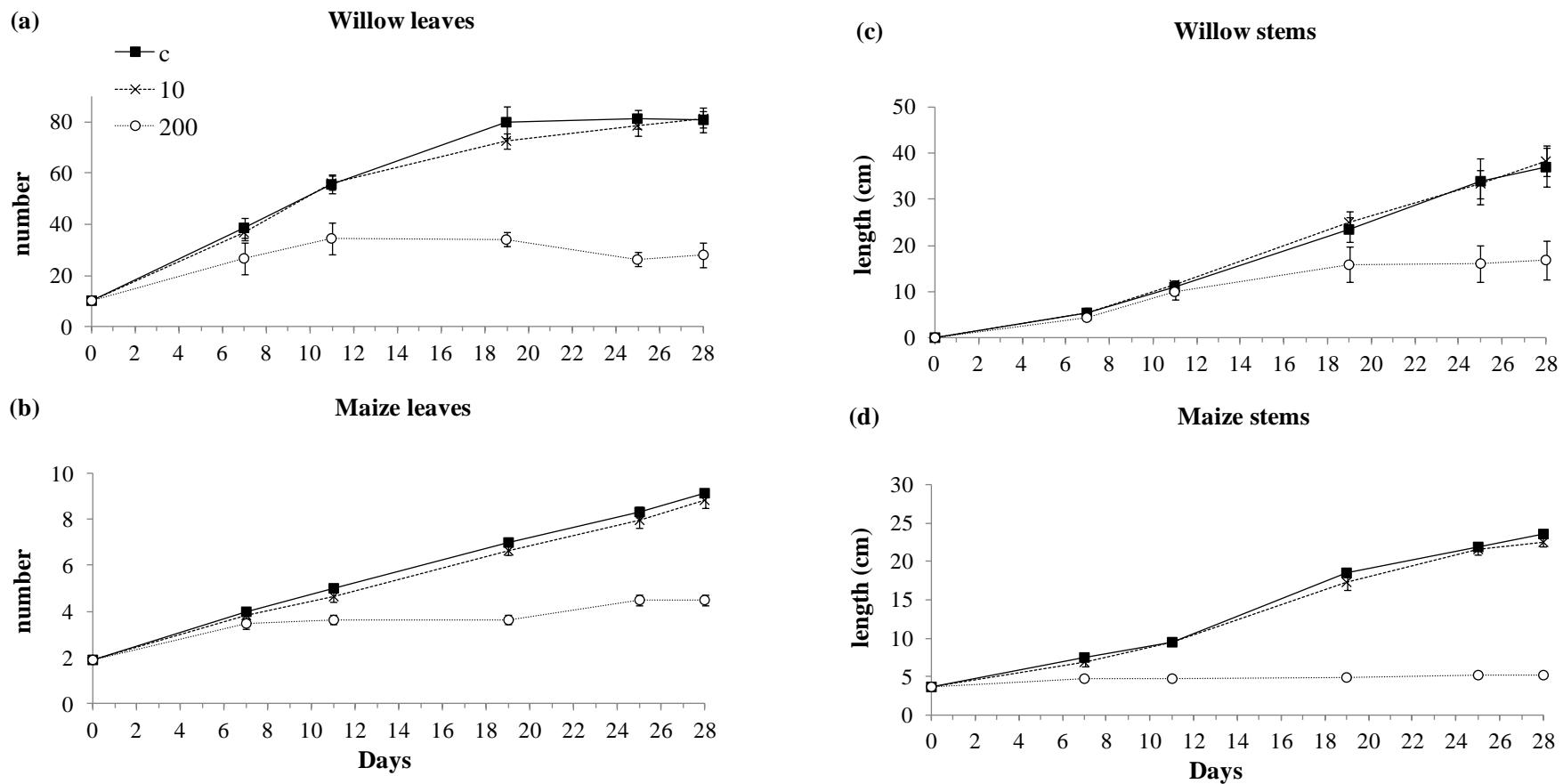


Fig. 4.1 Leaf number development (a, b) and stem lengths (c, d) during the experimental time in willow (a, c) and maize plants (b, d) exposed to 0 (c), 10 (10) and 200 (200) mg kg⁻¹ SDZ. Values denote mean \pm SE. Error bars not shown are smaller than symbols.

Table 4.1 Final biometrics and dry matter contents (%) of willow and maize plants. Control, 10 and 200 denote treatments 0, 10 and 200 mg SDZ kg⁻¹. Values denote mean \pm SE; HSD post-hoc test ($p<0.05$). Letters, when present, indicate significant difference among SDZ treatments ($p<0.05$).

Sample	Stem length cm	Leaves number	Leaves and stems g (fm ¹)	Roots				Dry mass			
				Area cm ²	Volume cm ³	Total length cm	Root %	Leaf %	Stem %	SRL ³ cm g ⁻¹ dm	
Willow											
Control	37.0 \pm 4.1 ^a	80.8 \pm 4.7 ^a	22.5 \pm 1.3 ^a	1.6 \pm 0.3	87.1 \pm 9.7 ^a	2.1 \pm 0.9 ^a	416.7 \pm 81.0 ^{ab}	9.96 \pm 1.3	20.98 \pm 0.5	25.98 \pm 0.3 ^a	2604
10	38.4 \pm 3.3 ^a	81.2 \pm 3.2 ^a	22.8 \pm 1.6 ^a	1.6 \pm 0.3	97.1 \pm 16 ^a	1.3 \pm 0.3 ^a	600.8 \pm 77.5 ^a	10.38 \pm 1.1	20.77 \pm 0.7	27.08 \pm 0.4 ^a	3534
200	16.9 \pm 4.3 ^b	28.0 \pm 4.8 ^b	3.2 \pm 0.8 ^b	0.9 \pm 0.1	40.2 \pm 4.6 ^b	0.5 \pm 0.1 ^b	256.1 \pm 22.2 ^b	5.57 \pm 3.2	21.63 \pm 1.5	17.23 \pm 2.8 ^b	5122
Maize											
Control	23.5 \pm 0.5 ^a	9.2 \pm 0.2 ^a	102.2 \pm 4.2 ^a	6.8 \pm 0.7 ^b	156.7 \pm 28.6 ^b	6.8 \pm 1.4 ^a	371.5 \pm 98.2 ^a	8.77 \pm 2.0	12.58 \pm 0.2 ^b	6.34 \pm 0.1 ^b	619
10	22.5 \pm 0.6 ^a	8.8 \pm 0.3 ^a	97.0 \pm 6.4 ^a	9.9 \pm 1.4 ^a	252.9 \pm 38.5 ^a	9.0 \pm 1.7 ^a	580.7 \pm 73.0 ^a	8.68 \pm 0.7	13.62 \pm 0.3 ^b	6.16 \pm 0.5 ^b	675
200	5.2 \pm 0.2 ^b	4.5 \pm 0.2 ^b	0.2 \pm 0.0 ^b	0.8 \pm 0.1 ^c	17.1 \pm 1.2 ^c	0.4 \pm 0.0 ^b	60.8 \pm 4.9 ^b	5.69 \pm 2.1	78.53 \pm 9 ^{a,2}		1520

¹ fresh mass

² sum of dry mass of leaves and stems; significant difference to the respective sum of other treatments (control and SDZ 10 mg kg⁻¹)

³ specific root length (cm g⁻¹ dm)

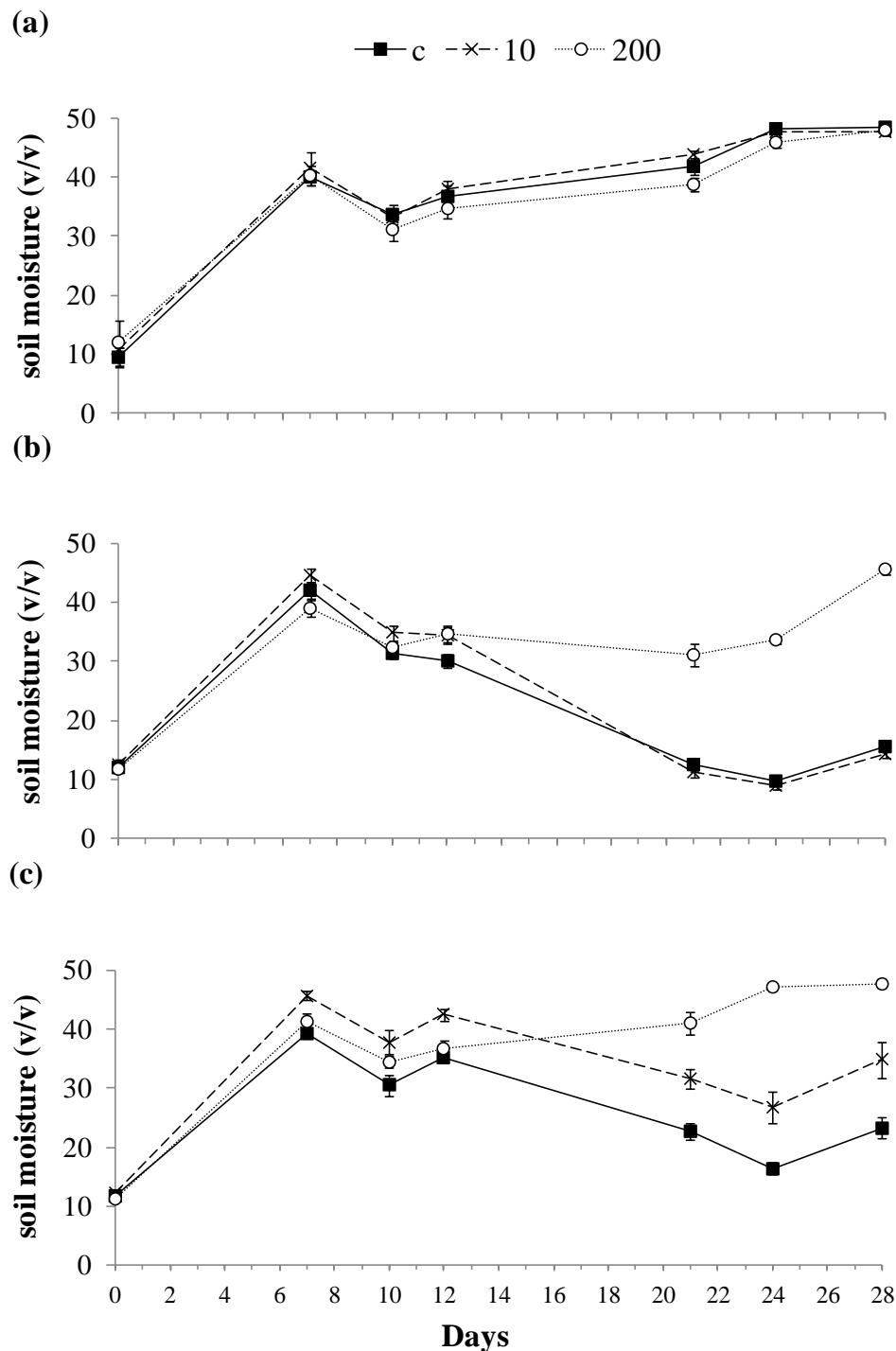


Fig. 4.2 Soil moisture (v/v) in pots without plants (a), with willow (b) and maize plants (c). C, 10 and 200 denote treatments with 0, 10 and 200 mg kg⁻¹ SDZ. Values denote mean \pm SE. Error bars not shown are smaller than symbols.

SDZ content in plants and soil

Within the experimental duration of 40 d 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 4.2). 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 4.2). 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 BCF 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 4.2). 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).

Table 4.2 Concentrations of SDZ and of two hydroxyl-metabolites in bulk and rhizosphere soil, at the rhizoplane and in plant tissues (mg SDZ kg⁻¹ dm) and resulting bioconcentration factor (BCF) and translocation factor (Tf %) after 40 d of experiment. Values denote mean ± SE.

Sample	SDZ soil conc. ¹	Soil		Rhizoplane mg kg ⁻¹	Plant			BCF	Tf %
		bulk	rhizosphere		roots	stems	leaves		
		SDZ							
Willow	10	0.12 ± 0	0.23 ± 0	<LOD ²	333 ± 68	<LOD	<LOD	33.3	<LOD
	200	29.4 ± 3	27.9 ± 1	600 ± 52	5464 ± 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	5331 ± 210		708 ± 184 ⁵	26.7	13.3
Control	10	0.34 ± 0							
	200	22.4 ± 2							
OH-SDZ^{3,4}									
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 ⁵		
Control	10	0.07 ± 0.0							
	200	1.71 ± 0.1							

¹ soil spiking concentration (mg kg⁻¹)

² <LOD = below limit of detection

³ relative quantification based on calibration for SDZ

⁴ sum of 4-OH-SDZ and 5-OH-SDZ

⁵ leaves and stems together

Element content in plants

In Table 4.3 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 d. 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 4.3). 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 4.3). 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.

Chlorophyll content

In this study SPAD values and total chlorophyll content of leaves from plants grown in SDZ-contaminated soil were determined (Table 4.3). 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 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 (Supplementary material, Fig. S2) and the death of maize plants.

Table 4.3 Total C and N (%), C:N ratio, amount of K, Ca (g kg^{-1} dm), K:Ca ratio, SPAD values (non dimensional) and total chlorophyll ($\mu\text{g g}^{-1}$ fm) in willow and maize tissues. Control, 10 and 200 correspond to soils treated with 0, 10 and 200 mg SDZ kg^{-1} . Values denote mean \pm SE; HSD post-hoc test ($p<0.05$). Letters, when present, indicate significant difference among SDZ treatments ($p<0.05$).

		Willow			Maize		
		Control	10	200	Control	10	200
C total	Roots	39.7 \pm 0.6	38.5 \pm 0.1	38.9 \pm 0.6	36.9 \pm 1.0	36.1 \pm 0.8	37.6 \pm 0.8
	Stems	41.6 \pm 0.1	42.4 \pm 0.1	41.4 \pm 0.5	35.2 \pm 0.3 ^a	36.0 \pm 0.8 ^a	32.6 \pm 0.2 ^b
	Leaves	41.6 \pm 0.2 ^a	41.0 \pm 0.3 ^{ab}	40.5 \pm 0.4 ^b	39.8 \pm 0.2 ^{ab}	40.3 \pm 0.2 ^a	38.7 \pm 0.0 ^b
N total	Roots	1.86 \pm 0.1 ^b	1.71 \pm 0.0 ^b	2.83 \pm 0.0 ^a	0.99 \pm 0.1 ^b	0.91 \pm 0.1 ^b	2.14 \pm 0.2 ^a
	Stems	1.39 \pm 0.1 ^b	0.93 \pm 0.1 ^b	3.83 \pm 0.5 ^a	0.92 \pm 0.1 ^b	1.24 \pm 0.4 ^b	6.00 \pm 0.3 ^a
	Leaves	3.52 \pm 0.0	3.33 \pm 0.0	3.40 \pm 0.1	2.66 \pm 0.1 ^b	3.10 \pm 0.3 ^b	4.84 \pm 0.0 ^a
C:N	Roots	21.6 \pm 0.9 ^a	22.6 \pm 0.5 ^a	13.8 \pm 0.5 ^b	40.1 \pm 5.2 ^a	40.6 \pm 2.5 ^a	18.4 \pm 2.0 ^b
	Stems	30.5 \pm 1.9 ^b	46.7 \pm 3.4 ^a	11.5 \pm 1.3 ^c	42.4 \pm 6.0 ^a	27.5 \pm 7.4 ^a	5.5 \pm 0.3 ^b
	Leaves	11.8 \pm 0.2	12.3 \pm 0.2	12.0 \pm 0.5	15.0 \pm 0.3 ^a	13.6 \pm 1.3 ^a	8.0 \pm 0.0 ^b
K	Roots	12.1 \pm 0.2	13.6 \pm 2.9	n.a. ¹	16.1 \pm 1.1	14.4 \pm 1.1	n.a.
	Stems	12.1 \pm 0.3	11.2 \pm 0.4	9.6 \pm 1.5	63.7 \pm 2.7 ^a	57.6 \pm 4.7 ^a	43.5 \pm 2.4 ^b
	Leaves	21.4 \pm 0.1 ^a	17.4 \pm 0.6 ^b	23.4 \pm 0.9 ^a	27.4 \pm 0.3 ^b	28.5 \pm 2 ^b	55.1 \pm 0.7 ^a
Ca	Roots	4.6 \pm 0.1	5.3 \pm 0.9	n.a.	2.4 \pm 0.2	2.6 \pm 0.2	n.a.
	Stems	3.9 \pm 0.3	3.3 \pm 0.3	4.8 \pm 0.7	2.1 \pm 0.1 ^b	2.7 \pm 0.2 ^b	8.6 \pm 0.5 ^a
	Leaves	9.7 \pm 0.4 ^b	10.5 \pm 0.5 ^b	15.8 \pm 0.5 ^a	0.64 \pm 0.1 ^c	2.4 \pm 0.2 ^b	8.1 \pm 0.0 ^a
K:Ca	Roots	2.6 \pm 0.1	2.5 \pm 0.2	n.a.	6.8 \pm 0.2	5.6 \pm 0.2	n.a.
	Stems	3.2 \pm 0.1 ^{ab}	3.5 \pm 0.1 ^a	2.1 \pm 0.2 ^b	30.7 \pm 0.4 ^a	21.7 \pm 0.3 ^b	5.2 \pm 0.2 ^c
	Leaves	2.2 \pm 0.1 ^a	1.7 \pm 0.1 ^b	1.5 \pm 0.1 ^b	43.3 \pm 1.2 ^a	12.7 \pm 0.4 ^b	6.8 \pm 0.1 ^b
SPAD	Leaves	35.6 \pm 0.7	36.9 \pm 0.6	31.4 \pm 3.3	39.5 \pm 1.1	36.4 \pm 1.2	n.a.
	Total chlorophyll	2870 \pm 147 ^{ab}	3130 \pm 160 ^a	2014 \pm 482 ^b	1511 \pm 26.5	1309 \pm 145	n.a.

¹ n.a. = not analyzed due to shortage in sample material resulting from strongly inhibited plant development

Morphological root alterations

After 40 d 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. 4.3a-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. 4.3d-f).



Fig. 4.3 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.

