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Antibiotic effects on seed germination and root development of tomato (*Solanum lycopersicum* L)

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ABSTRACT

Antibiotics are emerging pollutants released into the environment through wastewater and manure or effluents from livestock plants. Compared to the wide literature on the effects of antibiotics on the development of drug-resistant bacteria and on the adverse effects on animals and human beings, the effects on plants are less investigated. Here we evaluated the effects of four antibiotics (cloramphenicol: CAP, spiramycin: SPR, spectinomycin: SPT, vancomycin: VAN) belonging to different chemical groups, on seed germination and root development of tomato (*Solanum lycopersicum* L. cv. San Marzano). Specifically, seed germination and root elongation kinetics, as well as the number of mithotic figures in root apical meristem, were studied in relation to different concentrations of each antibiotic (0, 0.1, 1, 10, 100, 1000 mg L^{-1}) for 10 and 7 days, respectively. Results showed that seed germination was not affected, but root development (root elongation kinetics and cell division) was impaired at concentrations from 10 mg L^{-1} (SPT) and 100 mg L^{-1} (CAP) to 1000 mg L^{-1} (SPR and VAN).

1. Introduction

Antibiotics constitute a new class of contaminants of emerging concern with adverse effects on the ecosystems and potential risks for human health, related to non-voluntary exposure or selection of antibiotic-resistent bacteria (Halling-Sorensen et al., 1998; Martinez, 2009; Du and Liu, 2012; Roose-Amsaleg and Laverman, 2016). These compounds are extensively used for the treatment of both human and veterinary infections, as well as in agriculture and aquaculture, with production and consumes increasing worldwide (Sarmah et al., 2006; Pan and Chu, 2016a; Roose-Amsaleg and Laverman, 2016). As bioactive molecules, their half-life in animals is usually short, ranging from days to few weeks, therefore most of them are excreted through urines and feces (Sarmah et al., 2006; Lofrano et al., 2017) in metabolized and non-metabolized forms. Thus, they can enter soil, surface and groundwater through soil manure amendments and effluents from wastewater treatment plants originally not designed to deal with these micropollutants (Sarmah et al., 2006; Li, 2014; Bondarczuk et al., 2016; Kaczala and Blum, 2016; Lofrano et al., 2017). Considering that antibiotics are administered to livestock not only to treat diseases, but also as growth promoters through food (Phillips et al., 2004; Landers et al., 2012), their final concentrations in soils and water can be exceedingly high, ranging between μ g kg^{-1} – mg kg^{-1} and μ g L^{-1} – mg L^{-1}, respectively (Xie et al., 2011; Yang et al., 2011; Yan et al., 2013).

The number of studies focusing on the toxicological assessment of antibiotics in the environment is constantly increasing, with the aim to bridge the various knowledge gaps (i.e. relevant endpoints to be considered) associated with these issues. Boxall (2004) and Kümmerer (2009) represent two comprehensive reviews on the ecotoxicity of antibiotics. Conversely, little is known about the effects of antibiotics, and generally pharmaceuticals, in relation to plants (Bártíková et al., 2016). Only few studies investigated the toxic effects on plants, involving growth reduction and metabolic disorders (Migliore et al., 1995; Hillis et al., 2011; Chen et al., 2011; Bártíková et al., 2016; Pan and Chu, 2016b; Pino et al., 2016). The effects vary in relation to the species, the molecule and the end-points (Martí et al., 2011; Corbel et al., 2015; Pino et al., 2016). Li et al. (2011) reported that in wheat (*Triticum aestivum*) exposed to oxytetracycline (10, 20, 40, 80 mM) root number was more affected than chlorophyll content and photosynthesis, with IC_{50} = 7.1 mM. Hillis et al. (2011) studied the effects of 10 antibiotics,

Abbreviation: CAP, Cloramphenicol; SPR, Spiramycin; SPT, Spectinomycin; VAN, Vancomycin; ANOVA, Analysis of variance; MANOVA, Multivariate analysis of variance; H_0, Null hypothesis; λ_g, Length of lag phase - seed germination; λ_e, Length of lag phase - root elongation; μ_g, Growth rate - seed germination; μ_e, Growth rate - root elongation; A_g, Maximal growth - seed germination; A_e, Maximal growth - root elongation; *MI*, Mithotic Index; IC_{50}, Half maximal inhibitory concentration

