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Sulphadimethoxine inhibits *Phaseolus vulgaris* root growth and development of N-fixing nodules

Marilena Sartorius [a], Anna Riccio [a], Michele Cermola [a], Paolo Casoria [b], Eduardo J. Patriarca [a], Rosarita Taté [a,*]

[a] *Institute of Genetics and Biophysics "A. Buzzati-Traverso", CNR, Via P. Castellino 111, CP 80131, Naples, Italy*
[b] *Department of Environmental Sciences, University "Parthenope", Centro Direzionale di Napoli Isola C4, CP 80143, Naples, Italy*

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ABSTRACT

Sulphonamides contamination of cultivated lands occurs through the recurrent spreading of animal wastes from intensive farming. The aim of this study was to test the effect(s) of sulphadimethoxine on the beneficial N-fixing *Rhizobium etli–Phaseolus vulgaris* symbiosis under laboratory conditions. The consequence of increasing concentrations of sulphadimethoxine on the growth ability of free-living *R. etli* bacteria, as well as on seed germination, seedling development and growth of common bean plants was examined. We have established that sulphadimethoxine inhibited the growth of both symbiotic partners in a dose-dependent manner. Bacterial invasion occurring in developing root nodules was visualized by fluorescence microscopy generating EGFP-marked *R. etli* bacteria. Our results proved that the development of symbiotic N-fixing root nodules is hampered by sulphadimethoxine thus identifying sulphonamides as toxic compounds for the *Rhizobium*–legume symbiosis: a low-input sustainable agricultural practice.

1. Introduction

Sulphonamides are antibacterial compounds widely used in livestock breeding either as feed additives or during mass therapy (Halling-Sørensen et al., 1998; Jørgensen and Halling-Sørensen, 2000). Routinely, sulphonamides, such as sulphadimethoxine (SDM), are administrated orally to cattle, poultry and swine at a dose ranging between 80 and 100 mg kg^{-1} body weight. Indeed, by using this administration manner, only a relative low percentage of SDM is absorbed by the animals, whereas the majority (up to 90%) is eliminated uncharged in their excreta (Bevill, 1988; Sarmah et al., 2006). Animal wastes are often collected and applied to arable land. As a consequence of their elevated chemical stability sulphonamides accumulate in the soil and other environmental compartments where their activity is maintained for a long time (Jørgensen and Halling-Sørensen, 2000). Besides this, sulphonamides can also be released into the environment via aquaculture. Hence, current research is oriented to identify the environmental risk encountered by the use of antibiotics (Kemper, 2007).

The effects of SDM on plant species and aquatic organisms have been studied under controlled laboratory conditions (Migliore et al., 1995, 1996, 1997; De Liguoro et al., 2007). It has been shown that SDM affected the growth of different plants, including *Amaranthus retroflexus*, *Hordeum distichum*, *Panicum miliaceum*, *Plantago major*, *Pisum sativum*, *Rumex acetosella*, and *Zea mays* (Migliore et al., 1995, 1996, 1997). Indeed, the SDM-induced alterations were dependent on the organ analysed (root, stalk and leaves), the phenotype evaluated (dimension, weight, pigmentation and geotropism) and, predominantly, on the timing/stage (days after germination) at which the analysis is performed (Migliore et al.,1995, 1996, 1997).

Plants belonging to the *Fabaceae* family including beans, lentils, lupins, peanuts and peas (usually named legumes), are cultivated for either human/animal consumption or oil production. Most relevant, legumes are able to enter in symbiosis with bacteria belonging to *Rhizobiaceae* family, leading to the formation of new plant organs, *i.e.* the N_2-fixing root nodules (van Rhijn and Vanderleyden, 1995; Patriarca et al., 2004; Cooper, 2007). Inside the nodules, the atmospheric nitrogen is converted/reduced into ammonium by the intracellular form of *Rhizobium*, the bacteroids. The ammonium produced is released and used by the plant in order to grow (Patriarca et al., 2002). Hence, nodulated legumes may grow efficiently also in nitrogen-deficient soils reducing the requirement of pollutant and costly nitrogen fertilizers. Based on these features and considering the high protein levels contained by their seeds, legume plants acquired huge agriculture relevance mostly in developing countries. In the case of *Rhizobium etli–Phaseolus vulgaris* symbiosis, development of root nodules is a morphogenetic event involving divisions and differentiation of plant cells as well as proliferation of bacteria and their differentiation into intracellular N-fixing bacteroids (Tatè et al., 1994; Cermola et al., 2000; Ferraioli et al., 2004). Hence, we speculated that the rhizobia–legume symbiotic interaction, and particularly nodule development, could

* Corresponding author. Tel.: +39 081 6132470; fax: +39 081 6132760.
E-mail address: rori@igb.cnr.it (R. Taté).