DISCUSSION

SDZ in soil

Soil spiking concentrations of SDZ strongly declined within 40 d and the soil extractable SDZ diminished to concentrations that are frequently detected in arable soils (e.g., Höper et al. 2002; Hamscher et al. 2003). 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 non-linearity 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 d willow removed 0.16% of the total amount of SDZ spiked to soil at the low 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 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).

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 5464 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 d 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., *Zea 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 (Trapp et al. 1990; Kumar 2005; Pilon-Smits 2005). 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 (Supplementary material, 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 (Li et al. 1997; Burken 2003). 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, from the data it remains unclear, whether OH-SDZ in plants originated from plant metabolism or root uptake from soil.

Effects on plants

Biometric analyses evidenced that SDZ has the potential to adversely affect *Salix fragilis* L. and *Zea 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., Hamscher et al. 2002;

Christian et al. 2003; Aust et al. 2008). However, even at an environmental relevant soil concentration of 10 mg kg^{-1} SDZ led to alterations in root morphology. Correspondingly, sulfadimethoxine had similar effects on *Salix 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 (Migliore et al. 1995; Boonsirichai et al. 2002). 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 $200 \text{ mg SDZ kg}^{-1}$. 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 $200 \text{ mg SDZ kg}^{-1}$. 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 disturbed. Consequently, lack of K probably compromised plant nutrition, growth, tropism, enzyme homeostasis and osmoregulation (Schachtman and Schroeder 1994; Taiz and Zeiger 2009).

To conclude, this study focused on some of the ecological consequences of sulfonamide contaminated waste application on agricultural lands. Considering that the lower concentration tested may be expected in arable soils as the upper level of sulfonamide contamination, it is possible to state that the overall physiological parameters analyzed in this study showed the potential of SDZ to negatively influence the growth of important crops such as maize. 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.

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CHAPTER V

Root associated soil microbial community composition and enzyme activities of willow and maize plants in the presence of sulfadiazine

The experimental works described in this chapter are part of the following scientific manuscript: Michelini L., Reichel R., Ghisi R., Thiele-Bruhn S. (2012). Willow and maize root associated soil microbial community composition and enzyme activities in the presence of the antibiotic sulfadiazine. Archives of Environmental Contamination and Toxicology. Submitted the 12th of November 2012- currently under review

PREFACE

Antibiotics usually act on particular metabolic pathways of living cells; hence, as a consequence of their presence in the environment, also non-target organisms sensitive to this pharmaceutical mode of action can be affected (Henschel et al. 1997). At present, several studies exist on the SA effects on soil microbial communities following the entrance of the active molecules into soils through manure (Zielezny et al. 2006; Kotzerke et al. 2008; Hammesfahr et al. 2011b; Liu et al. 2012). However, the influence of growing plants and especially plant roots on these effects was not addressed in the cited publications and there is a lack of studies on that topic. For example, Ollivier et al. (2010) investigated microbial genes and transcripts of the N-cycle in the rhizosphere and reported that respective effects of sulfadiazine (SDZ) differed among plant species. As is well known, plants strongly influence the structural and functional diversity of soil microbial consortia due to the peculiar root exudation and rhizodeposition processes (Sørensen 1997; Smalla et al. 2001; el Zahar Haichar et al. 2008). The molecules released from the roots interact with bacteria in the rhizosphere and their composition varies depending on the species, cultivar, and physiological status of the plant (Sørensen 1997; Heuer et al. 2002). Therefore, we hypothesized that the presence of growing plants substantially influences antibiotic effects on the soil environment.

With the present study we aimed to examine if antibiotics differently alter the activity and community structure of selected important microbial groups present in bulk soil (identified as the fraction of soil not directly investigated by roots), rhizosphere soil (the first 2-3 mm of soil attached to the root apparatus) and plant roots. For this purpose, *Salix fragilis* L. and *Zea mays* L. plants were grown in topsoil from an Orthic Luvisol spiked with SDZ, a sulfonamide antibiotic widely used to treat infectious diseases of animals by numerous Gram-positive and Gram-negative bacteria (De Liguoro et al. 2007). Since the activation of microbial growth through the addition of nutrient substrates very much governs the effects of bacteriostatic sulfonamides (Hammesfahr et al. 2008; Hammesfahr et al. 2011a), no manure was added in this study, in order to investigate if the activating source could be represented by the vegetal organism. Sulfadiazine influence on soil microorganisms was investigated by determination of a set of enzymatic activities of the C-, N- and P-cycle, and the community structure of total bacteria and *Pseudomonas* through 16S rRNA gene PCR-denaturing gradient gel electrophoresis (DGGE).

Abbreviations

B = bulk soil; R = rhizosphere soil; M = maize; W = willow; C = control without sulfadiazine; SDZ = sulfadiazine; 10, 200 = soil spiked with sulfadiazine at nominal concentrations of 10 and 200 mg kg⁻¹; Ps = *Pseudomonas*; TC = total microbial community; dm = dry mass

MATERIALS AND METHODS

Soil and plant sampling

For this study two different plant species were selected: (i) the monocotyledon *Zea mays* L., widely grown as fodder plant used for animal breeding, and thus typically fertilized with manure that may contain pharmaceutical antibiotics; (ii) *Salix fragilis* L., a dicotyledon plant, with high potential for soil phytoremediation and use in short rotation coppice or forestry for fast woody biomass production. Details on plant growth conditions and the soil used were previously reported (Michelini et al. 2012). Briefly, genetically identical *Salix fragilis* L. cuttings (about 20-cm long and 1-cm of diameter) from the experimental farm of the University of Padova at Legnaro (Italy) and *Zea mays* L. seeds (cultivar PR39K13 Pioneer Hi-Bred, Buxtehude) were grown in tap water for 10 d and then transferred in an Orthic Luvisol topsoil material. This soil, not previously fertilized with manure, was collected from an arable field at Merzenhausen (Germany). It consisted of clay 15.4%; silt 78.2%; sand 6.4%; with a pH (CaCl₂) of 6.3; the total organic carbon was 2.1%; and the maximum water holding capacity (w/w) was 45.8%. Before plant allocation, soil was air-dried, sieved (4 mm screen), to ensure physical homogeneity, and mixed with approximately 28 mg N, 14 mg P and 34 mg K kg⁻¹ soil by addition of NPK fertilizer. Polypropylene Kick-Brauckmann pots (25.5 cm height and 28.5 cm external diameter) were filled with 7 kg soil and each one was split in two parts by a PE sheet. Each half was planted with one plant and soil was spiked with SDZ sodium salt (99.0% minimum, CAS: 547-32-0, Sigma Aldrich, Germany) at nominal concentrations of 0 (control), 10 and 200 mg kg⁻¹ dm. The experiment was conducted with six independent replicates per treatment group. Plants were cultivated in a greenhouse under natural photoperiod for 40 d (7 April to 16 May 2011) and every pot was irrigated twice per week with the same amount of water (200-500 ml per pot, depending on the plant requirements). At the end of the exposure period, samples of bulk soils were collected and the root apparatus of every plant was vigorously shaken to separate the root-adhering rhizosphere soil from bulk soil. Prior to soil sampling, each pot was moistened to about

55% of water holding capacity to ensure similar conditions for subsequent analyses of enzyme activities. Root and soil samples were stored at -20°C until analyses. Dry mass of soil was determined after drying at 105°C for 24 h.

Soil enzyme activities

Enzyme activities from rhizosphere and bulk soil were measured with a fluorimetric microplate enzyme assay according to a method developed by Marx et al. (2001) and further described by Ernst et al. (2009). Three replicates per group were analyzed and each measurement was repeated four times. The following enzyme substrates based on methylumbelliferyl (MUB) and 7-amino-4-methylcoumarin (AMC) were analyzed: L-leucine-AMC for leucine-aminopeptidase (EC 3.4.11.1), MUB-β-D-cellulobioside for β-cellulbiohydrolase (EC 3.2.1.91), MUB-phosphate for acid phosphatase (EC 3.1.3.2), MUB-β-D-glucopyranoside for β-glucosidase (EC 3.2.1.3), MUB-N-acetyl-β-D-glucosaminide for chitinase (EC 3.2.1.14) and MUB-β-D-xylopyranoside for xylosidase (EC 3.2.2.27) activities. These enzymes were selected as they are highly relevant in nutrient pathways in the soil environment. In particular, leucine-aminopeptidase is involved in the N-cycle, β-cellulbiohydrolase, β-glucosidase, chitinase and xylosidase in the C-cycle, and the acid phosphatase in the P-cycle.

For analyses, moist soil samples (corresponding to 0.5 g dry mass) were mixed with 50 ml autoclaved water in a sterile glass jar by vortexing for 2 min to achieve a homogenous suspension. Subsequently, aliquots of 50 µl were dispensed into black microtiter well plates (Biozyme Scientific, Hess. Oldendorf, Germany). Afterwards, 50 µl of appropriate buffer were added into each well. In particular, these buffers have been: 100 mM MES, pH 6.1, for glycosidases and acid phosphatase and 50 mM TRIZMA, pH 7.8, for the leucine-aminopeptidase (Marx et al. 2001). At the end, 100 µl substrate solutions were added to achieve a final volume per microtiter of 200 µl. All chemicals have been purchased from Sigma Aldrich Chemicals (Taufkirchen, Germany). Plates were kept at 30°C in the dark for 120 min and the fluorescence was measured using a Victor 3 MultiLabel Reader (Perkin Elmer, Germany) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Final enzyme activities are expressed as nmol MUB (AMC) g⁻¹ dry soil h⁻¹.

DNA extraction

Microbial DNA was isolated from bulk and rhizosphere soil using FastDNA® SPIN Kit for Soil and FastPrep® Instrument (MP Biomedicals, Santa Ana, CA). Furthermore, DNA was purified from solution using the Geneclean® Spin Kit (Qbiogene, Inc., Carlsbad, California). For root bacterial DNA extraction (both endophytes and rhizoplane bacteria) the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) was used. Three replicates per group were analyzed. Obtained supernatants were stored at 4°C and amplified within 24 h.

PCR and DGGE of soil and roots

The PCR amplification of 16S rRNA gene fragments was done using the total community (TC) primer set F968GC/R1378 (Heuer et al. 1997) and the primer pair F311Ps/R1459Ps for *Pseudomonas* (Ps) (Milling et al. 2005). The PCR products, differing in melting properties, were separated using a DCode System for DGGE (Bio-Rad Laboratories, Inc.). PCR samples were loaded onto polyacrylamide gel (6-9%, w/v) in 1 x TAE buffer. Gels were prepared with denaturing gradients ranging from 26 to 58% (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run at 58 °C for 6 h at 220 V (Heuer et al. 2002). After electrophoresis, the gels were silver stained and photographed on a UV transillumination table (BioDocAnalyze, Biometra, Germany). Gels were analyzed using image analysis BIOGENE software (Vilber-Lourmat, Marne-la-Vallée, France). Comparisons were based on relative molecular mass calculations, which were derived from a standard lane. The band patterns were linked together using the BIOGENE database and exported as binary data for further statistical analysis.

Data analysis

Dendograms of DGGE binary data were calculated based on UPGMA and the Pearson correlation as a measure of similarity. Analyses were performed with the multivariate analysis software package PC-ORD Version 5.02 (MjM Software, Gleneden Beach, Oregon, USA). For enzyme activities, open source software R (R Development Core Team, 2008), utilizing “car” and “agricolae” packages, was used for statistical analyses. Significant differences ($p<0.05$) among groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences test for comparisons. Non-rotated principal component analysis was calculated for enzyme activity patterns using IBM SPSS Statistics 20 (IBM Germany, Ehningen, Germany).

RESULTS

Soil enzyme determination

Table 5.1 and Fig. 5.1 show the enzyme activities in bulk and rhizosphere from soils planted with willow or maize that were spiked with 0, 10 and 200 mg SDZ kg⁻¹, respectively. Results revealed that the impact of the antibiotic was concentration dependent, its effect varied between soil compartments (i.e. bulk or rhizosphere soil) and plant species (i.e. willow or maize). The activities of β -cellobiohydrolase and xylosidase were similar in soil planted with maize and willow, while the other enzyme activities were higher in soil planted with maize. In most cases, SDZ at a spiking level of 200 mg kg⁻¹ substantially and significantly ($p<0.05$) reduced all enzyme activities in bulk and rhizosphere soil. Effects were even stronger in bulk soil compared to rhizosphere soil, even if differences were not statistically relevant, and in soil planted with willow compared to soil planted with maize. In particular, a significantly stronger decrease in the activity of acid phosphatase ($p<0.05$) was found in the samples from bulk soil planted with willow compared to bulk soil of maize (27% and 19% less than the corresponding control). The general trend of the investigated enzyme activities was the following for the two plant species (W, M) and the two investigated soil compartments (B, R). For willow planted soil it was BW10 \geq BWC >> BW200 and RW10 \geq RWC \geq RW200. Similar trends were recorded in maize planted rhizosphere soil, while the sequence was different for bulk soil (except for β -glucosidase and leucine-aminopeptidase, Table 5.1): BMC \geq BM10 > BM200 and RM10 > RMC \geq RM200. In contrast, SDZ at a spiking level of 10 mg kg⁻¹ had no significant effects, although it tended to increase the enzyme activities compared to the control of bulk soil in willow planted pots (100.7-105.3%) and in rhizosphere soil of both willow (103.7-114.5%) and maize (102.5-116.2%). Generally, enzyme activities were not different between bulk soil and rhizosphere soil in control treatments without SDZ. However, inhibiting effects of SDZ at a spiking level of 10 mg kg⁻¹ were only determined in maize bulk soil but not in the rhizosphere soil, yet without showing any statistical difference.

These findings are summarized in the cluster dendrogram in Fig. 5.1. Cluster analysis revealed a separate clustering of the enzyme activity pattern of willow and maize planted soils sharing less than 50% similarity, which is especially due to the different activity of acid phosphatase ($p<0.05$) between willow and maize as stated above. Enzyme activities in maize planted soil became clearly clustered when spiked with SDZ at 200 mg kg⁻¹,

while the same concentration caused enzymatic activities in willow planted bulk soil that were totally different (0% similarity) to all other samples.

Considering the investigated enzymes, the highest activity reduction following SDZ exposure was found for leucine-aminopeptidase in the bulk soil and xylosidase in the rhizosphere soil, with an average decline of activity of 24% compared to controls for the first enzyme and 19% for the latter. On the opposite, β -glucosidase was the enzyme least affected by SDZ application.

Total community and *Pseudomonas* DGGE profiles

Denaturing gradient gel electrophoresis was applied to both bulk and rhizosphere soils to analyze the fragments generated from specific 16S rRNA-PCR. Banding patterns were nearly identical among replicates and corresponding DGGE profiles are presented in Fig. S3-S8 (Supplementary material). On TC and Ps DGGE gels, structural community changes were indicated by the appearance or loss of certain DGGE bands, as well as by higher or lower band intensities. The latter aspect was not evaluated, due to the varying photo quality of DGGE gels. The responses were particularly clear at the root apparatus level and more obvious for maize than willow (Supplementary material, Fig. S5, S8). The plant-specific differences were more evident on TC than Ps DGGE, indicating the higher diversity of total bacteria. The TC and PS band patterns evidently changed with increasing nominal SDZ concentration, which was mostly documented by the loss of bands indicative for the control treatment and the appearance of significant bands. The latter was especially pronounced in the DGGE lanes of the 200 mg kg⁻¹ treatments.

Overall changes of DGGE banding patterns are illustrated by the dendograms from cluster analysis (Fig. 5.2, 5.3 and S9, S10 of Supplementary material). Samples clustered according to the three main influencing factors: plant type, soil or plant compartment and the administered SDZ dose. The plant type had the strongest influence on the bacterial community structure formed after 40 d of experiment. Soils, either planted with willow or maize, shared less than 25% similarity in TC and Ps DGGE banding patterns (Supplementary material, Fig. S9, S10). Within these plant clusters, bacterial community structures clearly differed between roots, rhizosphere soil and bulk soil (Fig. 5.2 and 5.3). In more detail, root associated communities shared down to 0% similarity with the structure of the microbial communities from the two soil compartments, while rhizosphere soil and bulk soil had a similarity of around 80% (TC) and 50% (Ps) in the corresponding DGGE information. However, among the two soil compartments, cluster

separation due to the effect of SDZ on the bacterial community structure was stronger in the rhizosphere compared to bulk soil and this was especially pronounced in the presence of maize (both TC and Ps) and willow (TC) (Fig. 5.2a-b and 5.3a).

The effect of SDZ on the structural diversity of bacterial communities led to cluster separation. The clustering depended on the SDZ spiking level. Also, effects of SDZ on DGGE banding patterns of both TC and Ps increased from bulk soil to the plant root apparatus, as can be seen from the dendograms (Fig. 5.2, 5.3 and S9, S10 of Supplementary material). Depending on the SDZ spiking concentration, individual cluster formation was more pronounced for Ps than for TC and clearer for soil planted with maize than willow. Replicates of 10 mg SDZ kg⁻¹ treatments frequently clustered more closely to the control and clearer effects on microbial structure were determined for bacterial communities treated with the highest dose of 200 mg SDZ kg⁻¹.

From cluster analyses of enzyme activities and bacterial community structures, it appeared that the shifts in structural diversity (DGGE) were greater than the shifts in functional diversity (enzymes), with clusters sharing generally at least 50% similarity in enzyme activities and often less than 25% in DGGE banding patterns (Fig. 5.1 and S9, S10 of Supplementary material).