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at concentration ranging from 1 to 10,000 μ g L^{-1}, on seed germination and root elongation of lettuce (*Lactuca sativa*), alfalfa (*Medicago sativa*) and carrot (*Daucus carota*), proving the absence of toxic effects on germination up to the highest concentrations and, conversely, the usefulness of root elongation as a sensitive endpoint, due to root physical interaction with soil contaminants (Klaine et al., 2003). Similarly, Chen et al. (2011) reported inhibition of root elongation in wheat, Chinese cabbage (*Brassica campestris*) and corn (*Zea mays*) in relation to chloramphenicol concentrations. Only one study highlighted effects on seed germination even at low concentrations of penicillin (0.004 mg L^{-1}), enrofloxacin (1 mg L^{-1}) and oxytetracycline (10 mg L^{-1}) on soybean (*Glycine max*), of enrofloxacin (0.1 mg L^{-1}) on sunflower (*Helianthus annuus*), and of penicillin (4 mg L^{-1}) and oxytetracycline (100 mg L^{-1}) on corn (Eluk et al., 2016).

The potential effects of antibiotics on plant growth and development still need to be fully assessed. Such studies would be of paramount importance considering the possible effects of antibiotics on food webs, ecosystem dynamics and human health. Tomato (*Solanum lycopersicum* L.) is one of the most important crops worldwide (Baldantoni et al., 2016) and is considered a model species for ecotoxicological tests (Kapustka and Reporter, 1998). The plant can uptake and accumulate tetracyclines and sulfonamides, which impair its growth (Ahmed et al., 2015), and is sensitive, in terms of root elongation, to quinolones, 14-ring macrolides and phenicols (Pan and Chu, 2016b). However, phytotoxicity or resistance toward other widely employed antibiotics still need to be evaluated. To this aim, the effects of four antibiotics belonging to different chemical groups, glycopeptides (vancomycin, VAN), 16-ring macrolides (spiramycin, SPR), aminogylcosides (spectinomycin, SPT) and phenicols (cloramphenicol, CAP), were evaluated on *S. lycopersicum* cv. San Marzano through seed germination and root development (elongation and cell division in apical meristem) analyses.

2. Materials and methods

2.1. Experimental design and laboratory analyses

Seed germination, root elongation and cell division were evaluated according to internal protocols.

The antibiotics Table 1, supplied by Sigma-Aldrich (Milan, Italy), were dissolved in distilled and sterile water and then diluted to the desired concentrations, nominally 0, 0.1, 1, 10, 100, 1000 mg L^{-1}. The concentrations of CAP, SPR and VAN were evaluated spectrophotometrically using a Cary 50 UV–Vis spectrophotometer (Varian, Palo Alto, USA), whereas those of SPT using a Focus GC equipped with a Flame Ionization Detector (Thermo Fisher Scientific, Waltham, USA).

Tomato seeds were exposed to 5 mL of antibiotic solution in Petri dishes during 10 and 7 days for the seed germination and the root elongation tests, respectively. Seed germination was evaluated on batches of 100 seeds (subdivided in 5 Petri dishes with 20 seeds each) for each concentration and antibiotic, kept at 25 ± 0.5 °C in the dark. Germinated seeds were counted twice per day, early in the morning and in the evening. Root elongation was evaluated on 20 seeds (1 per Petri dish) for each concentration and antibiotic, kept at 25 ± 0.5 °C in the dark. Seeds were imaged once per day with a Coolpix S8300 camera (Nikon, Tokyo, Japan) and root length was derived through image analysis using the Fiji software (Schindelin et al., 2012). After 7 days, roots were excised 1.5 cm from the apex, fixed in cold MeOH:acetic acid 3:1 v:v and kept at − 18 °C. Mithotic cells were counted on the Feulgen stained (10' at 60 °C hydrolisis, 60' Feulgen reaction) root apices, squashed in a drop of 50% acetic acid, using a Dialux 20 microscope (Leitz, Germany) at 250x and 400x magnification using phase contrast. The Mithotic Index (*MI*) was defined as the number of mithotic cells per root meristem.

