Evaluation of Toxicity of Three Antibiotics to Germination and Early Growth of *Trifolium alexandrinum* Seeds

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Abstract Antibiotics are one of the most important emerging contaminants as they have been detected in several environmental compartments including soil. Compared to the wide literature on the effects of antibiotics on aquatic organisms, knowledge and understanding of their potential effects on terrestrial environment is still scarce, especially when mixtures are taken into consideration. In this study, a screening-level phytotoxicity assay was used to evaluate the effects of three antibiotics (Oxytetracycline, Levofloxacin, Ampicillin) individually and in mixtures, on seed germination, root and shoot elongation of Egyptian clover (*Trifolium alexandrinum* L.) After five days exposure time, the acute effects of individual antibiotics and their binary and ternary mixtures were assessed using three endpoints, namely percentage of seed germination, shoot length and root elongation. The results of the study indicated that plant germination was insensitive to the individually tested antibiotics, with no significant decreases up to the highest treatment concentration of 100 mg L^{-1}. Root elongation was observed as the most sensitive end point in individual antibiotics. However, increase in the root and shoot elongation was observed in mixtures relative to the individual antibiotics. The results show the importance of evaluating the toxicity of antibiotic mixtures, since they might have distinct effects when compared to the individual compounds.

Keywords: Phytotoxicity, Antibiotic mixtures, *Trifolium alexandrinum*, Germination

1. Introduction

Antibiotics are considered as contaminates of emerging concern because of their uncontrolled release and exposure in the environment causing detrimental effects on ecosystem and potential risks to human health (Polianciuc et al., 2020). They reach the environmental matrices via multiple sources, such as improper disposal or through wastewater after excretion (Zafar et al., 2021). Since these contaminants are not completely removed in the wastewater treatment plants (WWTPs), antibiotics and their metabolites can be released directly into the aquatic environment and, eventually, may contaminate drinking water (Felis et al., 2020). Furthermore, biosolids produced in WWTPs or animal manure can be used as fertilizers in agriculture, and reclaimed wastewater can be used for irrigating the crops. Considering multiple entrance sources of antibiotics in the soil. The final concentrations in soil and water can be extremely high, varying between μg kg^{-1} – mg kg^{-1} (Yan et al., 2013) and could bioaccumulate in organisms, raising questions about the potential adverse effects due to long-term exposure (Christou et al., 2019). Compared to the increasing number of studies focusing on the toxicity assessment of antibiotics in the environment, there are only a few studies that have reported the toxic effects on plants (Rede et al., 2019, Mukhtar et al., 2020, Wahid et al., 2021). Moreover, the results differ depending on the species, the tested compound and the endpoints (Christou et al., 2019, Wahid et al., 2021). Few studies highlighted effects on seed germination at environmentally relevant concentrations (Minden et al., 2016; Eluk et al., 2016; Rede et al., 2019). Although there is some literature on toxic effects of single antibiotic on plants, the current knowledge on the effects of their mixtures is still sparse, especially in plants (Rede et al., 2019). With this background, the aim of present work was to assess the effects of three antibiotics belonging to different chemical groups, Oxytetracycline (OXY), Levofloxacin (LEV) and Ampicillin (AMX) on *Trifolium alexandrinum* through single and combined (binary and ternary) tests. The phytotoxic impacts were assessed via different endpoints, namely seed germination percentage, root and shoot elongation after an exposure time of five days.

2. Methodology

2.1. Selected plant, soil and chemicals

Egyptian clover seeds (*Trifolium alexandrinum* var. Punjab Berseem) were obtained from Awan garden center (Islamabad, Pakistan) and stored in the dark at 4 °C. Seeds were surface sterilized and acute toxicity tests were performed on soil collected from an undisturbed site having no background antibiotic concentration of selected antibiotics. The physicochemical properties of the soil are mentioned in Table 1.
OXY (purity 99%), LEV (purity 99.9 %) and AMP (purity 99.4%) were purchased from Sigma-Aldrich, Germany. Fresh stock solutions of pharmaceuticals were

prepared in HPLC grade water obtained from *CarloErba Reagents (France)*.

