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Research Article

Effect of veterinary antibiotics on the seed germination of *indica* rice varieties

Dipshika Motwani N. and Preeti Mehta*
Department of Plant Biology and Plant Biotechnology, Women's Christian College (Autonomous), 51 College Road, Chennai 600 006, India.

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Abstract: The effect of three antibiotics with different action modes (namely, tetracycline, amoxicillin and trimethoprim) on three different rice (*Oryza sativa* L.) varieties. Each antibiotic test had 6 treatments with tetracycline and amoxicillin concentrations of 0, 1, 10, 100, 1000, 10000 mg/L. The trimethoprim test also had 6 treatments with varying concentrations of 0,1,10, 50,100, 500 mg/L. The range of phytotoxicity of the antibiotics was on three types of indigenous rice species at the concentration of EC50 0.2 > 10,000 mg/L. None of the antibiotics caused a significant decrease in seed germination for three types of rice plant species. Compared with seedling vigor index, shoot and total length measurements, root elongation was consistently the most sensitive end point. The additional use of screening assays for evaluating environmental impacts of antibiotics can provide insight into relative species sensitivity and serve as a basis by which to screen the potential for toxic effects of novel compounds to plants.

Key words: Germination, seedlings, *indica* rice, antibiotics, phytotoxicity.

Abbreviations : VA – Veterinary antibiotics; FGP - Final germination percentage; GE % - germination energy percentage; DAS – days after sowing; CRD - completely randomized design; LSD - least significant difference.

Introduction

Rice (*Oryza sativa* L.) is life and is one of the most important cereals in this world (Savitha *et al.*, 2015). It is the staple of more than half of the world's population. Worldwide, more than 3.5 billion people depend on rice for more than 20% of their daily calories. Paddy production in 2017 is now predicted to be at 758.8 million tones. India is the second largest producers of rice accounting for 20% of all world rice population. There are about 10,000 varieties of rice in the world out of which about 4,000 are grown in India. India's annual paddy production is around 85-90 million tonnes. West Bengal is the state which is the largest producer of rice in India, with a production of 146 lakh tonnes and a yield of 2600 kilograms per hectare.

Veterinary antibiotics (VAs) have been widely used to treat diseases and to protect the health of animals for several decades As most VAs undergo metabolic changes in animals, approximately 90% of them are excreted in urine and 75% in animal feces in one dose. Thus, VAs may be excreted as the parent compounds and/or metabolites, which enter into the environment through the spreading of animal manure on agricultural land, direct deposition by grazing livestock, and the discharge of wastewater (Minden *et al.*, 2017). They cause damages to the ecosystem when discharged into the environment, but there is a lack of information on their toxicity to plants and animals.

The use of antibiotics in veterinary medicines represents a widespread practice (Ghava *et al.*, 2015). Extensive use of antimicrobials in veterinary medicine results in environmental exposure (Vaclavik *et al.*, 2004). Veterinary antibiotics are widely used to prevent infections in and promote growth in farm animals (Pan and Chu *et al.*, 2016, 2017a,b). Antibiotics are specifically designed to control bacteria in human or animals and help to protect their health (Liu *et al.*, 2009). They are also incorporated into animal feed to improve growth rate and feed efficiency (Sarmah *et al.*, 2006). Antibiotics also favour growth by decreasing degree of activity of the immune system, reduced waste of nutrients and reduce toxin formation (Nisha, 2008).

The environment may be exposed to veterinary medicine administered to livestock through the application of organic fertilizers to land (Kay *et al.*, 2004). Absorption of antibiotics in the animal gut is not complete and as a result substantial amounts of antibiotics are excreted in urine and faeces that end up in manure (Kumar *et al.* 2005). The assumed quantity of antibiotics excreted by animal husbandry adds up to thousands of tonnes per year (Kemper, 2008). Once, manure is applied to agricultural land to improve soil productivity crops would be exposed to antibiotics which may persist in soils from a few to several hundred days (Rani J. Bassil *et al.*, 2013).