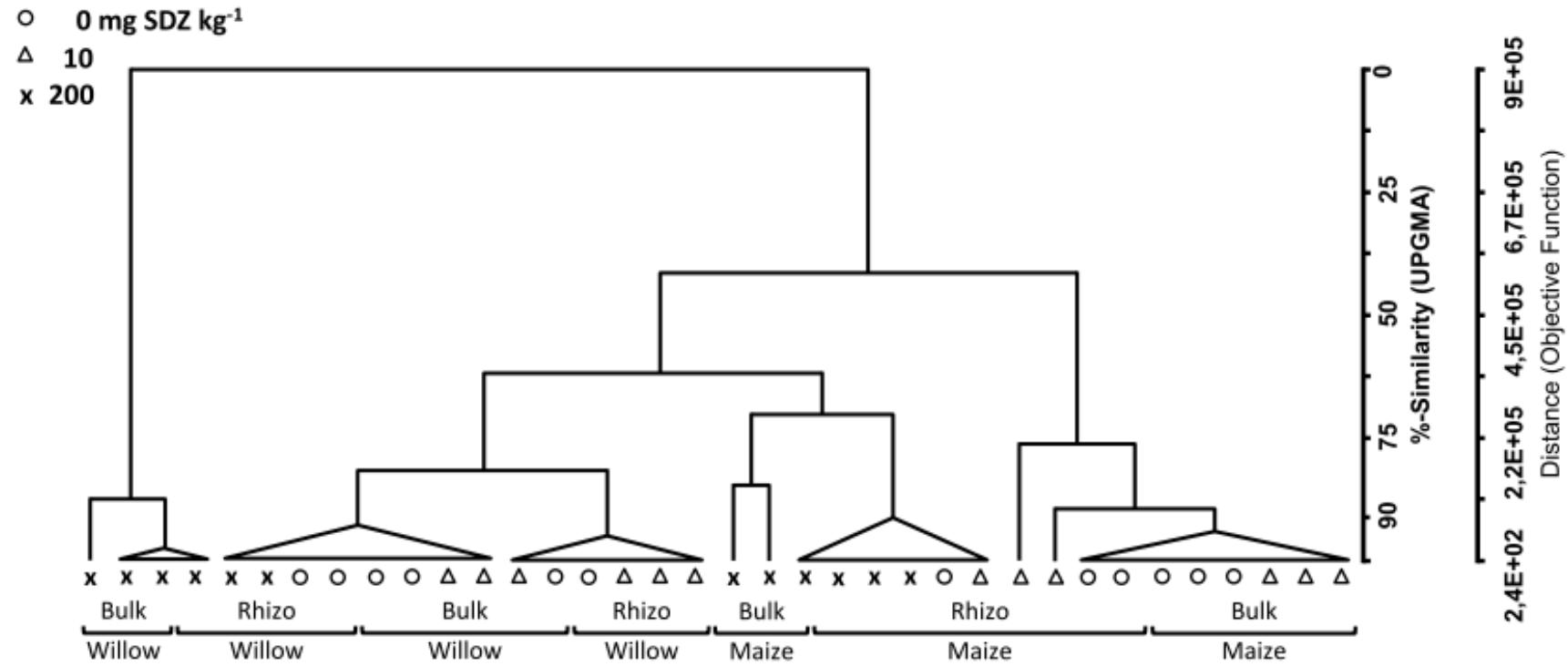


Fig. 5.1 Cluster dendrograms of soil enzyme activities from willow and maize planted bulk and rhizosphere soil. Clusters are combined at 90% similarity. Linkage method: GROUP AVERAGE (UPGMA); distance measure: Euclidean (Pythagorean).

Table 5.1 Enzyme activities in bulk (B) and rhizosphere (R) soil of willow and maize plants. Data are expressed as nmol MUB (AMC) g⁻¹ dry soil h⁻¹. C, 10 and 200 correspond to soil spiking levels of 0 (control), 10 and 200 mg SDZ kg⁻¹. Different letters indicate significant differences at $p<0.05$.

Enzyme	Bulk soil (B)			Rhizosphere soil (R)		
	control	10	200	control	10	200
Willow (W)						
leucine-aminopeptidase	1327.0 ± 61.4 a	1336.3 ± 45.1 a	883.8 ± 55.0 b	1303.9 ± 78.8	1352.6 ± 10.5	1101.6 ± 106.1
β-cellulobiohydrolase	194.9 ± 11.6 a	201.2 ± 7.8 a	146.5 ± 8.9 b	199.8 ± 5.3 ab	220.9 ± 11.2 a	173.7 ± 13.8 b
acid phosphatase	1455.5 ± 55.8 a	1468.2 ± 39.2 a	1063.8 ± 38.3 b	1372.8 ± 54.6 ab	1501.8 ± 13.8 a	1236.3 ± 83.4 b
β-glucosidase	226.4 ± 14.4 a	238.3 ± 5.9 a	164.1 ± 14.2 b	217.4 ± 4.0	248.9 ± 13.2	202.7 ± 23.7
chitinase	283.7 ± 13.6 a	289.1 ± 13.6 a	219.2 ± 13.2 b	270.7 ± 8.7 ab	301.2 ± 4.9 a	242.9 ± 11.9 b
xylosidase	178.8 ± 11.7 a	187.9 ± 6.9 a	129.5 ± 7.8 b	177.5 ± 2.1 ab	190.3 ± 2.4 a	150.8 ± 11.1 b
Maize (M)						
leucine-aminopeptidase	1484.6 ± 17.2 a	1448.6 ± 33.2 a	1123.2 ± 69.3 b	1415.4 ± 56.1	1506.7 ± 108.4	1260.6 ± 30.0
β-cellulobiohydrolase	203.0 ± 1.6 a	198.2 ± 6.1 a	162.9 ± 6.9 b	195.6 ± 5.6 ab	211.0 ± 12.4 a	173.3 ± 3.2 b
acid phosphatase	2021.9 ± 11.2 a	1985.9 ± 37.5 a	1641.5 ± 69.9 b	2004.5 ± 54.1 ab	2082.0 ± 119.8 a	1769.3 ± 51.4 b
β-glucosidase	243.8 ± 11.2	257.6 ± 27.1	195.5 ± 11.2	227.2 ± 8.3	263.9 ± 28.0	219.5 ± 18.6
chitinase	305.0 ± 0.6 a	294.4 ± 7.8 a	244.1 ± 6.5 b	293.0 ± 10.6 ab	302.9 ± 14.6 a	258.3 ± 6.3 b
xylosidase	180.0 ± 1.0 a	182.3 ± 7.5 a	143.6 ± 4.6 b	178.7 ± 8.7 ab	183.1 ± 9.9 a	155.8 ± 2.9 b

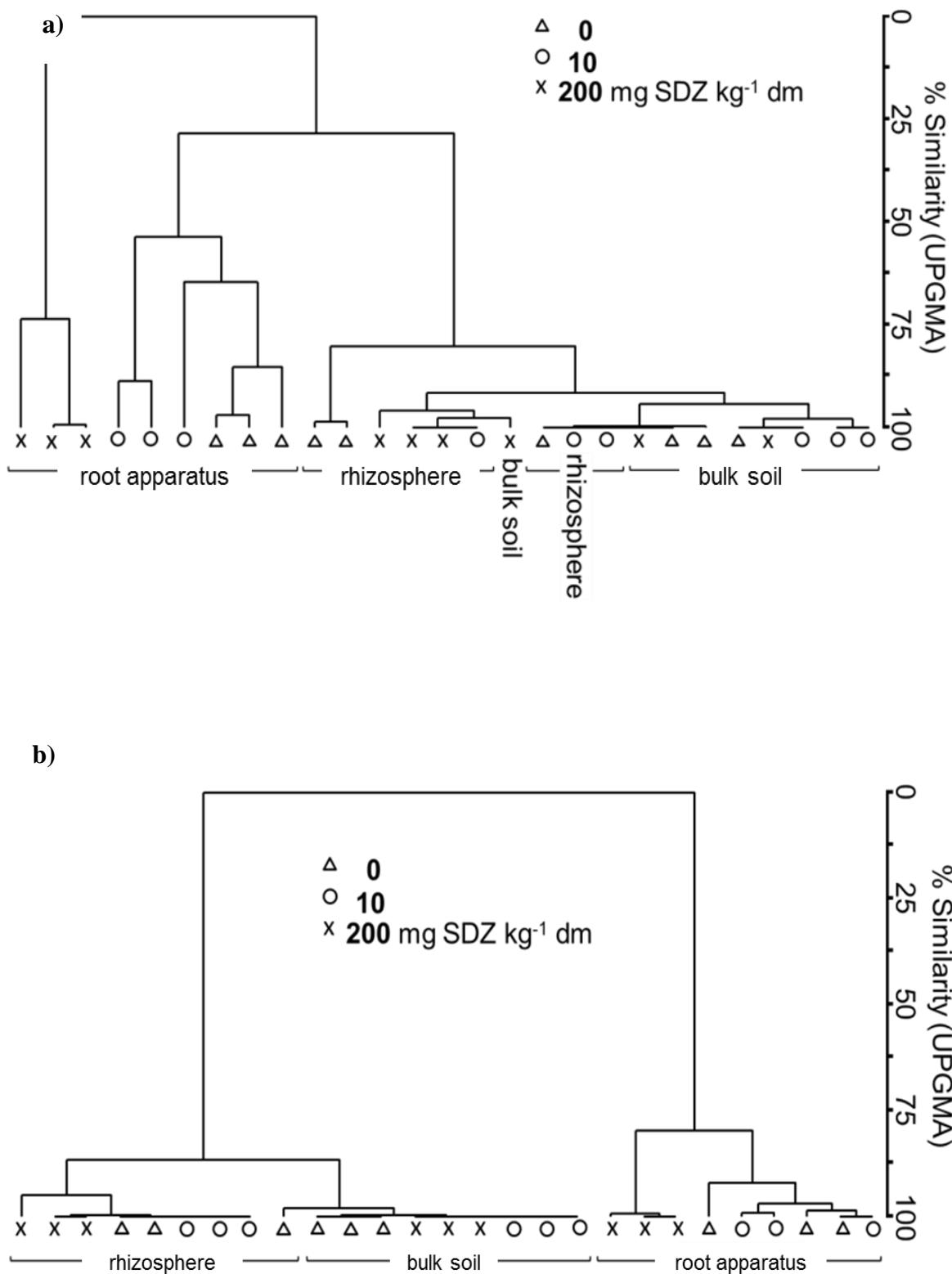


Fig. 5.2 Cluster dendograms of total bacterial community 16S rRNA gene DGGE patterns of micro-compartments, i.e. bulk soil, rhizosphere soil and root apparatus, from soil planted with maize (a) and willow (b). Total bacterial community primer set was used for PCR amplification.

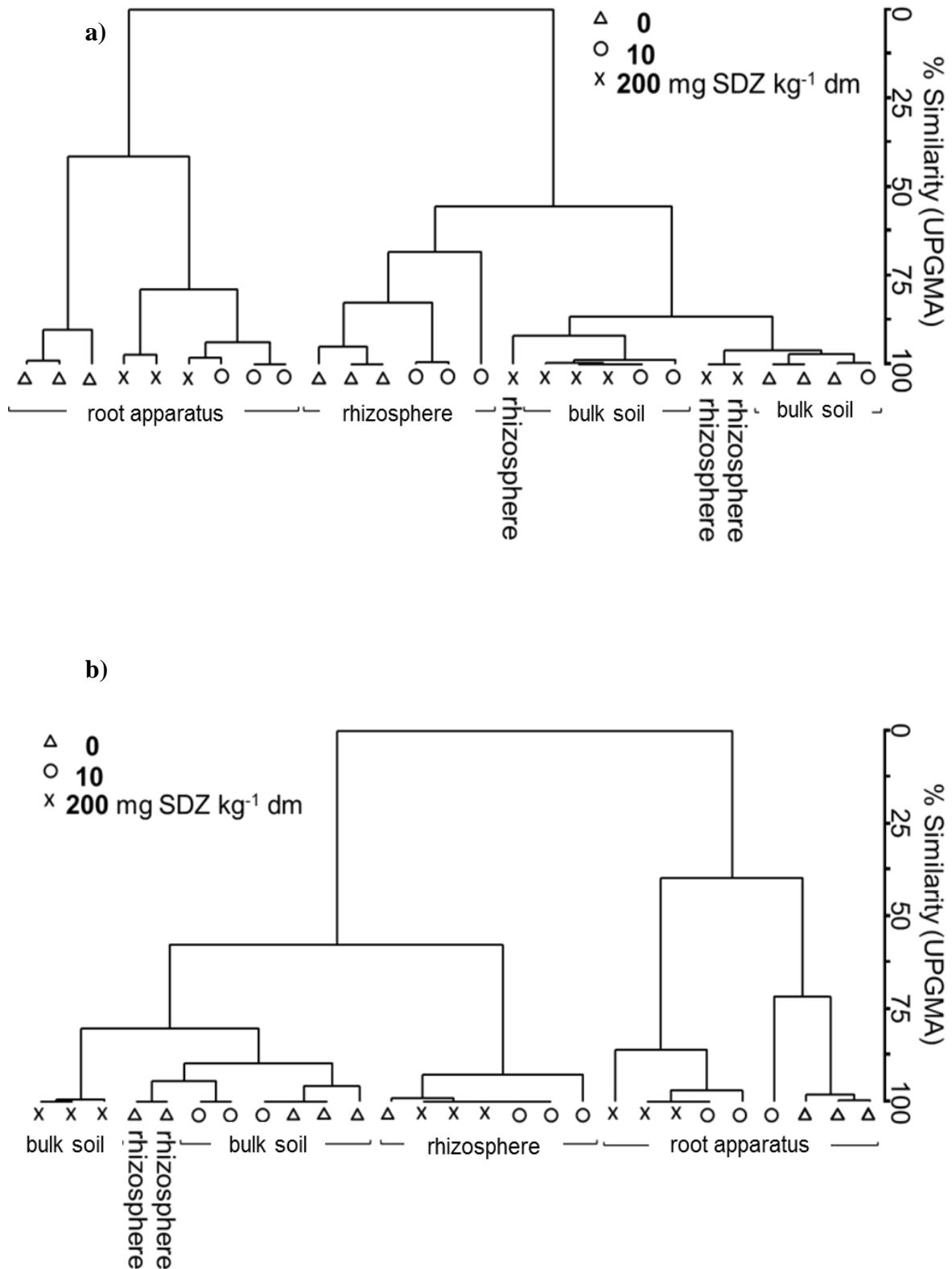


Fig. 5.3 Cluster dendograms of *Pseudomonas* specific 16S rRNA gene DGGE patterns of micro-compartments, i.e. bulk soil, rhizosphere soil and root apparatus, from soil planted with maize (a) and willow (b). *Pseudomonas* specific primer set was used for PCR amplification.

DISCUSSION

The fate of SDZ in soil and growing maize and willow plants, respectively, was previously reported (Michelini et al. 2012). Briefly summarized, the mild-solvent extractable SDZ fraction, which is operationally defined as the bioavailable fraction, decreased within 40-d pot experiments to a range from 1.2 to 16.5% of the spiking level of 10 and 200 mg kg⁻¹, respectively, in both bulk and rhizosphere soil of willow and maize plants. The SDZ concentrations were highest inside roots, where the antibiotic reached 333 and 5464 mg kg⁻¹ dm for willow exposed to 10 and 200 mg SDZ kg⁻¹ soil and 26.5 and 5331 mg kg⁻¹ dm, respectively, for maize. On the rhizoplane of willow and maize the antibiotic was detectable (600 and 699 mg SDZ kg⁻¹ dm, respectively) only at a spiking level of 200 mg kg⁻¹ soil. In both plant species SDZ altered root morphology and growth and affected the overall physiology.

It must be noted that the initial spiking levels of SDZ of 10 and 200 mg kg⁻¹, the latter meant to investigate effects potentially caused by SDZ, may appear to be higher than concentrations expected in the environment (Grote et al. 2004; Schmitt et al. 2005). However, at the spiking levels of 10 and 200 mg kg⁻¹, respectively, mild solvent-extractable mean SDZ concentrations of 0.2 and 28 mg kg⁻¹ were reached within the 40-d time period, thus approaching concentration levels found in arable field soils (Kim et al. 2011).

Enzymatic activities

The functional diversity of soil enzyme activities was dissimilar between plant species (Fig. 5.1), which is typically reported (Kourtev et al. 2003). Dissimilarity was mostly due to differences in the acid phosphatase activity as determined by principal component analysis (Supplementary material, Fig. S11). Sulfadiazine substantially affected enzyme activities of soil planted with both the plant species, leading to an intensified activity in bulk soil and especially rhizosphere soil samples spiked with 10 mg kg⁻¹ and a decreased activity in soil samples spiked with 200 mg kg⁻¹ (Table 5.1). Intensified enzyme activities due to antimicrobial action might be related to hormesis or cryptic growth (Thiele-Bruhn and Beck 2005) and are expected to go along with shifts in the microbial community structure that have been determined in this study (following paragraph). For example, ammonia oxidation was inhibited in soil spiked with 100 mg kg⁻¹ SDZ, while this function was taken over from bacteria by archaea at a lower spiking level of 10 mg kg⁻¹ (Schauss et al. 2009). However, enzymes tested here were all exoenzymes, whose activity

is not directly linked to microbial biomass and community structure (e.g., Knight and Dick 2004).

Enzyme activities of the N- and C-cycle were especially reduced, i.e. leucine-aminopeptidase (N-cycle) and xylosidase (C-cycle). Also other studies reported adverse, though in part inconclusive effects of sulfonamide antibiotics on soil enzymes. Corresponding to our findings, Kotzerke et al. (2008) reported that SDZ especially affected selected processes of N-turnover though not the nitrification. Hammesfahr et al. (2011b) and Toth et al. (2011) found, however, a reduced N-mineralization and nitrification in soil treated with SDZ and sulfadimethoxine, respectively, while ammonification was increased at a spiking level of 8.3 mg kg^{-1} . Moreover, Liu et al. (2009) noticed an inhibited phosphatase activity after addition of sulfamethazine and sulfamethoxazole sulfonamides to soil at concentrations in the range of 1 to 100 mg kg^{-1} . However, in the cited studies the antibiotic effects on microbial activities were investigated in the absence of plants and related interactions with soil. In the present work the SDZ effect on enzymes varied depending on the plant type, which is in line with findings of Ollivier et al. (2010) on SDZ effects in maize and clover planted soil.

Effects were less pronounced in rhizosphere compared to bulk soil. Obviously, plants were able to mitigate the adverse effect of the pharmaceutical antibiotic SDZ on soil enzyme activities. This might be attributed to two reasons, i.e. (i) effects on root growth and (ii) on root exudation, both going along with changes of the microbial structural diversity (following paragraph). The growth of willow and maize was affected in the presence of SDZ as reported by Michelini et al. (2012). In short, the root growth was abnormally stimulated at $10 \text{ mg SDZ kg}^{-1}$ soil, to 144% of the total root length for willow and 156% for maize compared to the control. At $200 \text{ mg SDZ kg}^{-1}$ soil, however, root growth and the whole plant performance were substantially altered and inhibited, leading to total root lengths of 61% and 16% for willow and maize, respectively.