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be highly susceptible to an SDM-induced inhibition of DNA replication.

The aim of this work was to evaluate under laboratory conditions the effect of SDM on the following processes: (i) seed germination, seedling development and growth of *P. vulgaris*; (ii) free-living growth of its symbiotic partner *R. etli* bacteria; and (iii) development of root nodules occurring during the symbiotic interaction. In particular, the impact of SDM on the bacterial capacity to induce nodulation and invade developing nodules, was assessed.

2. Materials and methods

2.1. Plant growth and SDM treatment

Seeds of *P. vulgaris* cv. Negro Jamapa were surface-sterilized with 5% (v/v) H_2O_2 at 25 °C for 30 min, washed extensively with sterile distilled water. Surface-sterilized seeds were transferred aseptically into Petri dishes (150 mm in diameter) containing agar–water (1%, w/v) and then incubated for germination in the dark at 23 °C for 72 h. Following germination, seedlings showing a rootlet of about 4–5 cm long were aseptically transferred into sterile plant-growth pouch (Mega International, Minneapolis, MN) containing 15 mL of a N-free Jensen medium (Vincent, 1970). Bean plants were maintained in a growth chamber at 28 °C during a 16-h light period and at 19 °C during the 8-h dark period; light supplied by fluorescent and incandescent lamps at an intensity of 250 μmol photons m^{-2} s^{-1}; relative humidity 65–75%. Plants were watered with sterile N-free Jensen medium.

Sulphadimethoxine (SDM) was purchased from Sigma Chemical (St. Louis, MO, USA), dissolved in distilled water and sterilized through a 0.2-μm Millipore filter. The effect of SDM on the germination process was assayed by incubating sterilized seeds of *P. vulgaris* on Petri dish containing agar–water supplemented with SDM at a concentration ranging from 10 to 300 mg L^{-1}. For every condition 180 seeds were assayed. As a control, 180 seeds were put to germinate in the absence of SDM; under this condition 95% of the assayed seeds germinated as usual. The effect of SDM on seedlings development as well as on the growth of common bean plantlets was assayed by germinating seeds on agar–water without added SDM; later on, the resulting germinated seeds were shifted into growth pouches containing Jensen medium supplemented with increasing concentrations (ranging from 10 to 300 mg L^{-1}) of SDM. At every SDM concentration assayed, the development of 60 seedlings (three seedlings per pouch, 20 pouches) was assessed. As a control, 60 seedlings were cultivated with Jensen medium in the absence of SDM. The concentration of SDM in the medium was maintained almost invariably as follows: every 2 d the plant-growth pouches were drained-off, exhaustively rinsed with sterile Jensen medium, and then watered with the same medium but supplemented with SDM at the required concentration. Twelve days-post planting (12 dpp) a number of plant features, namely the thickness of the leaves, as well as, the length of shoot and root systems were measured.