Table 1
Concentrations (mg L^{-1}), as mean ± s.d. of n = 3 replicate measures, of chloramphenicol (CAP), spiramycin (SPR), spectinomycin (SPT) and vancomycin (VAN) employed in seed germination and root development analyses. Solubility in water at 25 °C is also reported.

Nominal concentration	CAP	SPR	SPT	VAN
0	0.0	0.0	0.0	0.0
0.1	0.10 ± 0.04	0.04 ± 0.12	0.09 ± 0.12	0.5 ± 0.2
1	0.89 ± 0.08	0.90 ± 0.02	0.99 ± 0.03	1.5 ± 0.4
10	9.7 ± 0.2	8.53 ± 0.06	9.9 ± 0.2	11 ± 0.2
100	100 ± 2	68.0 ± 0.8	98 ± 2	102 ± 2
1000	1013 ± 24	748 ± 6	992 ± 11	1131 ± 16
Solubility in H_2O	$2.5{\cdot}10^3$	$<1{\cdot}10^3$	$1.5{\cdot}10^5$	$2.25{\cdot}10^2$

2.2. Data analysis

Seed germination and root elongation kinetics were described using the logistic, the Gompertz or the Richards models, choosing the one providing the best fit in terms of Akaike Information Criterium. The set of free parameters shared by the three models, namely the length of lag phase (λ_g for seed germination, λ_e for root elongation), the growth rate (μ_g for seed germination, μ_e for root elongation) and the maximum growth (A_g for seed germination, A_e for root elongation), were derived through nonlinear least-squares using the Gauss-Newton algorithm and employed as descriptors of the kinetics for the following analyses. The functions of the "grofit" package (Kahm et al., 2010) for the R programming language (Core Team, 2016) were employed in describing the seed germination and root elongation kinetics.

The overall differences in seed germination and root elongation kinetics among the concentrations of each antibiotic were evaluated, according to Rencher and Christensen (2012), through multivariate analyses of variance (MANOVAs) using λ, μ and A as dependent variables and the antibiotic concentration as fixed factor. *Post hoc* evaluation of the differences among the concentrations of each antibiotic was performed, following MANOVA rejection of the null hypothesis (H_0), through linear discriminant analysis with the superimposition of 95% confidence circles for each antibiotic dose. In addition, the differences in each parameter among the doses of each antibiotic were evaluated through one-way analyses of variance (ANOVAs) or Kruskal-Wallis H tests, according to the homoscedasticity or heteroscedasticity of the groups, respectively, evaluated through Breuch-Pagan tests. In the case of H_0 rejection, *post hoc* tests of Tukey HSD, following ANOVA, or Dunn, following Kruskal-Wallis H test, were employed. Finally, the differences in *MI* among the concentrations of each antibiotic were evaluated through Kruskal-Wallis H tests, followed by the Dunn *post hoc* test.

IC_{50} values for the seed germination, root elongation and cell division end points were calculated by fitting the concentration-response curves using log-logistic functions with lower asymptote equal to 0. In the case of the *MI* for CAP and SPR, the concentration-response curve was fitted using a Cedergreen-Ritz-Streibig model with $\alpha = 1$ and lower asymptote equal to 0.

Multivariate analyses were performed using the functions of the "stats" (Core Team, 2016) and "candisc" (Friendly and Fox, 2016) packages for the R programming language, whereas the univariate analyses with the functions of the "stats", "lmtest" and "drc" (Zeileis and Hothorn, 2002) packages.

3. Results and discussion

In relation to the three germination parameters (λ_g, μ_g, A_g), no significant difference (for $\alpha = 0.05$) was found among the concentrations of each antibiotic Fig. 1.

Conversely, in relation to the root elongation parameters (λ_e, μ_e, A_e), MANOVAs highlighted significant differences, primarily related to μ_e and A_e Fig. 2, among the concentrations of CAP (Pillai's trace = 0.642, $P < 0.001$), SPT (Pillai's trace = 0.656, $P < 0.001$) and VAN (Pillai's trace = 0.382, $P < 0.001$). According to the ANOVAs or Kruskal-Wallis H tests, CAP, SPT and VAN determined significant differences in