Since 5 to 25% of all photosynthetically fixed carbon is transferred to the rhizosphere through root exudates (Marschner 1995), we assume that the increased root biomass at $10 \text{ mg SDZ kg}^{-1}$ soil, combined with a continuing photosynthetic activity (Michelini et al. 2012), would result in an enhanced root exudation of enzymes and substrates for microbial growth. Among the six enzymes, activity of β -glucosidase was most increased ($\geq 114\%$ compared to control). Adopting data from Marx et al. (2005) for enzyme kinetics parameters (V_{max} , K_m), β -glucosidase exhibits highest sensitivity to substrate concentration of all investigated enzymes. Exudates produced from vegetal organisms can

change with species and the peculiar environmental conditions experienced by the plant (Walker et al. 2003), explaining the observed plant type influence on SDZ effects. In contrast, physiological damages of both plant species at 200 mg kg⁻¹ exposure hampered C assimilation and, most likely, limited exudation from the growth-inhibited, though intact roots.

SDZ effects on the bacterial community structure

The DGGE patterns of 16S rRNA genes of TC and Ps bacteria in roots, bulk and rhizosphere soil 40 d after soil spiking with SDZ showed dissimilarities between samples and treatments (Supplementary material, Fig. S3-S8). Results revealed differences in the DGGE patterns between TC and Ps. In most cases, effects on banding patterns of TC DGGEs were less conclusive than Ps. This is in disagreement with findings of Hammesfahr et al. (2008), though, reporting that Ps strains were less affected by SDZ at up to 100 mg kg⁻¹ soil. Changes of the bacterial community structure of both TC and Ps groups increased with the SDZ spiking level. Similar findings on the dependency of soil community structure alteration to SDZ doses have been previously reported (e.g., Schmitt et al. 2006; Hammesfahr et al. 2008; Demoling et al. 2009).

As for the case of enzymatic activities, the main factor leading to the clustering of DGGE banding patterns of the different samples has been the plant type (Supplementary material, Fig. S9, S10). This finding is attributed (i) to the different composition and characteristics of the root apparatus of the two plant species and, as a peculiarity of the 40-d pot experiments, (ii) to the more developed root apparatus of maize in comparison to willow (Michelini et al. 2012). Differences between samples were further increased after soil treatment with SDZ at both spiking levels with stronger effects on sample clustering in the presence of maize compared to willow. It is known that the peculiar influences of plants on soil (i.e. amount and balance of organic compounds exuded) are strictly related to the plant species (Haichar et al. 2008). Furthermore, microbial community composition of soils and especially of rhizosphere soil depends on the plant type (Smalla et al. 2001; Compant et al. 2010). The increased hypogaeal biomass of maize plants induced by SDZ in this study could have led to more nutrients available for endophytes and to an increased release of substances from roots (Přikryl and Vančura 1980).

A further factor, causing differences in bacterial community structures was the investigated micro-compartment. The microbial community structure of both TC and Ps substantially differed between bulk soil, rhizosphere soil and the root apparatus (i.e., inner

root and rhizoplane). Both rhizoplane and rhizosphere represent micro-compartments with specific properties resulting from the influences of root uptake and exudation (Bais et al. 2006), which affect the activity, growth and differentiation of bacterial and fungal populations (Jaeger et al. 1999; Yang and Crowley 2000). Correspondingly, the different bacterial communities reacted differently on SDZ antibiotic action. The strength of cluster separation due to effects of SDZ at different spiking levels followed the sequence root apparatus > rhizosphere soil > bulk soil (Fig. 5.2, 5.3). It has been previously demonstrated that antibiotics exert direct effects on the abundance and diversity of soil microorganisms and shifts from bacteria to fungi, bacteria to archaea or within bacterial groups have been reported (e.g., Zielezny et al. 2006; Hammesfahr et al. 2008; Schauss et al. 2009; Gutiérrez et al. 2010). A prerequisite, however, is the activation of soil microbial growth through the addition of a nutrient substrate (Schmitt et al. 2005; Thiele-Bruhn and Beck 2005; Demoling et al. 2009). Since root exudates and other rhizodeposits constitute a major source of organic carbon in soil (Kuzyakov and Domansky 2000), it becomes plausible that effects were strongest at the rhizoplane (root apparatus) and declined with increasing distance from the root. There is evidence that antibiotic induced changes of the soil microbial community structure go along with an increase in the antibiotic resistance level (Heuer et al. 2011). Since the rhizosphere hosts a number of opportunistic human pathogenic bacteria (Berg et al. 2005) and the share in antibiotic resistome of soil bacteria and human pathogens has been demonstrated (Forsberg et al. 2012), it is concluded that these increased adverse effects on rhizosphere and rhizoplane microbial communities might aggravate the risk of human and/or livestock exposure to soil-borne resistance.

Furthermore, this study reveals that direct antibiotic effects on the bacterial community structure and indirect effects through antibiotic induced alterations of the plants metabolism and morphology (Michelini et al. 2012) interact. This corresponds with findings of Hammesfahr et al. (2011a), who reported similar interactions between adverse antibiotic effects and manure fertilization. The SDZ dose-dependent effect, more pronounced in proximity to roots, was likely magnified by the root damages observed after the 200 mg SDZ kg⁻¹ treatment (Michelini et al. 2012). Root stresses, whether chemical or physical, influence the quantity of organic substances exuded (Rovira 1969). Comparing results from enzyme activities and the microbial community structure, it is striking that the functional diversity resulted more affected in bulk soil, whereas the structural diversity was more affected in the rhizosphere and especially at the root

apparatus. It is interpreted that the stronger shift of the faster growing community in the rhizosphere resulted in a stronger take-over of microbial functions by other microbial groups, which has been previously reported as functional redundancy (Heemsbergen et al. 2004). This was proven for SDZ when ammonium oxidation was shifted from bacteria to archaea (Schauss et al. 2009). On the other hand, antibiotic effects on enzyme functions in bulk soil cannot be easily compensated in the presence of a microbial community that is hardly growing due to a lack of nutrient substrate (Jenkinson and Ladd 1981).

To conclude, in this work it was demonstrated that SDZ can differently alter the activity and community structure of important microbial groups present in bulk and rhizosphere soil and root apparatus. In particular, the structural diversity of soil bacteria and especially *Pseudomonas* strains (16S rRNA gene banding pattern) was more sensitive to SDZ input to soil than the functional diversity (enzymatic activities). The latter were more pronounced in nutrient-limited bulk soil, while SDZ-induced effects on the microbial community structure were more pronounced in proximity of the roots (i.e., root apparatus and rhizosphere) than in bulk soil. This is related to direct antibiotic effects on the microbial community that is faster growing in the nutrient rich rhizosphere, and is thus more susceptible to the action of biostatic SDZ, and to indirect effects through adverse changes of the plant morphology and growth, and thus of the release of rhizodeposits to the soil. Antibiotic induced changes of the soil microbial community structure may go along with changes in the resistance level. When this happens in contact with crop plants, the risk of human and livestock exposure to soil-borne resistance through the food chain further increases.

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CHAPTER VI

Structural and functional alterations induced by sulfadimethoxine and sulfamethazine on *Hordeum vulgare* L. plants grown in nutrient solution

The experimental works described in this chapter are part of the following scientific manuscript: Michelini L., La Rocca N., Rascio N., Ghisi R. (2012) Structural and functional alterations induced by two sulfonamide antibiotics on barley plants. Plant Physiology and Biochemistry. Submitted the 12th of November 2012- currently under review

PREFACE

Considering previous results on the root architecture obtained after plant exposure to various SAs (Michelini 2012a; 2012b), in the present study the effects of two antibiotics on growth parameters, morphological structure and membrane integrity of barley roots were investigated, with the aim of scrutinizing more in depth the influences of veterinary antibiotics on plant development. For this reason, plants were grown in a nutrient solution containing 40 µM of sulfadimethoxine (SDM) and sulfamethazine (SZ), two SAs extensively exploited for routine mass treatment of farm animals (De Liguoro et al 2007; 2009). The dose 40 µM was chosen as it falls in the range of the SA concentrations, previously mentioned, meanly found in arable lands and manure. In addition, to have a wider picture of the effects of SAs on barley plants, the structural organizations, as well as the photosynthetic pigment levels, were compared in leaves of control plants and plants treated with the two contaminants.

Abbreviations: SA: sulfonamide; SDM: sulfadimethoxine; SZ: sulfadiazine; fm: fresh mass

MATERIALS AND METHODS

Plant material

Barley seeds (*Hordeum vulgare* L. cv. Express) were washed several times in tap water and germinated on moistened paper in the dark for 4 d. After seed germination, seedlings (approximately 3 cm roots and 3 cm shoots) were placed in pots containing 500 ml of 25 times diluted Hoagland's nutrient solution alone (Arnon and Hoagland 1940) and nutrient solution containing 40 µM (meanly 11500 µg l⁻¹) of two SAs. Active molecules used for the experiment were as follows: SDM (\geq 98.5 %, CAS: 122-11-2, Sigma-Aldrich, Milan, Italy) and SZ sodium salt (\geq 98 %, CAS: 1981-58-4, Sigma-Aldrich, Milan, Italy). Plantlets were then grown under a light/dark cycle of 12/12 h, at 23/20 °C temperature, 60–70% relative humidity and 400 µmol photons m⁻² s⁻¹ (PPFD). The solution was continuously aerated and renewed twice per week to prevent active molecule and nutrient depletion. Time exposure was 15 d. Three independent replicates per treatment were used, each of 10 barley plants.

Plant growth

During the whole experimental period, total root length and total area were measured to assess growth dynamics until the end of the exposure. For this aim a scanner-based image analysis system (WINRHIZO Pro, Regent Instruments, QC, Canada) was used. Furthermore, the total plant biomass was measured for each plant during the experiment. After plant harvesting, barley roots and shoots from each treatment were weighted to determine the fresh mass (fm). The final root apparatus architecture of plants exposed to the different treatments was documented through digital photos (Nikon D1X, 60 macro, Nikon, Tokyo, Japan).

Chlorophyll and carotenoid determination

At the end of the 15 d treatment, photosynthetic pigments were determined. To this purpose the central part of first leaves was sampled and pigments were extracted with dimethylformamide (CAS: 68-12-2, PROLABO, Paris, France) to a ratio of 1:100 (w:v). Chlorophylls and carotenoids were analyzed spectrophotometrically (PerkinElmer, Lambda 11, UV/VIS, Wellesley, MA, USA) and their concentrations were calculated using the extinction coefficients described by Wellburn (1994). Leaves from eight different plants were analyzed for each treatment.

Light microscopy

In order to assess the morphogenetic features after 15 d exposure to the two SAs, cross-sections of 3 roots (at 0.5 cm and 1.5 cm behind the root tips) and leaves (central part of the first leaf) were carried out. Tissue samples were fixed overnight at 4 °C in 6% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.9) and postfixed for 2 h in 1% osmium tetroxide in the same buffer. The specimens were dehydrated in a graded series of ethyl alcohol and propylene oxide and embedded in araldite. Thin sections (1µm) were obtained with an ultramicrotome (Ultracut, Reichert-Jung, Vienna, Austria), stained with equal volumes of 1% toluidine blue and 1% sodium tetraborate and observed under a light microscope (DMR5000, Leica, Wetzlar, Germany). Images were taken using a digital camera (DFC425C, Leica, Wetzlar, Germany).

Membrane leakage and potassium analysis

In order to evaluate the potential damage to membrane integrity exerted by SDM or SZ, another set of barley plants was cultivated and treated as previously described. After 15 d

of exposure to SAs, the root apparatus of each plant was rinsed several times with ultrapure water and then immersed in 500 ml ultrapure water at room temperature. The electrolytes released by roots were measured according to Huang et al. (2005). The electrical conductivity (EC) of the liquid medium was measured during 27 h, starting from the root immersion, using a conductivity meter HI 8333 (HANNA Instruments, Padova, Italy) for three pots each containing ten barley plantlets per treatment group. After 27 h roots were excised, gently dried and weighed. Membrane leakage was calculated as the increase of EC per unit fresh mass of root ($\mu\text{S cm}^{-1} \text{ g}^{-1} \text{ fm}$). Moreover, total K^+ concentration ($\mu\text{g l}^{-1}$) in the liquid medium was determined at the 27th h in all the samples through inductively coupled plasma atomic emission spectroscopy (ICP-AES, Spectrum CirosCCD, Kleve, Germany).

Statistical analyses

Open source software (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 (ANOVA) followed by Tukey's Honestly Significant Differences test for comparisons. Potassium root release was measured among samples through a non-parametric Kruskal Wallis test and a post-hoc Nemenyi-Damico-Wolfe-Dunn test ($p < 0.06$).

RESULTS AND DISCUSSION

Roots

Total biomass. The total plant biomass (Fig. 6.1a) of both control plants and plants selected for the treatment with the two SAs revealed values of approximately 0.2 g fm each before starting the experiment. Even during the period of SA exposure the total biomass of the three treatment groups showed trends towards constant growth. Masses of the samples treated with SDM and SZ showed a slight decrease compared to controls at the second analysis point, at 8 d after the beginning of the exposure and the decrease persisted through the whole period of treatment. However, these differences were not statistically significant. Moreover, the biomass of the epigeal apparatus of barley plants was not different among the three groups (approximately 0.45 g per plant at the end of the experiment), confirming that also the root biomass was comparable. Higher SDM concentrations, from 50 to 450 mg l⁻¹, induced strong inhibitions of the fresh mass

production of *Azolla filiculoides* Lam., *Lemna minor* L. and *Pistia stratiotes* L. grown in a nutrient solution (Forni et al. 2006).

Even if the SA treatment did not affect the total biomass of barley roots, important alterations of the whole root apparatus structure were found, with shorter primary root formation (Fig. 6.1b) and a less developed root area (Fig. 6.1c) in comparison to control plants. This phenomenon was visible in plants exposed to both the pollutants, with a much more pronounced effect after SZ than SDM exposure. In particular, looking at the total root lengths (i.e., primary and secondary roots together) (Fig. 6.1b), significant differences ($p < 0.05$) appeared at the 8 d from the beginning of the exposure and, at the end of the experimental time, plants exposed to SDM and SZ showed a total root length equal to 76% and 49% of the control group, respectively. Similarly, the root area values (Fig. 6.1c) were roughly consistent with those concerning the total lengths, showing a significant ($p < 0.05$) and much more visible inhibitory effect exercised by SZ. Therefore, it can be deduced from these results that the two SAs employed are able to cause a decrease in the root elongation rate, with a less compromising effect generated by SDM than by SZ.

Root elongation is often considered to be a primary effect measure in plant toxicity tests because the roots are the direct point of contact with the toxic medium and contaminants may enter the plant through them (Kapustka 1997). Evidence highlighted by the present study supports the use of root development analysis in surveying the plant response to pollutants in short-duration experiments (Kapustka 1997; Hillis et al. 2011).

Among the more evident SA effects on the plants used in this study there is the pollutant action on the overall root architecture. In fact, after 15 d from the beginning of the experiment, control plants showed very long and thin primary roots, while lateral ones were visible about 10 cm behind the root tips (Fig. 6.2a). Conversely, SDM and SZ treatments led to much shorter primary roots and numerous lateral ones a few mm from the apexes (Fig. 6.2b, c). Shorter roots were also noticed in *Lemna minor* L. plants exposed for 5 weeks to a flumequin antibiotic at concentrations from 50 to 1000 $\mu\text{g l}^{-1}$ (Cascone et al. 2004). The serious consequence on root elongation induced by both SZ and SDM might be explained by the inhibitory effect of these compounds on folic acid synthesis, a vitamin essential for many metabolic processes, including the biosynthesis of purine and pyrimidine bases of DNA and RNA (Stokstad and Jukes 1987). However, the shortening of the primary roots and the concomitant development of a large number of

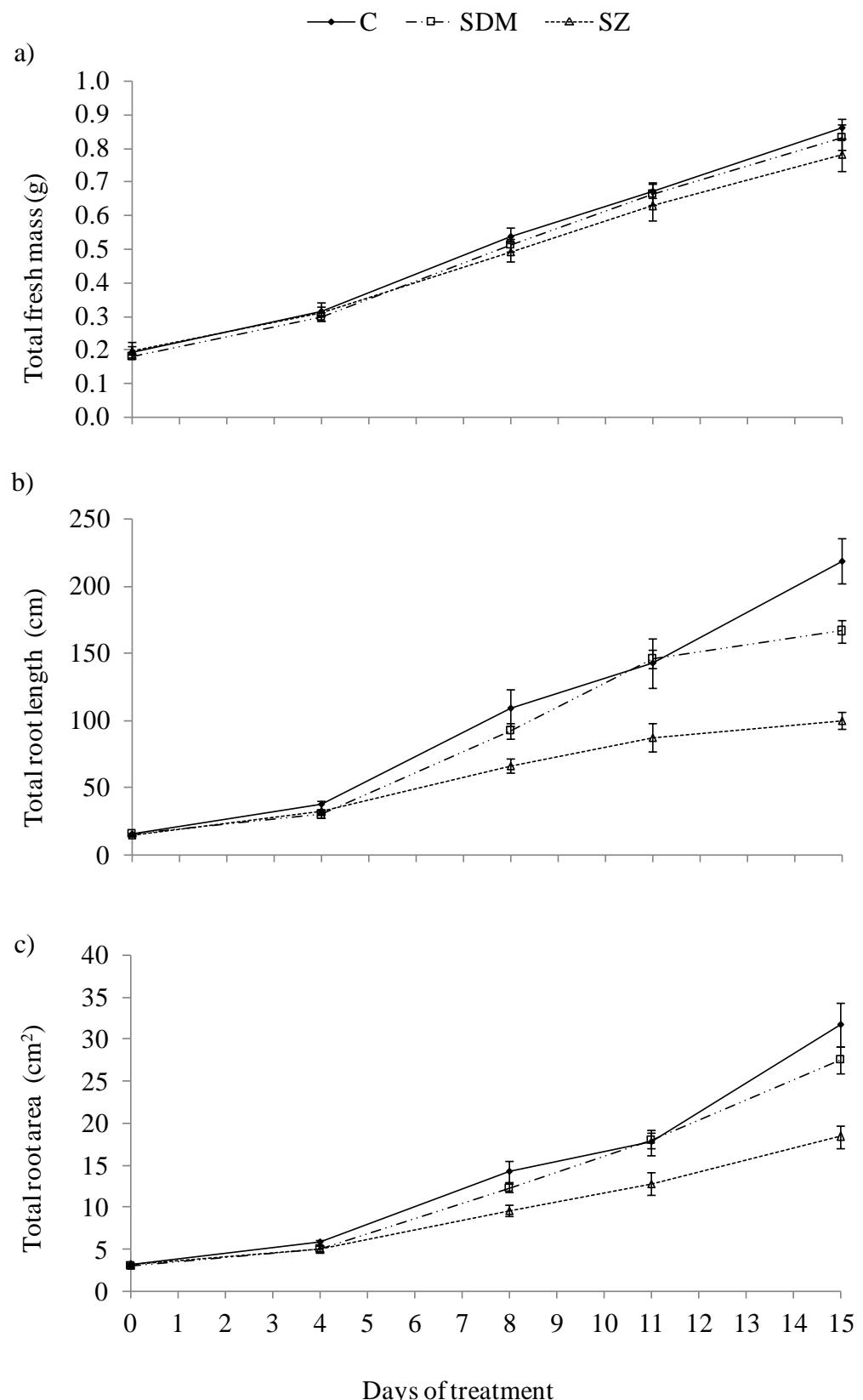


Fig. 6.1 Total fresh mass (a), total root length (b) and total root area (c) of barley plants exposed to 0 (C) or 40 μM of SDM or SZ at different times during the treatment period.