Table 1. Physical properties of soil used for experimentaion

Soil property	Values
Texture	Silty clay loam
Silt plus clay content (%)	82
O.M content (%)	0.9
Moisture content (%)	14.4
Dry bulk density (g/cm^3)	1.5
Ph	8.4
WHC (wt.%)	17
TDS (%)	0.44

2.2.Experimental design

Briefly, twenty seeds of Egyptian clover were placed onto 25 g of pre-dried soil in polystyrene petri dishes. 10 mL of antibiotic solution was added on the top of the soil using a Pasteur pipette. Petri dishes were sealed with parafilm and placed inside a greenhouse for an exposure time of five days. Each test was performed in triplicate and the average germination percentage, length of root and shoot was calculated.

2.2.1. Soil contamination with individual and mixture of antibiotics

Tests with each antibiotic was performed covering the environmentally relevant concentrations detected in local grade water and 25 g of soil was contaminated with 10 mL of diluted solution.

2.2.2. HPLC analysis of antibiotics

Antibitics related analsyses were perofmred using the method optimized and reported earlier by Zafar et al. (2021). Agilent 1260 Infinity II LC System (HPLC) equipped with quaternary pump having diode array detector (DAD), Eclipse (4.6 × 250 mm) C18, 5 µm analytical column, autosampler for auto injection, was used for detection of all the selected antibiotics.

2.3. Statistical analysis

Statistical analysis was performed using SPSS software, version 25.0. Mean, median, and standard deviation of the three studied endpoints were calculated in Microsoft excel. Data from acute ecotoxicity assays was analyzed through the application of a Kruskal-Wallis one-way ANOVA. A p value < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Effect of antibiotics on T. alexandrinum seeds

The exposure of *T. alexandrinum* seeds to oxytetracycline did not cause statistically significant effects on percentage of seed germination and shoot

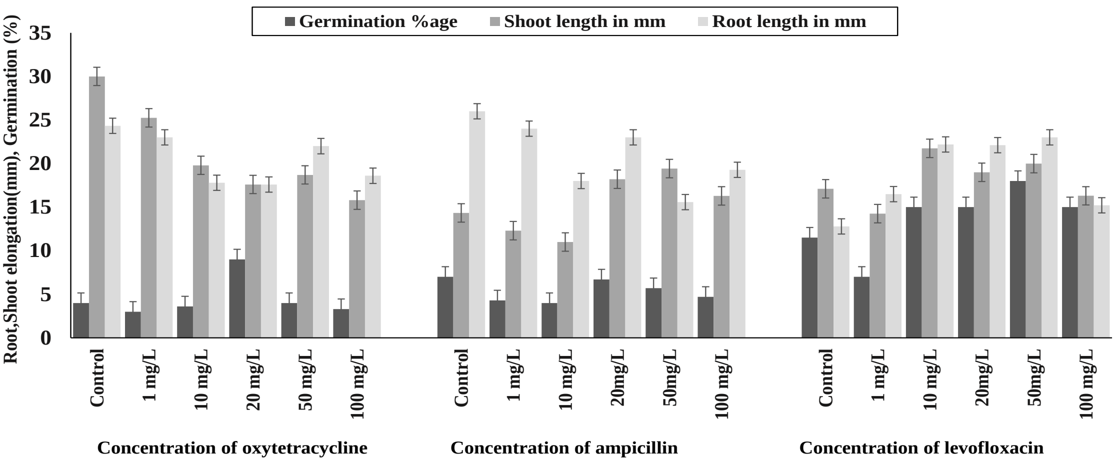

Figure 1. Effect of individual antibiotics on plant growth parameters upon exposure to different concentrations of antibiotics (mg L^{-1}).