2.2. Microbial growth conditions

The wild-type strain CE3 of *R. etli* (Noel et al., 1984) was grown on a rotary shaker (200 rev min^{-1}, G67 rotary shaker, New Brunswick, NY, USA) at 30 °C in TYR complete medium (per litre: tryptone, 5 g; yeast extract, 3 g; and $CaCl_2.2H_2O$, 0.88 g). RMM was used as a chemically-defined minimal medium (per litre: $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; K_2HPO_4, 1 g; KH_2PO_4, 1 g; $FeCl_3 \cdot 6H_2O$, 10 μg; $MnSO_4 \cdot H_2O$, 20 μg; $ZnSO_4 \cdot 7H_2O$, 20 μg; $CuSO_4 \cdot 5H_2O$, 20 μg; $CoCl_2$, 20 μg; Na_2MoO_4, 20 μg; biotin, 20 μg; calcium pantothenate, 20 μg; thiamine, 20 μg). RMM medium was supplemented with NH_4Cl (10 mM) and glucose (1%, w/v) as N and C sources, respectively. Antibiotics (purchased from Sigma Chemical, St. Louis, MO, USA) were added to the media in the following concentrations: tetracycline, 5 μg mL^{-1}; nalidixic acid, 20 μg mL^{-1}. *Escherichia coli* strains were grown at 37 °C in TY medium (per litre: tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g). Antibiotic concentration used for *E. coli* was tetracycline, 15 μg mL^{-1}. All media were solidified with 1.5% agar (w/v) (Difco Laboratories, NJ, USA). SDM resistance was evaluated by means of the liquid culture method. Different concentrations of SDM ranging from 10 to 300 mg L^{-1} were used and cell density (optical density, OD) was measured with a spectrophotometer. The OD value of starting cultures was 0.05 at 590 nm. Bacterial growth was monitored removing aseptically an aliquot (0.7 mL) of the culture 24 and 48 h after inoculation.

2.3. Bacterial transformation

R. etli was grown at 30°C with shaking in TYR medium, and competent cells for electroporation were prepared as previously described (Tatè et al., 1997). DNA of plasmids pAR66 (Patriarca et al., 1993) and pAR368 (see below) was purified by means of Wizard Plus SV Mini-Preps (Promega, Madison, WI, USA). Electroporation was performed with a Gene Pulser apparatus (BioRad Lab, Richmond, CA, USA). Electroporated cells were suspended in 1 mL of a complete medium (per litre: tryptone, 20 g; yeast extract, 3 g; KCl, 0.19 g; $CaCl_2$, 2.1 g; $MgSO_4$, 2.5 g; mannitol, 10 g). The cells were incubated for 4 h at 30 °C and then plated on TYR-agar containing appropriate antibiotics (tetracycline plus nalidixic acid). The colonies developed 3 d after incubation at 30 °C were purified by streaking on plates of complete TYR medium.

2.4. Nodulation test and preparation of fixed, sectioned material

The primary root of 5-day-old seedlings was inoculated with 100 μL of a bacterial suspension (1×10^6 cells mL^{-1}) in phosphate buffer (50 mM; pH 7.0). As a control, a group of seedlings were inoculated with phosphate buffer. The effect of SDM on the symbiosis was assayed by inoculating the main root of 5-day-old seedlings of *P. vulgaris* (developed in the absence of SDM) with strain CE3 (wild-type) of *R. etli*. The inoculated plantlets were grown in Jensen medium, with or without added SDM, and for every condition assayed, the nodulation ability of 36 plants (three plants per pouch, 12 pouches) was assessed. Twelve days-post inoculation (12 dpi) developed nodules were counted and their morphology was evaluated and photographed. For histological analysis, the roots were collected at 12 dpi and fixed as previously described (Tatè et al., 1994). Root pieces (ranging between 0.8 and 1 cm long) were excised with a razor blade and embedded in 4% (w/v) agarose (Fisher Molecular Biology, Trevose, PA, USA). Sections (50–80 μm) were obtained with a Leica Vibrating Blade Microtome model VT1000S (Leica, Nussloch, Germany).

2.5. Histological localization of β-galactosidase activity

Primary roots of inoculated plant, untreated or treated with different concentration of SDM, were collected at 12 dpi and incubated for 1 h at room temperature under a brief and gentle vacuum in a solution (pH 5.6): (glutaraldehyde, 2% (v/v); mannitol, 0.3 M; 2-N-morpholino ethanesulfonic acid, 10 mM). Fixed root fragments were washed three times with phosphate buffer (50 mM; pH 7.2) and immersed in staining solution: (phosphate buffer, 10 mM (pH 7.2), NaCl, 150 mM; $MgCl_2$, 1 mM; potassium ferricyanide, 5 mM; potassium ferrocyanide, 5 mM; Triton X-100, 0.03% (v/v); 5-bromo-4-chloro-3-indolyl β-galactopyranoside