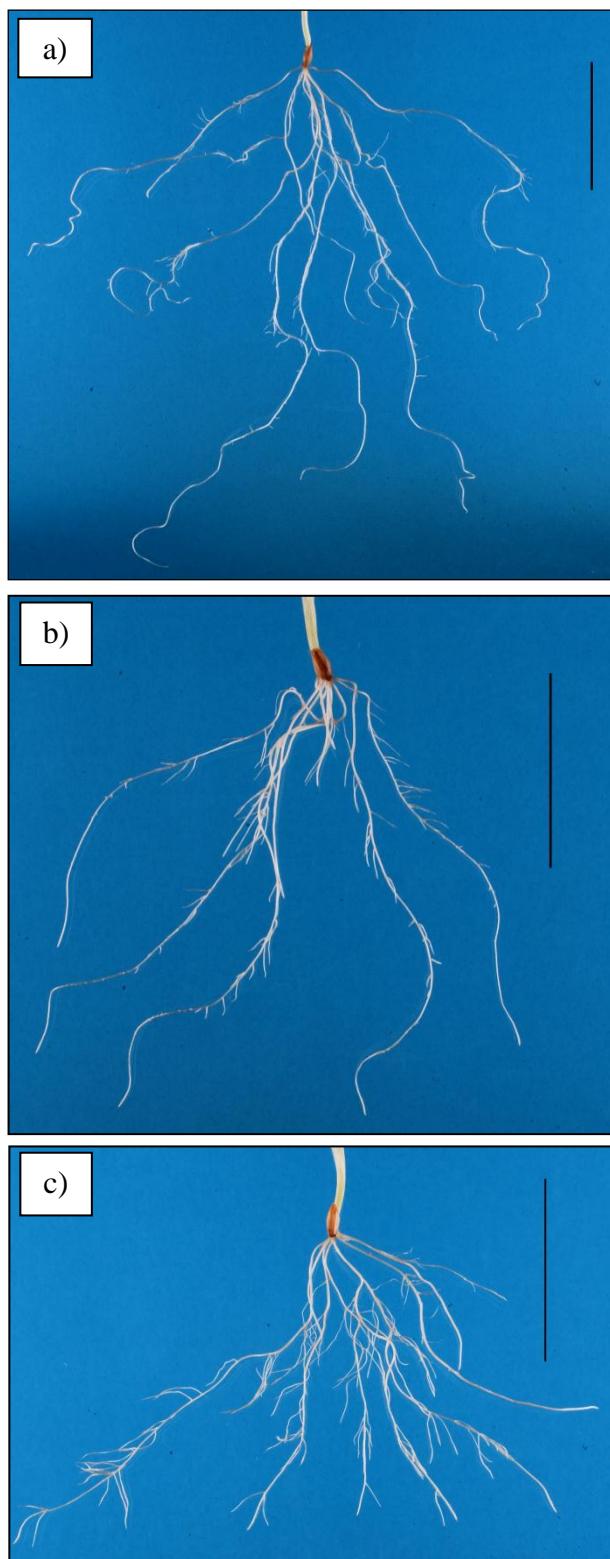


Fig. 6.2 Root apparatus morphology of control (a), SDM (b) and SZ (c) treated barley plants. The black lines correspond to 5 cm.

lateral roots in treated plants suggests that the two SAs, rather than inhibit the root growth, whose total biomass remains comparable with that of control plants, are able to affect the whole morphogenetic pattern of the root apparatus. According to Migliore et al. (1995) and Sartorius et al. (2009), the implication of such an effect could directly or indirectly involve the synthesis of plant hormones.

Inner root organization. In order to highlight differences in the development of primary roots due to the antibiotic addition to the nutrient solution, control and treated plants were compared through light microscopy analysis of thin cross sections cut from 0.5 and 1.5 cm behind the root tip.

At 0.5 cm behind the root tips control samples were at the beginning of their differentiation process (Fig. 6.3a, b). In fact, only few protoxylematic vessels, characterized by scarcely thickened cell walls could be seen in the central stele. Moreover, no root hairs are visible in the sections.

A more advanced stage of differentiation was found in SA treated barley plants. Roots exposed to SDM showed a larger central stele with a greater cell number and xylem vessels with more thickened cell walls (Fig. 6.3c, d). Roots coming from plants treated with SZ exhibited a degree of differentiation even greater than that found in the SDM treated ones (Fig. 6.3e, f). Around the central stele, with numerous thick-walled xylem vessels, a differentiated endodermis can already be observed, as well as sections of numerous root hairs, which are formed by specialized epidermal cells.

The dissimilar degree of root differentiation was even more evident in plants sampled at 1.5 cm behind the root tips. The central stele of control plants was surrounded by a differentiating endodermis (Fig. 6.4a), while in plants treated with both SAs a well-developed endodermis was present around the more expanded steles (Fig. 6.4b, c). Moreover, a higher number of xylematic vessels was detected in sample of SZ treated plants (Fig. 6.4c), in comparison to a lower number found in the SDM treated plants (Fig. 6.4b) and even lower in the control ones (Fig. 6.4a). Finally, in roots exposed to SZ the initiation of lateral roots coming out from the pericycle can be seen (Fig. 6.4c). All this shows that in SDM and even more in SZ treated plants the root differentiation is faster, visibly approaching the root apex.

The advanced stage of root differentiation in SA treated plants is also confirmed by the evident thickening of the lignified walls of xylematic vessels. As for SAs, other kind of abiotic agents were seen to cause root growth alterations and increased lignification. For

example, in Scots pine Cd accelerates differentiation and xylogenesis without causing injury to root tip (Schützendübel et al. 2001). Moreover it was noticed that Cd stimulated a premature root development in barley, involving xylogenesis and root hair formation. This was correlated with a shortening of the root elongation zone and, therefore, with root growth reduction (Ďurčeková et al. 2007). Considering our biometrical and structural results, it is presumed that the inhibitory effect of the two active molecules on the root, namely length shortening, was due to an early achievement of the final stage of development. This involves a shortening of the division phase of the root growth. In fact, it is well known that above the apical meristem there is the so-called "postmitotic zone" (Baluska et al. 1996), whose cells undergo a "preparatory phase", critical for the subsequent growth of the root. Alterations and reductions of this area may interfere with root growth leading to early differentiation of the radical structures. In our case this might be due to SA interactions with folic acid synthesis, as previously hypothesized. Similar effects on root morphogenesis and its growth were also found in rice plants exposed to heavy metal stress conditions (Rascio et al. 2008) and in onion plants treated with metabolic inhibitors (De Tullio et al. 1999).

Root thickening and initiations of lateral roots closer to the root tips were also recorded in plants exposed to Al (even in this case interactions with DNA expression were supposed to be at the origin of the structural modifications) (Čiamporová 2002). Moreover, in previous studies (Michelini et al. 2012a; 2012b) it was shown that two SAs (sulfadimethoxine and sulfadiazine) were able to considerably alter the root morphology of willow (*Salix fragilis* L.), stimulating the development of an abnormal number of lateral roots.

Lux et al. (2011) stated that the accelerated root differentiation leading to the earlier maturation of the endodermis in response to Cd exposure is of functional significance in protecting the shoot from excessive heavy metal loads. Accordingly, the faster root development and endodermis differentiation observed in SA treated barley plants could be interpreted as acclimation responses that reduce the apoplastic movement of SAs to the xylem, giving rise to a sort of barrier to the movement of SAs towards the shoot. It was demonstrated several times that the content of these compounds is much lower in the aerial parts of the plants than in the roots (Migliore et al. 1995; Michelini et al. 2012a; 2012b). In addition, the earlier differentiation of the root tissues in SA treated plants, besides advancing the gain of primary structure shown by the root hair appearance, also

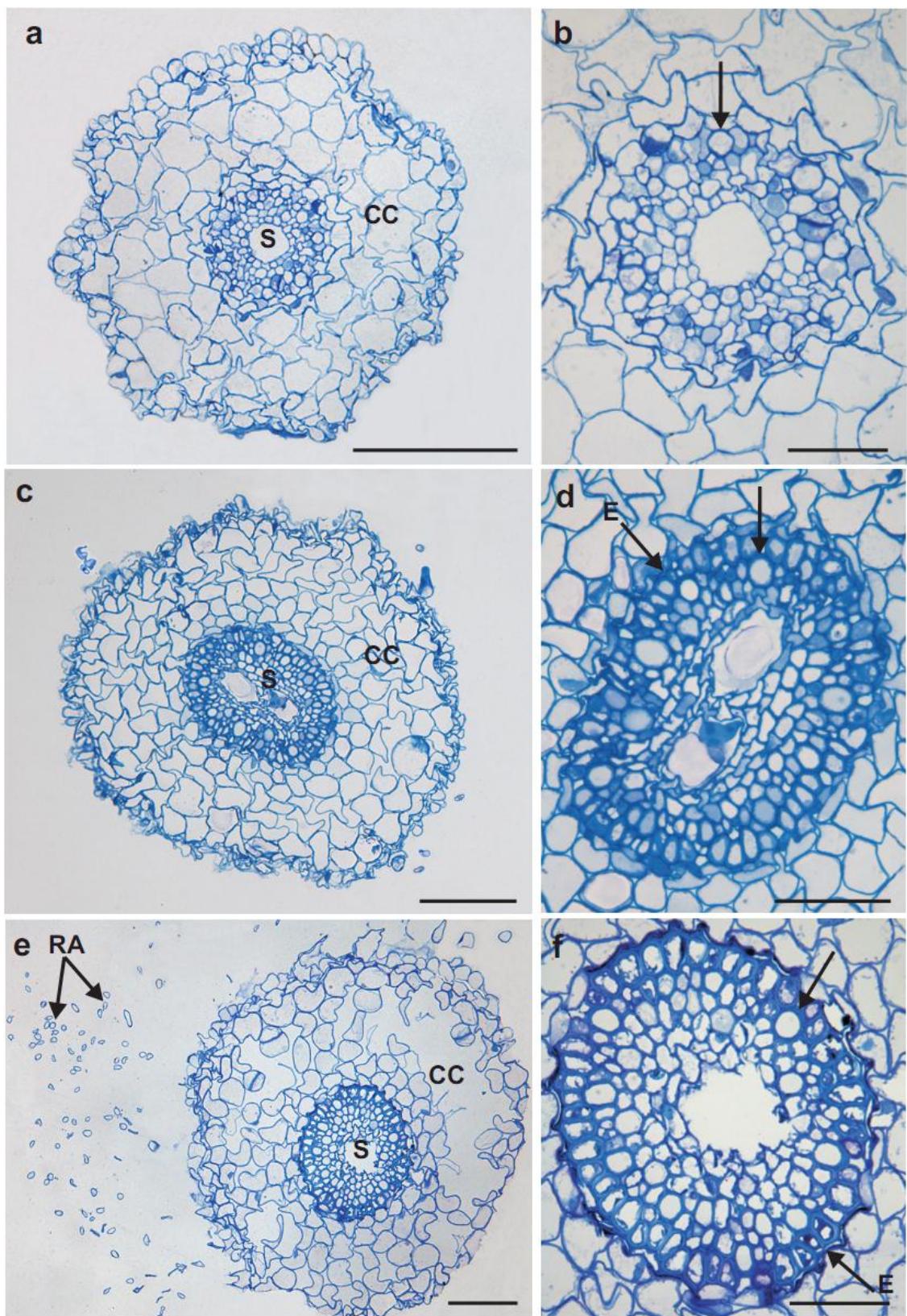


Fig. 6.3 Light microscope micrographs of thin transversal sections from barley roots taken 0.5 cm behind the tip: (a, b) root from a control plant, (c, d) root from a SDM treated plant, (e, f) root from a SZ treated plant. (S: stele, RA: root hairs; E: endodermis, CC: cortex cells, arrows: protoxylematic vessels). (a, c, e: bars = 100 µm; b, d, f: bars = 50 µm).

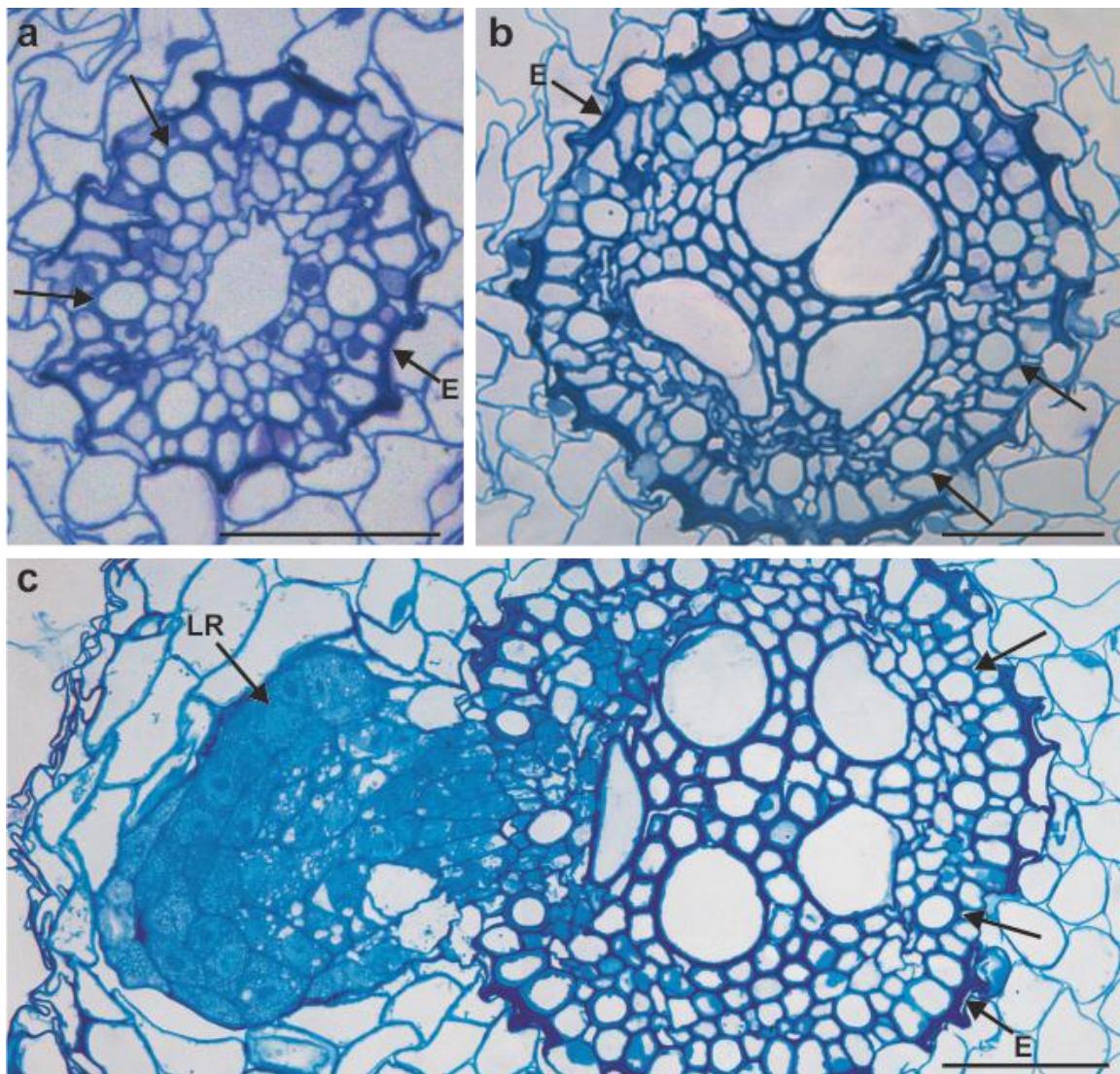


Fig. 6.4 Light microscope micrographs of thin transversal sections from barley roots taken 1.5 cm behind the tip: (a) root from a control plant, (b) root from a SDM treated plant, (c) root from a SZ treated plant. (E: endodermis, LR: lateral root, arrows: protoxylematic vessels). (Bars = 50 µm)

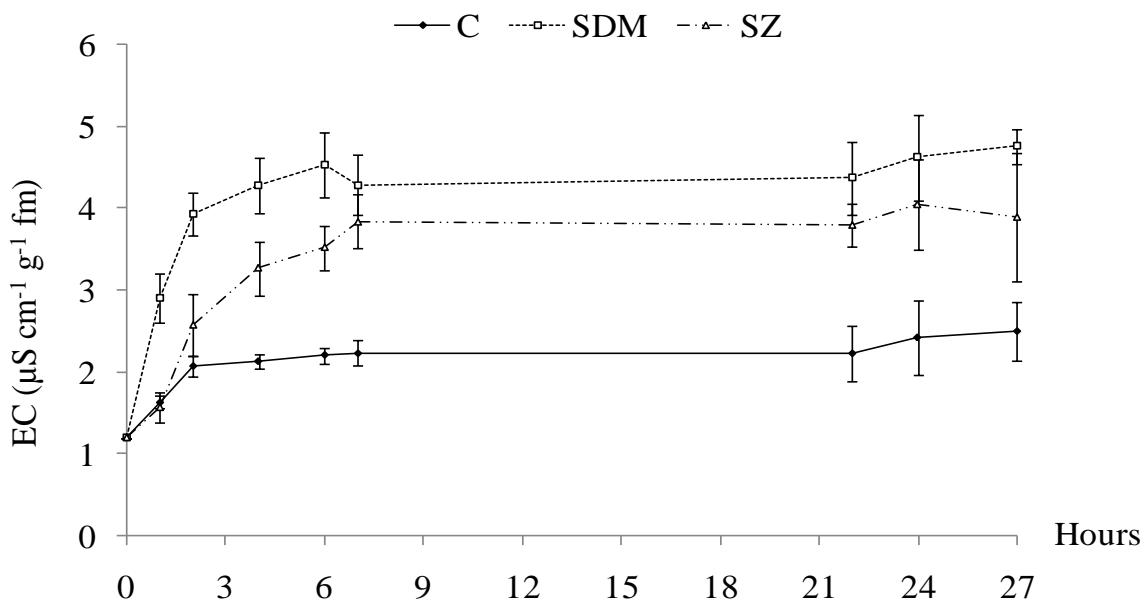
anticipates the consequent disruption of rhizodermis, and its replacement on barley root surface with the underlying exodermis (Gierth et al. 1999). This event may have an additional acclimation function in SA exposed plants since the mature exodermis, with suberized cell walls, gives rise to an apoplastic barrier at the root surface (Enstone et al. 2002), thus reducing the extension of root region involved in nutrient as well as in pollutant uptake from the soil.