wastewater channels, previously (Zafar et al., 2021). Assays with all the three antibiotics were conducted in concentration range between 1 and 100 mg L^{-1} (1, 10, 20, 50, 100 mg L^{-1}). To study a more realistic scenario, Egyptian clover seeds were exposed to twenty different mixtures of antibiotics. For the fifteen binary mixtures and five ternary mixture, the nominal concentration of each antibiotic was 1, 10, 20, 50 or 100 mg L^{-1}. The concentration of stock solutions was confirmed by liquid chromatography. Dilutions were prepared in HPLC

length, although root length was observed as the sensitive end point as decrease in root length was observed at 100 mg L^{-1} (Fig. 1), which is consistent with the results reported by Hillis et al. (2011), where they tested the effect of oxytetracycline on three plant species and reported significant decrease in root lengths of *D. carota* and *L. sativa* at concentrations of 1000 and 10,000 µg L^{-1}, respectively. Exposure of ampicillin to *T. alexandrinum* seeds significantly stimulated the root growth at the lowest exposure concentration of 1 mg L^{-1} (Fig. 1). However, no significant effect on germination percentage was observed compared to control. Low toxicity of penicillin is described in the literature for legumes when exposed to a concentration range of between 1 and 10,000 μg L^{-1} (Hillis et al., 2011). Root length was the most sensitive end point for *T. alexandrinum* exposed to levofloxacin where significantly decreased root growth at a concentration of 1 mg L^{-1} was observed (Fig. 1).

3.2. Effect of antibiotic mixtures on T. alexandrinum seeds

In order to assess the possible influence on the observed phytotoxic effects of antibiotic mixtures, different proportions of the tested antibiotics were considered and the sum of concentrations of antibiotics was maintained as 1 ppm or 1000 μg L^{-1} in all tested mixtures.
Binary mixtures of AMP and OXY (BM1), OXY and LEV (BM2) and AMP and LEV (BM3) did not show statistically significant differences to the three endpoints relative to the control (Fig. 2). However, some differences were observed when compared to the individual antibiotics. Overall, an increment in the shoot length of mixtures was observed in BM1 as compared to the individual antibiotics. Root length increase was observed in BM2 relative to the individual antibiotics. In BM3, the highest increase in the root length was observed at the concentration of 50 mgL^{-1}, which was statistically significant. Detailed results on antibiotic mixtures would be shared in the full-length paper.

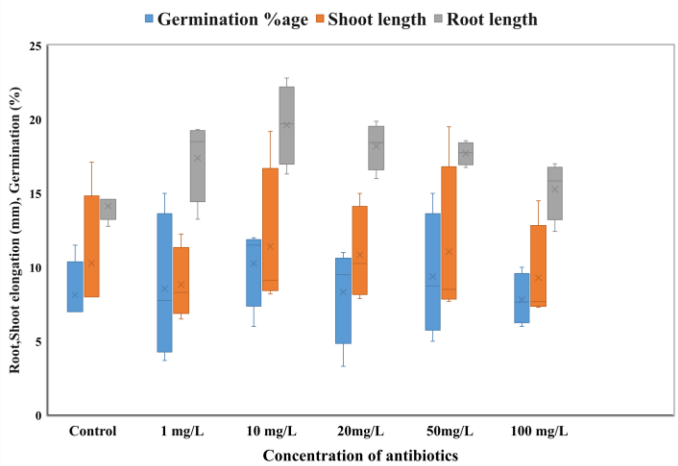

Figure 2. Comparison between average response of antibiotic mixtures on plant growth parameters upon exposure to different concentrations (mg/L).

The combination of the three antibiotics did not affect endpoints when results were compared to the control ($p < 0.05$), but root elongation of *T. alexandrinum* increased compared to the individual antibiotics (Fig. 2). The shoot length upon exposure to ternary mixtures presented an increase as compared to all the individual antibiotics AMP, OXY and LEV ($p < 0.05$) (Fig. 2).

4. Conclusion

It can be conldued form the results that the risk of individual antibiotics and their mixtures at environmentally relevant concentrations is negligible. However, it highly depends on the type and interaction of the antibiotics present in the environment. Furthermore, phyto-toxicological research is needed to better understand the possible effects of environmentally relevant concentration of antibiotics and their mixtures on plants in terms of their productivity and, food security.

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