Electrolyte leakage and K⁺ analysis. Electrolyte leakage is commonly used as an indicator of stress injury or senescence (Liu and Huang 2000). The amount of electrolyte

leakage from root tissues of control and SA treated barley plants is given in Fig. 6.5. Starting from the first experimental hour, both the SAs induced a significant increase ($p < 0.05$) in the release of electrolytes by roots compared to untreated plants. In particular, after 27 h the monitored electrical conductivity of the aqueous solution in which roots were immersed was 92% higher than control for SDM treated plants and 56% for SZ treated ones.

These results were supported by K^+ concentrations measured in the aqueous solution after 27 h of root immersion (Fig. 6.5). The K^+ concentrations in the medium of control plants were found to be much lower than those of both the SA-treated plant media ($p < 0.06$), with values that were nearly halved.

This increased ion leakage suggests a loss of membrane integrity, which might be due to SA insertion inside them. The $\log K_{ow}$ values of SDM and SZ, a parameter which indicates the tendency of a molecule to bioaccumulate, thus 1.63 for SDM and 0.89 for SZ (Díaz-Cruz et al. 2006), suggest that the two compounds have an affinity for the lipidic phase of cell membranes. Such an assumption leads to the hypothesis of an accumulation of SAs with a consequent destabilization of the normal structure and function of membranes. An increase in electrolyte leakage from rice shoot, probably due to membrane lipid peroxidation, was seen following exposure to polycyclic aromatic hydrocarbons (Li et al. 2008). Moreover, damage to plasma membranes was already reported as a result of exposure to organic compounds such as herbicides (Duke and Kenyon 1993). Accordingly, from a recent work (Michelini et al. 2012b) a disturbance was hypothesized in the formation and/or regulation of lipid membrane components following the treatment of maize and willow plants with sulfadiazine, a sulfonamide antibiotic. Whatever the mechanism responsible for these effects on membranes is, it is clear that SA-induced cellular leakage can lead to a failure in root functions, such as water transport and ion supply (Huang et al. 2005), thereby compromising plant productivity.



	1h	2h	4h	6h	7h	22h	24h	27h	K ⁺ (µg l ⁻¹)
C	b	b	b	b	b	b	b	b	66 ± 10 a
SDM	a	a	a	a	a	a	a	a	114 ± 10 b
SZ	b	b	ab	a	a	ab	ab	ab	127 ± 40 b

Fig. 6.5 Electrical conductivity ($\mu\text{S cm}^{-1} \text{g}^{-1} \text{fm}$) of aqueous media of control (C), SDM and SZ treated barley plants measured after different periods of root immersion in ultrapure water. Different letters, in the table below, indicate statistical differences respectively for hourly EC values ($p < 0.05$) and for K⁺ concentrations ($p < 0.06$) in the aqueous media, the latter analyzed at the end of the 27 h period.

Leaves

Photosynthetic pigments and leaf structure. To evaluate possible alterations induced by the two drugs at the level of photosynthetic apparatus, the content of chlorophylls and carotenoids and the ratio chlorophyll *a*/chlorophyll *b* were determined after 15 d of SA exposure.

The amounts of chlorophyll *a*, chlorophyll *b* and their ratio, as well as the carotenoid contents were found to be not much different among the three kinds of plants (Fig. 6.6). In particular, control and SZ treated plants had quite comparable values. Conversely, plants exposed to SDM showed slightly but significantly lower values ($p < 0.05$), with a drop of 17% in total chlorophyll and 16% in total carotenoid amounts, compared to

control plants. Such data indicate a higher toxicity of SDM against the photosynthetic apparatus, possibly imputable to its higher affinity for the cell membranes. It is well known that chloroplasts are the major target of biotic and abiotic stresses and that the pigment decrease is the first signal of some photosynthetic organelle damage (Mostowska 1997). Thus the slight reduction of chlorophyll and carotenoid amounts found in leaves suggests that the photosynthetic apparatus of barley plants was not noticeably altered by the presence of these drugs in the growth medium. A study performed with *Lemna minor* L. plants exposed for 5 weeks to flumequin antibiotic did not highlight evident changes in the final amount of chlorophyll *a* following 50, 100, 500 and 1000 µg l⁻¹ treatment, while chlorophyll *b* content in treated plants decreased noticeably (Cascone et al. 2004).

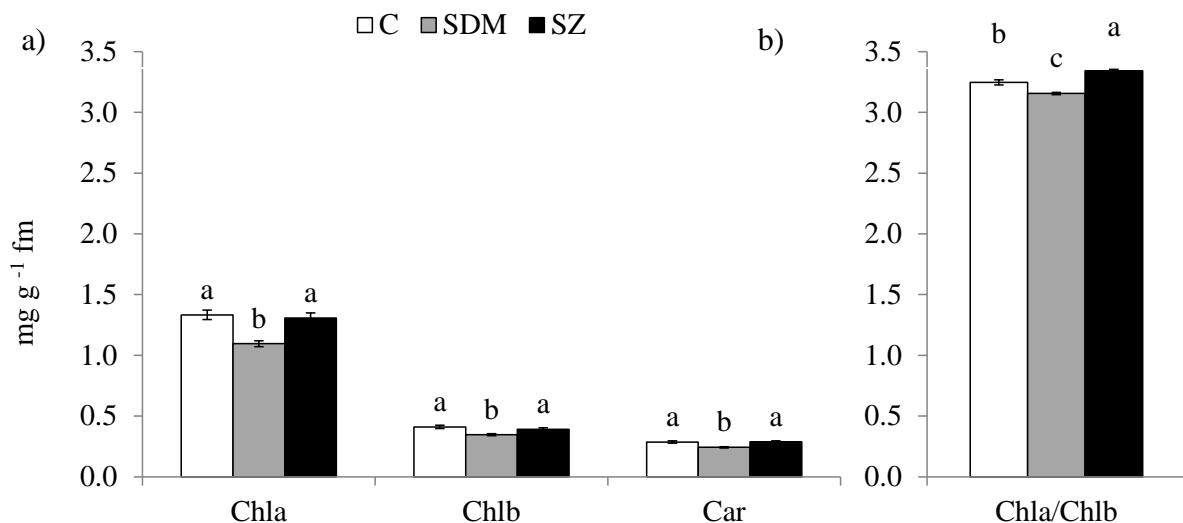


Fig. 6.6 Photosynthetic pigments from leaves of control (C), SDM and SZ treated barley plants. (a) Chl *a*: Chlorophyll *a*; Chl *b*: chlorophyll *b*; Car: total carotenoids; (b) Chlorophyll *a*/Chlorophyll *b*: Chla/Chlb. Different letters indicate significant difference ($p < 0.05$).

In order to check the status of leaf tissues from a morphological point of view, leaves from control and SA treated plants were also compared through a light microscopy analysis of thin cross sections. As it can be seen from micrographs (Fig. 6.7a-c), the inner organization of the leaves was found to be similar in plants grown in all the three experimental conditions, with numerous chloroplasts in the mesophyll cells, without evident differences between the control and the SA treated groups.

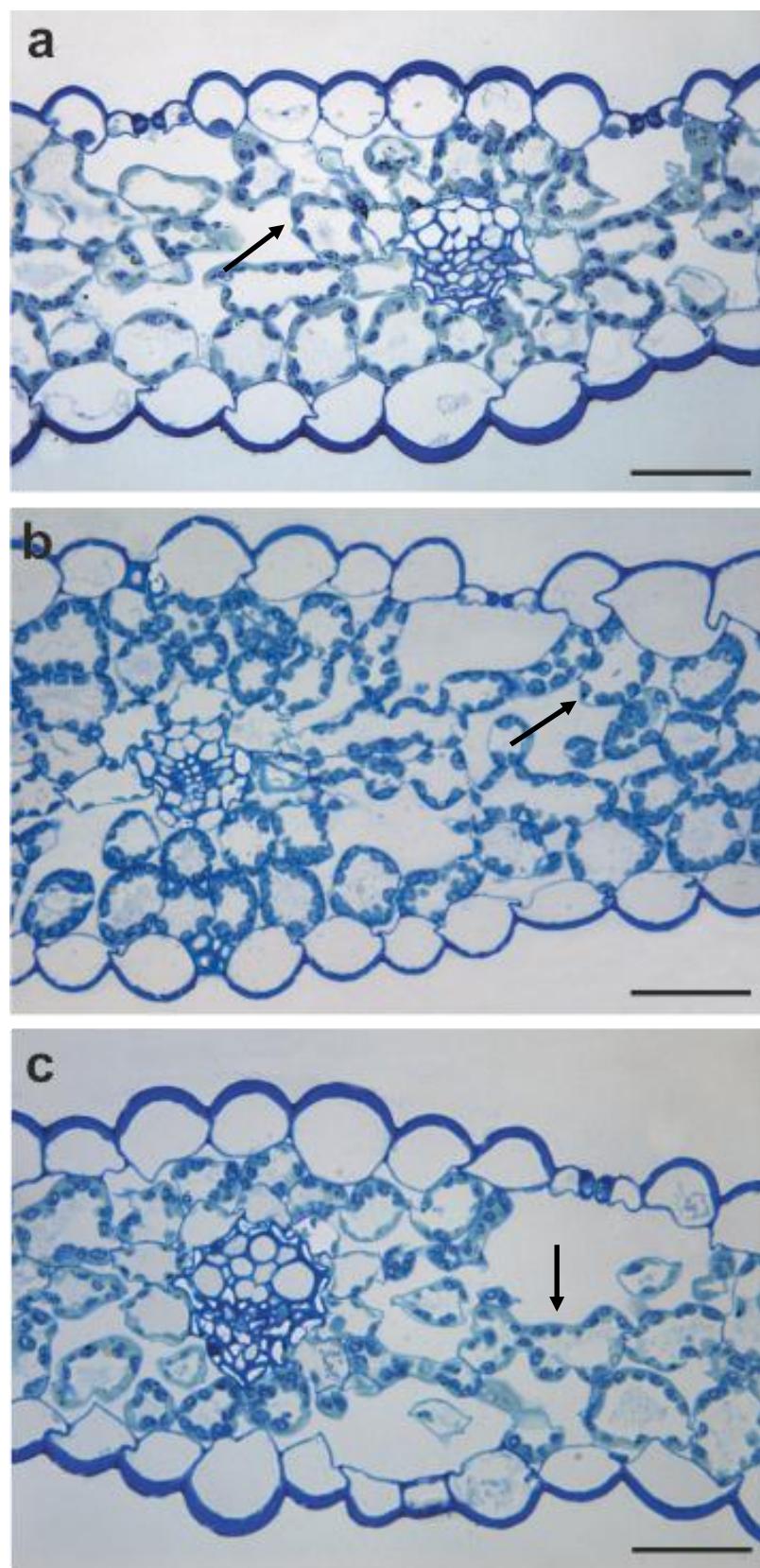


Fig. 6.7 Light microscope micrographs of thin transversal sections of leaves from control (a), SDM (b) and SZ (c) treated barley plants. Arrows: chloroplasts. (Bars = 100 μm).

The missing or scanty response of barley leaves to SA treatment might be explained with the fact that their tissues are not directly exposed to pollutants supplied in the growth media. The aerial organs, in fact, suffer only the effects of contaminants moved to the shoots from the roots, where a high percentage of them can be retained (Gent et al. 2007; Rascio et al. 2008; Isleyen et al. 2012).

However, it cannot be excluded that higher concentrations of SAs and/or longer experimental times might cause evident damages also to the leaves.

As a whole, from results presented in this study, it is possible to conclude that i) over a short period, such as two weeks, the two active molecules act primarily at a root level, as the shoot apparatus shows no important signs of SA-induced stress, and ii) not only the root architecture and inner structure were affected, but even the root function was compromised by the two antibiotics.

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CHAPTER VII

General conclusions

Conclusions

In the past few years, there have been frequent detections of antibacterial agents in the environment. To better investigate the relations of such organic molecules with plants, rhizosphere and soil related microorganisms, different experiments were carried out under laboratory and greenhouse conditions. Results showed that:

- 1) considering the possible accumulation of sulfonamides into and/or on root apparatus of plants and the possible aptitude to phytoremediate such contaminants, outcomes of the performed research highlight differences depending on both the active molecule and the plant species tested.

In particular, from a study carried out with SDZ as the active molecule, the accumulation was found to be higher for the woody plant, *Salix fragilis* L., compared to that of the crop, *Zea mays* L., both grown on a soil matrix. For the two plant species, accumulation was found to be dominant in the root apparatus (spiking doses 10 and 200 mg kg⁻¹), even if a small part of the antibiotic moved to stems and leaves (spiking dose 200 mg kg⁻¹). Antibiotic retention in roots could be due (i) to derivate formation and/or to protein binding, thereby allowing the plant to stabilize the organic compound in the root cortex to limit its flow through the Caspary strip to the vascular tissues and (ii) to an inhibited leaf transpiration.

A further investigation showed that *Salix fragilis* L. plants, exposed to SDM, did not show any antibiotic presence in leaves, even when the active molecule was present in the nutrient solution at doses similar to those found in fresh manure (i.e., from 155 to 620 mg l⁻¹).

The active molecules used were detected in the green tissues only in the case of species exposed to SDZ, probably because of the chemical-physical properties characterizing the tested molecules. In particular, while the log *K_{ow}* of SDM is 1.63, that of SDZ is much lower, -0.09, suggesting a higher tendency of the latter compound to dissolve in water. Therefore, it is possible to speculate that, having entered the root through the apoplast, SDZ may move up to the leaves within the water flow, differently to SDM whose chemical-physical characteristics limit such behavior. Moreover, it is worth noting that among the studies, performed in different laboratories, the methods adopted to extract the antibiotics from vegetal tissues and to analyze them varied. However, the underlying aim of this study was not that to investigate the differences of extraction protocols.

In conclusion, considering that further studies are required to better elucidate the effective SA removal by *Salix fragilis* L. from soils and waters, the preferential accumulation seen in roots and rhizoplane may allow the application of this willow species for SA phytoremediation purposes. In fact, both SA phytoextraction (i.e., the use of plants to clean up pollutants via accumulation in harvestable tissues) and phytostabilization (i.e., the use of plants to stabilize pollutants in soil) by willow could limit their diffusion by erosion agents and their interaction with other organisms living in soil. Conversely, considering both the SA accumulation of *Zea mays* L. and the adverse effects caused by SDZ on its growth, this species is not recommended for remediation aims. However, its cultivation should be discouraged in SA polluted soils to avoid eventual food contamination.

- 2) By investigating the effects on plant development and physiology caused by the exposure to various SAs and concentrations, it was evident that responses varied considerably with the species used in performing the laboratory trial, even if a common trait of exposed plants was an altered architecture of the root apparatus. A woody plant such as *Salix fragilis* L. was proved to be not extremely affected, after a period of one month exposure to SAs, even at particularly high doses. Considering outcomes from biometric, photosynthetic and gas exchange parameters, the dose at which no adverse effects were observed was of 1 mg SDM L^{-1} of nutrient solution. However, no particularly negative effects were detected at the dose of 10 mg SDZ kg^{-1} of soil, when considering analyzed data such as water uptake, contents of nutrients and chlorophylls, biomass production and root architecture, for the same woody species but grown on soil.

This discrepancy of one order of magnitude may be explained by considering that the active molecule used has been different and that the soil matrix may have hampered the direct contact of the active molecule with roots. It is well known that once in the soil, covalent and polar bonds, and hydrophobic interactions with soil organic matter and mineral surfaces may cause the soil sorption of SAs. Conversely, plants grown in the liquid medium were directly in contact with SAs and, therefore, more vulnerable to them.

Moreover, another important result showed that after exposing *Salix fragilis* L. cuttings to 10 mg SDM L^{-1} of nutrient solution, the plant tolerance to SDM

increased with the exposure duration, probably because of the onset of some acclimation mechanisms, leading to suggestion of the possible use of *Salix fragilis* L. for long-term field projects.

In contrast, the growth of important crops such as *Zea mays* L. was evidently affected by SAs. In fact, physiological parameters of *Zea mays* L. (e.g., water uptake and nutrient accumulation) were disturbed when cultivated on a soil matrix with a spiking dose of 10 mg SDZ kg⁻¹.

Based on the fact that an altered root architecture was often found in plants exposed to both SDM and SDZ, a further experiment was carried out using *Hordeum vulgare* L. plants treated with 11.5 mg l⁻¹ of SDM and SZ, separately, in nutrient solution. The aim of such trial was to explore in depth the SA impacts on the structure and function of root apparatus. Results showed effects on root architecture, e.g. abnormal lateral root number and position, accompanied by improved differentiation degree and thickened cell walls compared with control plants. Moreover, even the membrane integrity of barley turned out to be compromised by the two SAs, SZ being the more toxic antibiotic to plants.

To conclude, achieved results suggest that SAs, when present at environmental relevant doses, are well tolerated by the woody species *Salix fragilis* L., but not by important crops such as *Zea mays* L. and *Hordeum vulgare* L., whose agronomic performances could be compromised.

- 3) Scrutinizing the sulfonamide impact on selected important microorganisms present in soil and plant roots, obtained results lead to the conclusion that the effects of an antibiotic, such as SDZ, can alter both the functional and structural diversity of the soil microbial community. In particular, the structural diversity (DGGE) was seen to be much more sensitive than the functional one (enzymatic activities) to SDZ input to soil. In addition, SDZ-induced effects appeared more evident in proximity to roots (i.e., root apparatus and rhizosphere) than in bulk soil, probably because plants, as important sources of available organic carbon through exudates, favor the root and rhizosphere microbial growth, and to indirect effects through adverse changes of the plant morphology and physiology, thus of the rhizodeposit release into the soil.

Overall achievements have highlighted the adverse ecological consequences caused by SDZ in arable lands. In fact, if a delicate task such as the peculiar plant-

microbe interactions appears disturbed, as a consequence, numerous intrinsic processes (e.g., carbon sequestration, ecosystem functioning and nutrient cycling) may be compromised, as well as the crop production itself. Moreover, the combined adverse antibiotic effects on root associated microorganisms alert about the risk of human and livestock exposure to soil-borne resistance through the food chain.

Such considerations point to a need to become aware of this kind of environmental pollution, which has yet to be examined close up. In view of possible phytoextraction and/or phytodegradation aims, more investigations should be carried out, especially under field conditions and after longer periods of SA exposure, focusing the attention on the toxic effects of antibiotic medicines on both plant physiology and living organisms (e.g., towards insects and grazing animals).

Finally, the frequent and simultaneous presence of more than one pharmaceutical product in soils and waters should not be overlooked or underestimated, thus leading to possible synergistic effects on organisms living in the polluted environments.

Supplementary material

Table S1 Gradient program for separation and detection of sulfadiazine and its metabolites from background contaminants on a Sunfire C18 HPLC column.

Time (min)		% Solvent A	% Solvent B
0-2		70	30
2-4	linear	0	100
4-8		0	100
8-8.2	linear	70	30
8.2-9		70	30

Table S2 Compound dependent parameters for the sensitive determination of the antibiotic sulfadiazine and its two metabolites in extracts of plant tissues and soil.

Antibiotic/ Metabolite	Precursor Ion	Source parameters		Fragment Ion	Mass analyzer parameters		
		DP (m/z)	EP (V)		CEP	CE (V)	CXP
SDZ	251.15	26	4	65.1	22	59	2
				92.0	22	43	4
				108	13	35	8
N-Acetyl-SDZ	293.20	70	10	65.1	38	29	6
				134	14	10	3
				198	14	10	3
4-OH-SDZ	267.21	41	9.5	156	10	21	4
				201	10	25	4
				173	10	23	4
SDM	279.01	35	36	186	16	23	4
				204	16	23	4
				124	16	35	4
				156	16	25	4

DP: Declustering potential; EP: Entrance potential; CEP: Collision cell entrance potential; CE: Collision energy; CXP: Collision cell exit potential

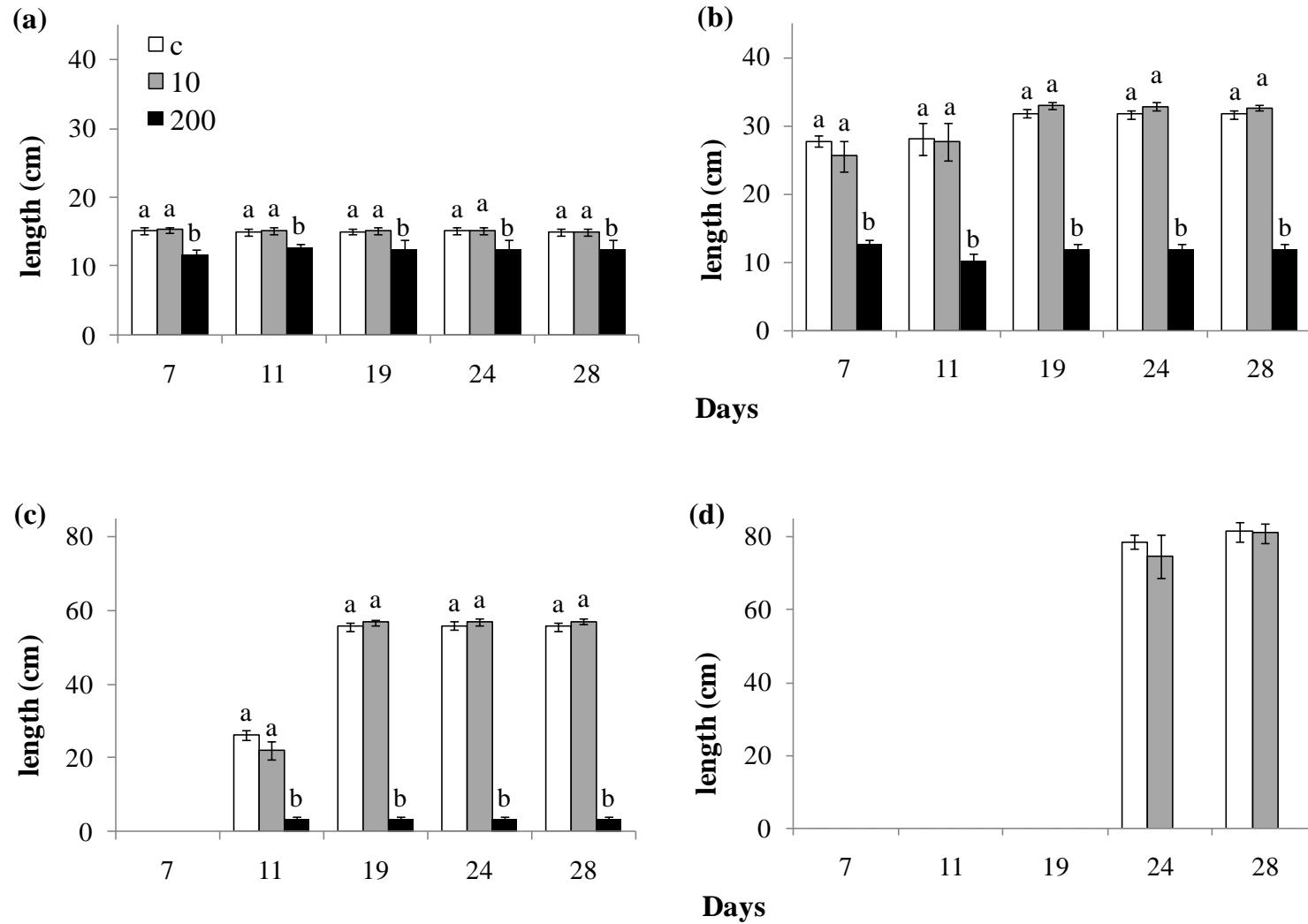


Fig. S1 Length and development of the second (a), third (b), fourth (c) and fifth (d) leaves of maize plants exposed to 0 (control), 10 and 200 mg SDZ kg⁻¹ soil. Values denote mean \pm SE; HSD post-hoc test ($p<0.05$). Letters, when present, indicate significant difference among SDZ treatments ($p<0.05$).

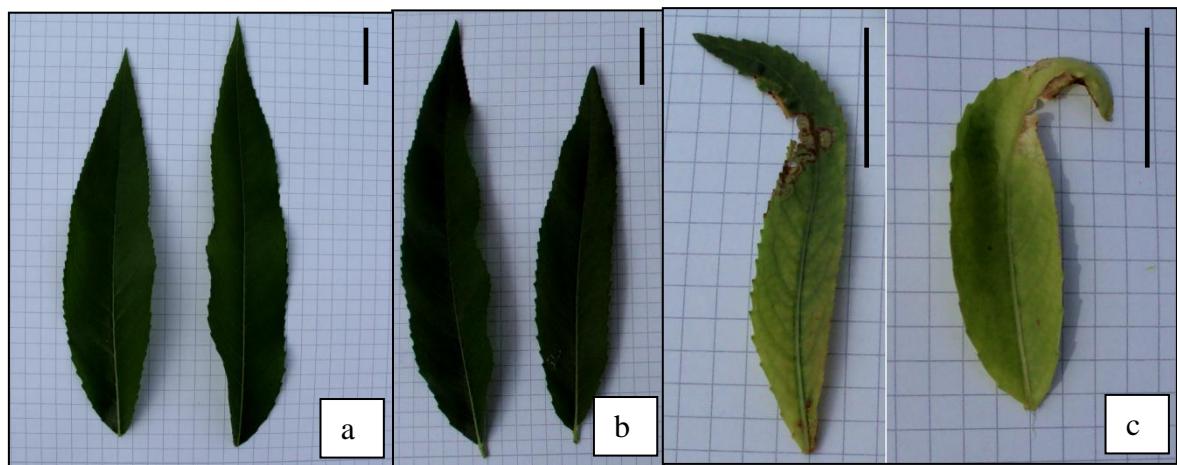


Fig. S2 Adult leaves of *Salix fragilis* L. plants exposed to soil concentrations of 0 (a), 10 (b) and 200 (c) mg SDZ kg⁻¹ soil. Black lines correspond to 2 cm.

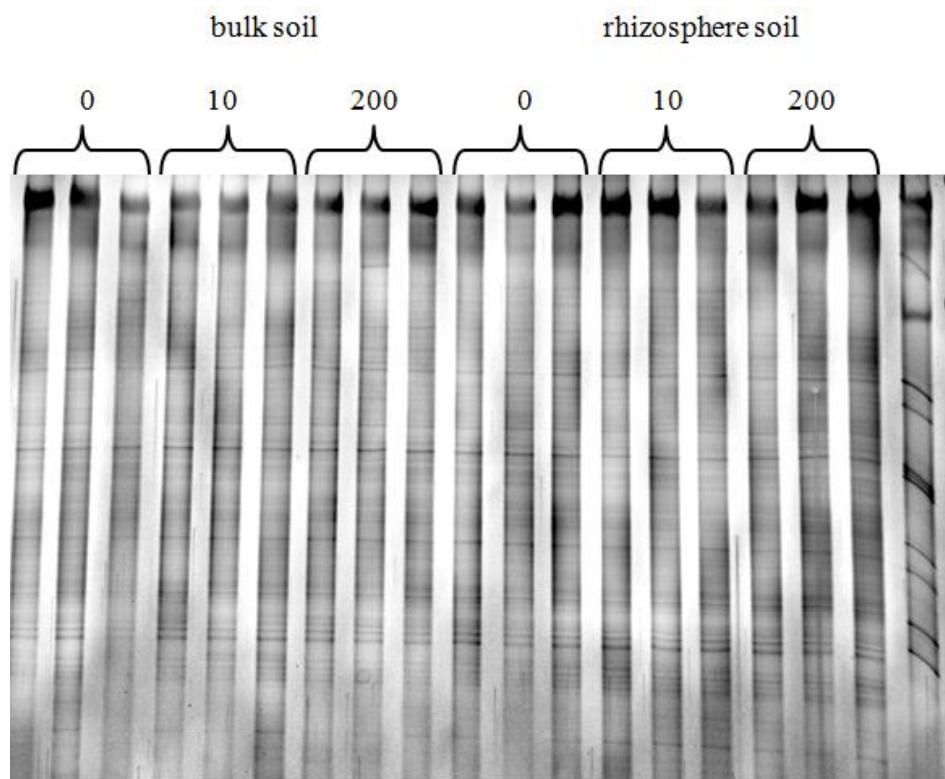


Fig. S3 TC DGGE of bulk and rhizosphere soil from pots planted with willow plants exposed to SDZ 0, 10 and 200 mg kg⁻¹ soil.

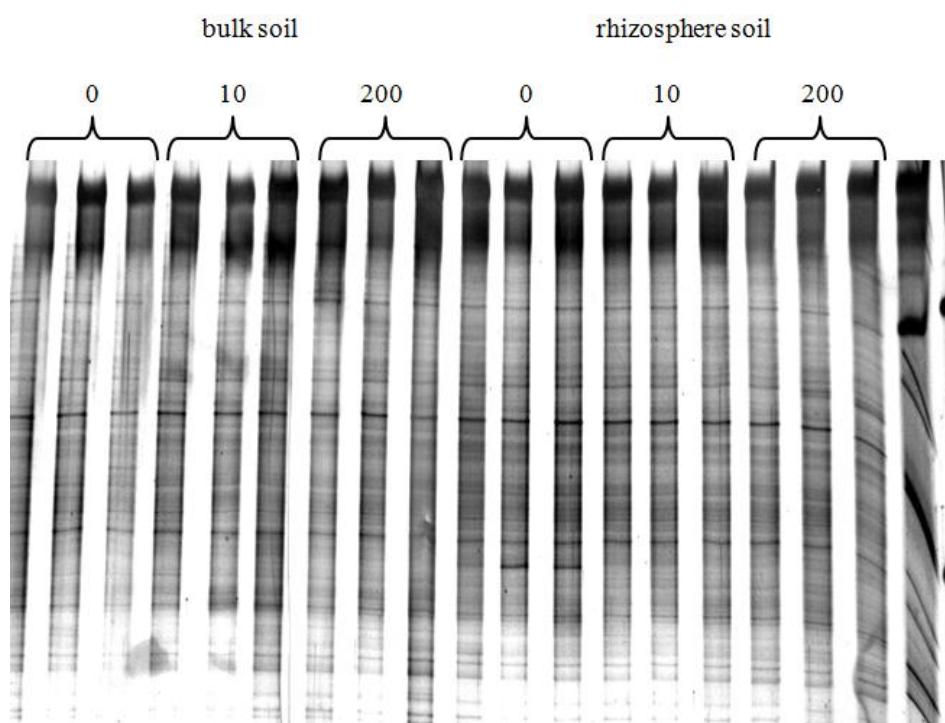


Fig. S4 TC DGGE of bulk and rhizosphere soil coming from pots containing maize plants exposed to SDZ 0, 10 and 200 mg kg⁻¹.

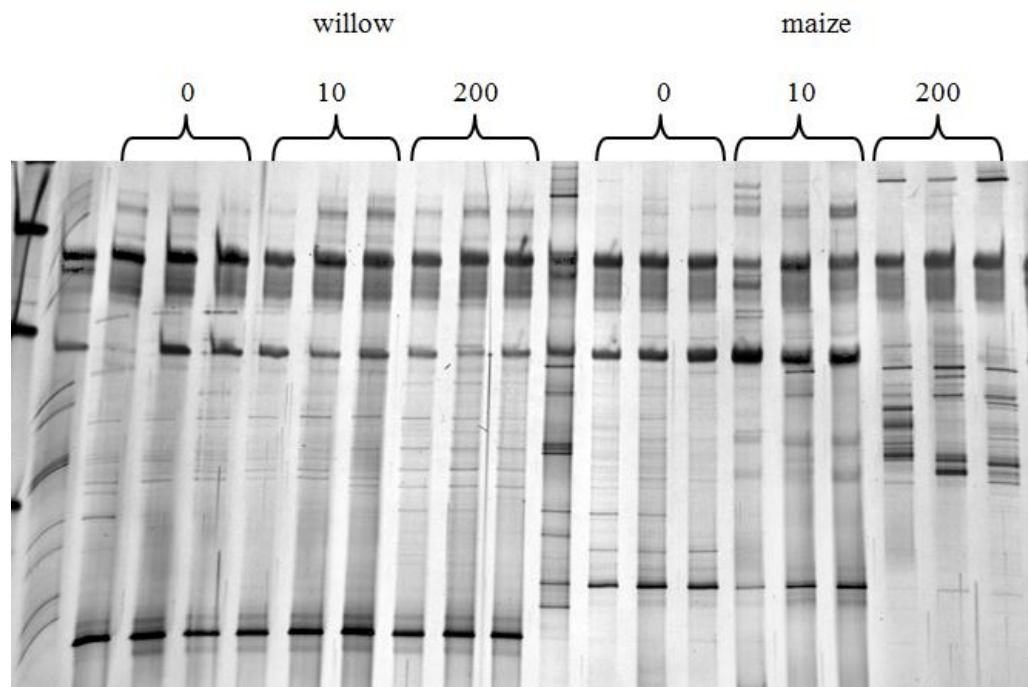


Fig. S5 TC DGGE of willow and maize root apparatus exposed to SDZ 0, 10 and 200 mg kg⁻¹.

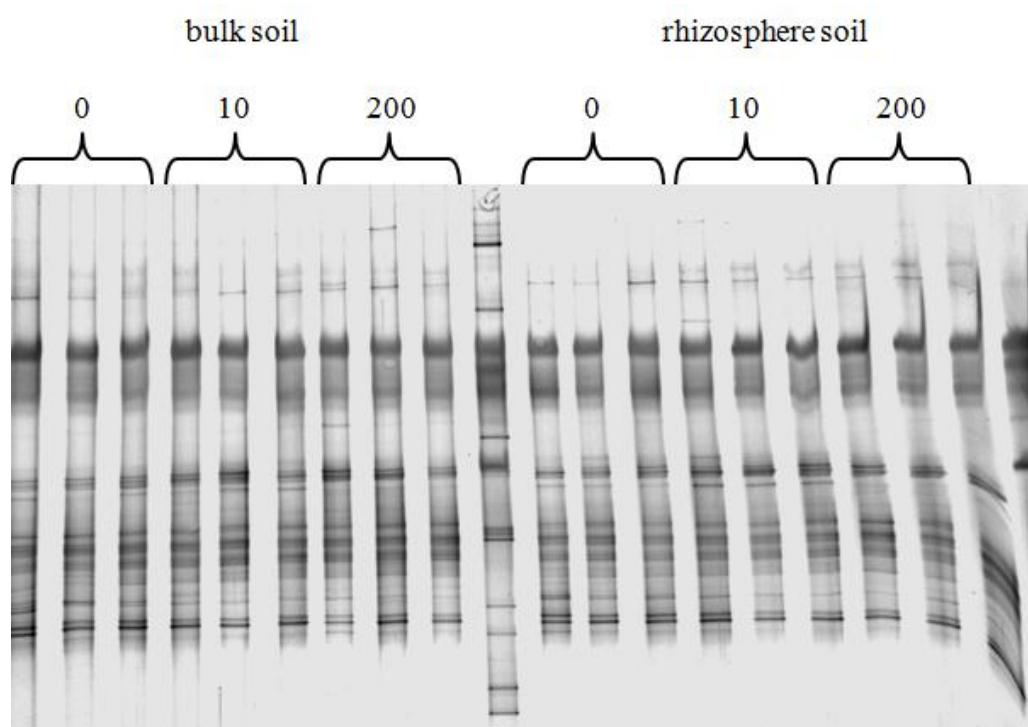


Fig. S6 Ps DGGE of bulk and rhizosphere soil coming from pots containing willow plants exposed to SDZ 0, 10 and 200 mg kg⁻¹.

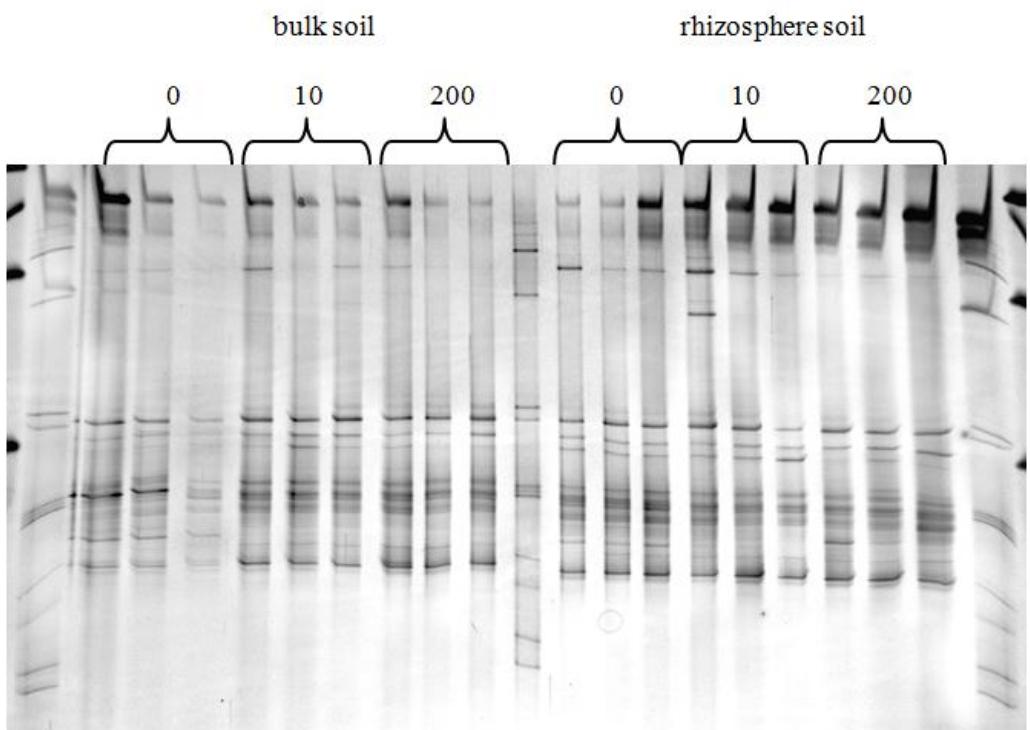


Fig. S7 Ps DGGE of bulk and rhizosphere soil coming from pots containing maize plants exposed to SDZ 0, 10 and 200 mg kg⁻¹.

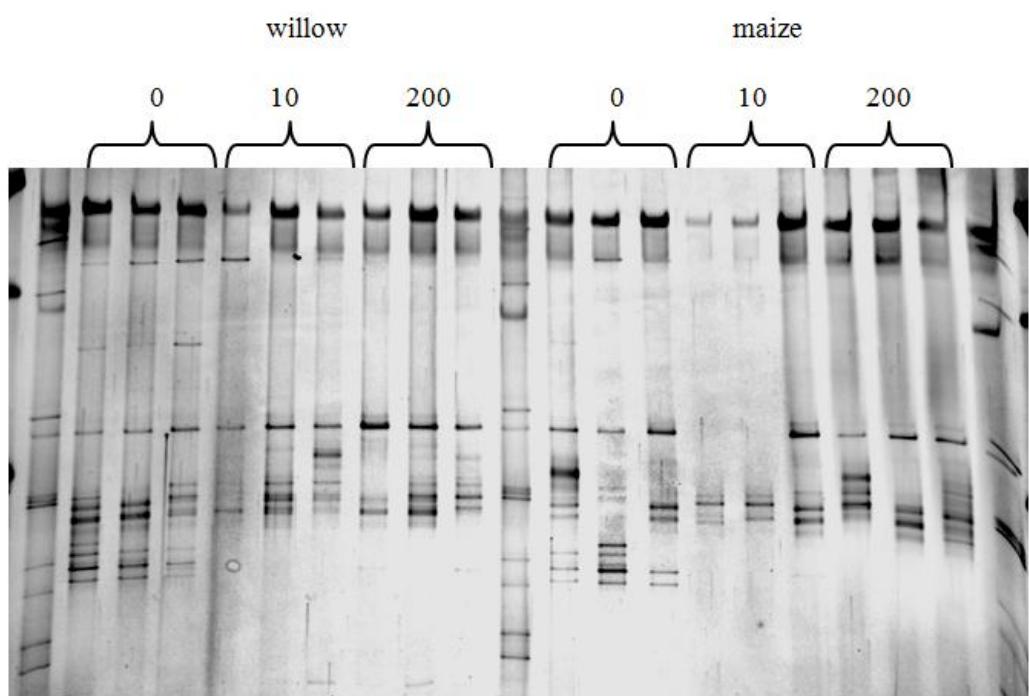


Fig. S8 Ps DGGE of willow and maize root apparatus exposed to SDZ 0, 10 and 200 mg kg⁻¹.

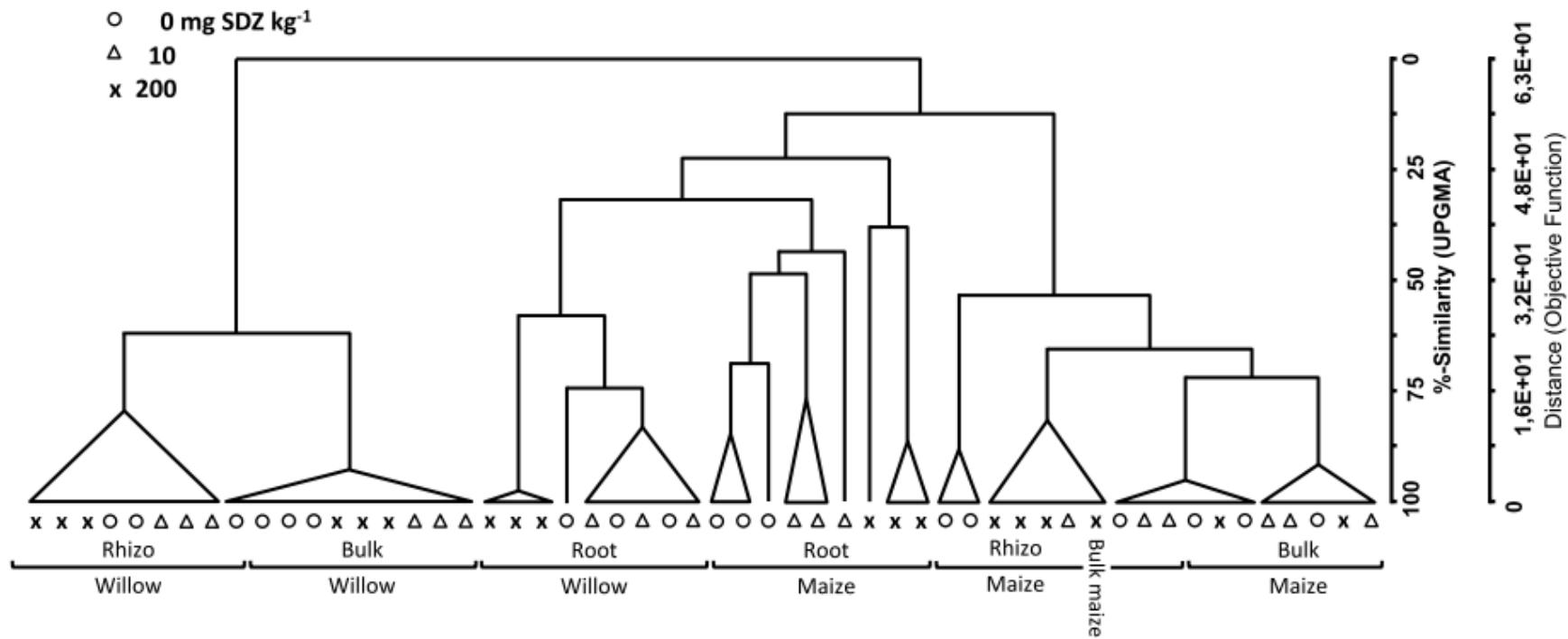


Fig. S9 Cluster dendrograms of total bacterial community 16S rRNA gene DGGE patterns of willow and maize roots. Clusters are combined at 90% similarity. Linkage method: GROUP AVERAGE (UPGMA); distance measure: Euclidean (Pythagorean).

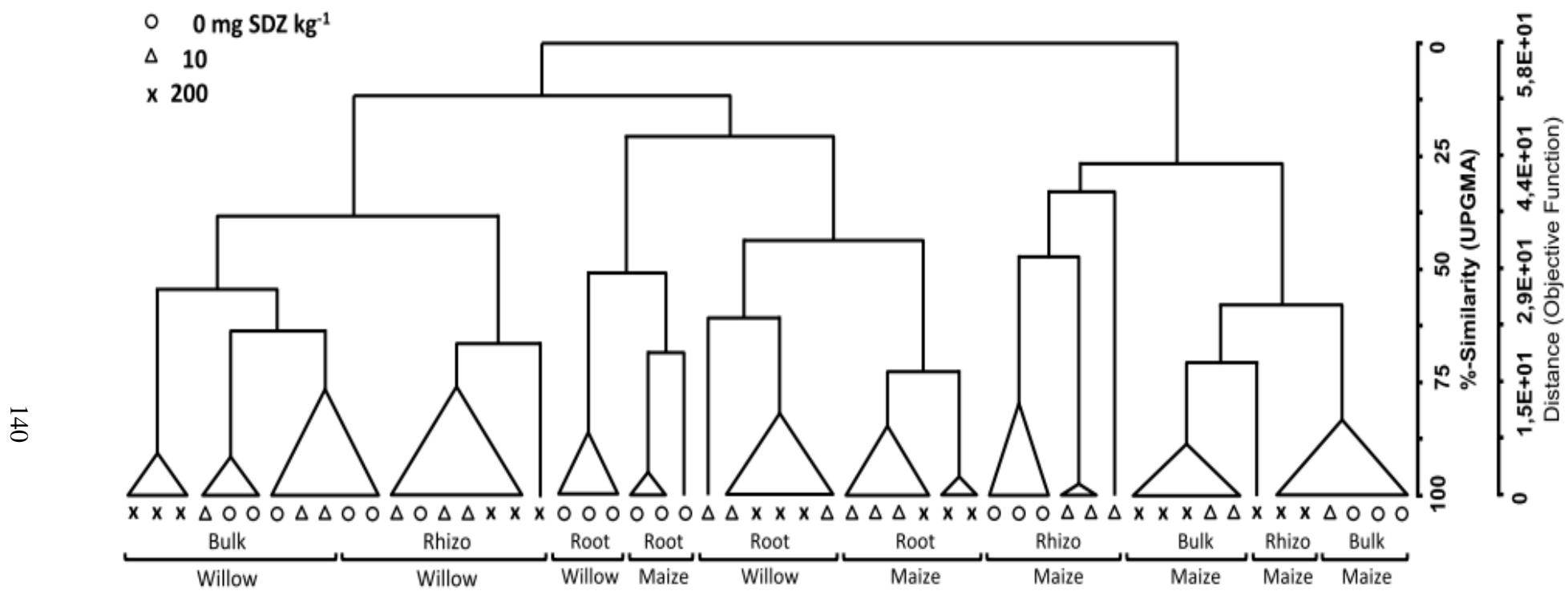


Fig. S10 Cluster dendrograms of *Pseudomonas* 16S rRNA gene DGGE patterns of willow and maize roots. Clusters are combined at 90% similarity. Linkage method: GROUP AVERAGE (UPGMA); distance measure: Euclidean (Pythagorean).

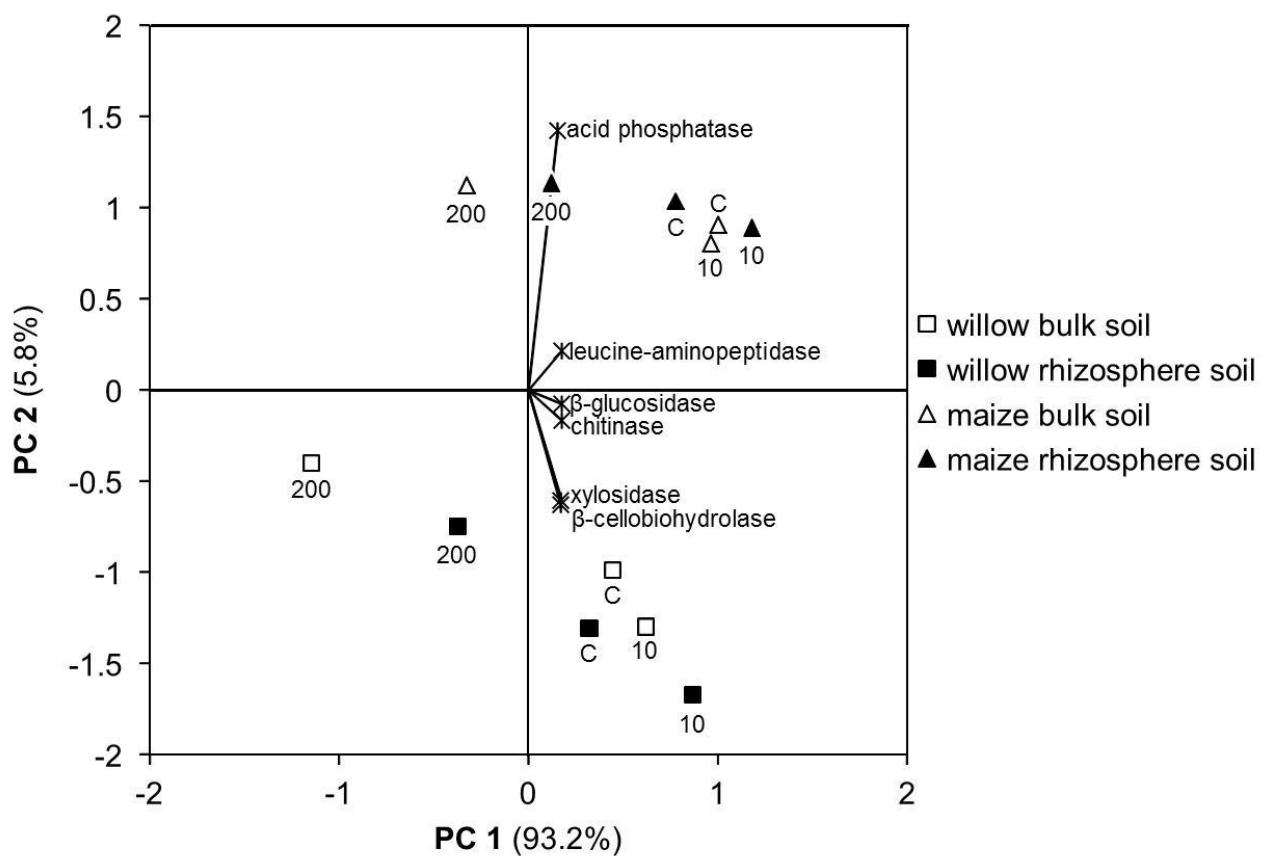


Fig. S11 Principal component analysis and loadings of soil enzyme activities from willow and maize planted bulk and rhizosphere soils exposed to SDZ at spiking concentrations of 0 (C), 10 and 200 mg kg⁻¹.

Acknowledgments

I would like to express my gratitude to Prof. Rossella Ghisi, who continuously supervised my work during the three years of PhD. I've particularly appreciated the positive working atmosphere allowed by my supervisor.

Sincere thanks go to Prof. Dr. S. Thiele-Bruhn, for supervising my work at the University of Trier and thereafter.

I've appreciated very much the help of people involved in the preparation of manuscripts resulting from researches carried out at the University of Padova and at the University of Trier. In particular, I would like to thank Prof. Nicoletta La Rocca, Prof. Nicoletta Rascio, Prof. Francesca Capolongo, Prof. Willy Werner, Franco Meggio PhD, Rüdiger Reichel PhD student, Guglielmo Gallina PhD and Stefania Ferro PhD.

Big thanks go also to the DAFNAE department staff, in particular to Mrs. Anna Rita Trentin for her technical assistance in laboratory. Moreover, the support of Soil Science department, University of Trier, was highly appreciated, especially the very accurate assistance of E. Sieberger in conducting the DGGE and enzymatic activities measures, of P. Ziegler and M.-O. Aust.

Huge thanks go to Marina Canapero and Lissa Marie Veilleux for their kind assistance in the English language.

Veneto Agricoltura Agency and the “Ing. A. Gini” Foundation are acknowledged for funding my PhD.



Salix fragilis (A.M.)