***Corresponding Author:**
Dr. Preeti Mehta,
Dept of Plant Biology & Plant Biotechnology,
Women's College,
Chennai 600 006, India.
E-mail: preetimehta264@gmail.com

Animal-used antibiotics were mainly found in a region with significant agricultural activity. The presence of antibiotics in the environment can adversely affect the soil system (Pan and Chu *et al*., 2016). Plants uptake the antibiotics contained in the manure-treated soil; and lead to bio-accumulation of metabolites which can affect the growth of these plants. Various antibiotics in the soil can be accumulated in plant tissues and have either detrimental or enhancing effects on functional traits of crop and wild plant species (Minden *et al*., 2017). Since the beginning of the "antibiotic era", the effects of antibiotics on plant growth have been tested, in general with inhibitory results (Ghava *et al*., 2015). Whether these effects also occur for lower antibiotic concentrations remains largely unclear (Minden *et al*., 2017). These effects depend on the nature of the crop species and the kind of antibiotic used. Most studies on the environmental fate of antibiotics focus on aquatic environments or wastewater treatment plants. Little is known about the behavior of antibiotics on vegetables at environmentally relevant concentrations in agricultural soil (Kang *et al*., 2013, Ahmed *et al*., 2017). To address this knowledge gap, the effect of three antibiotics with different action modes (namely, tetracycline, amoxicillin and trimethoprim) on three indigenous rice varieties were studied.

Materials and Methods

Seeds of three *indica* rice varieties were collected and used for this study. TKM13 and Ponni were collected from Thiruvallur district, Tamil Nadu and IR64 from Kurnool, Andhra Pradesh. Healthy, uniform seeds of all varieties were surface sterilized with 1% sodium hypochlorite for 3 min, 70% ethanol and distilled water and surface dried using Whatman paper. Thirty seeds for each cultivar in each treatment were allowed to germinate on a filter paper in 9 cm diameter Petri dishes.

Each antibiotic test had 6 treatments with tetracycline and amoxicillin concentrations of 0, 1, 10, 100, 1000, 10000 mg/L, . The trimethoprim test had 6 treatments with concentrations of 0, 1, 10, 50, 100, 500 mg/L. Each treatment including controls was carried out in three replicates. 10 ml of appropriate solution was applied to each Petri dish. The Petri plates were arranged in completely randomized design (CRD) with three replicates for each treatment. Germination room temperature was maintained at 25 ± 1°C in the dark with 8 h photoperiod and a relative humidity of 70%. Petri plates were periodically checked and respective solutions were applied to compensate evaporation (Swathi *et al*., 2017). Seeds were considered germinated when the radicle had extended for at least 2mm. Seedling shoot and root length of randomly selected seedlings from each replication were measured at the time of harvest (4 and 7 days after treatment application) by using a scale. After final count, final germination percent (FGP) and germination energy percentage (GE %) were calculated by the following formulae (Ruan *et al*., 2002, Hassan *et al*., 2013).

GE (%) = Number of germinated seeds at 4 DAS x 100
Total number of seed tested

FGP = Number final germinated seeds x 100
Total number of seed tested

Seedling vigour index (V) was computed according to Hassan *et al*., 2013 as follows:
V= L x FG where L is the mean seedling length (total shoot and root length in mm) and FG is final germination percentage (%). This experiment was repeated twice to determine the consistency of results of various varieties against different levels of antibiotic concentrations.

Data were analyzed using Analysis of Variance method (ANOVA) (http://faculty.vassar.edu/lowry/anova1u.html) and least significant difference (LSD) for individual parameters.

Viability test : The viability and quality of seeds used for the experiment were tested using triphenyltetrazolium reagent (Himedia, Mumbai). This was done according to Peters, 2000. Three replicates of 5 seeds were soaked in water for 24 h, cut along the margin without damaging the embryo and soaked in 1% solution of 2,3,5 – triphenyltetrazolium chloride (TTC) solution at 25 ± 1°C in the dark. The seeds were removed from the TTC solution after 24 h and the embryos were observed. The embryos of viable seeds appeared reddish in colour.

Statistical Analysis:
All experiments were performed in three replicates. The end points were evaluated after 7 days for 3 indigenous rice varieties using one-way analysis of variance (ANOVA) to identify significant effects with a type I error rate (a) of 0.05.

Averages and standard deviation of the growth inhibition were calculated. The averages of growth inhibition were compared by Tukey's test and P-values were determined to evaluate the differences among treatments as the following, averages and standard deviation of the relative concentrations was calculated the regression analysis, averages may be compared by Tukey's test at = 0.05, following one-way ANOVA and plotting % growth data versus the concentration of the antibiotic was analyzed by linear regression to calculate the EC50. Dunnett's adjustment (a = 0.05), which allows for unplanned comparisons between all means and the control was also determined. The raw data were analyzed using regression models using GraphPad Prism 7.

Results and Discussion

Viability test :

The viability test using 1% triphenyltetrazolium revealed that the 3 indigenous rice varieties were viable initially. All the embryos were stained red.

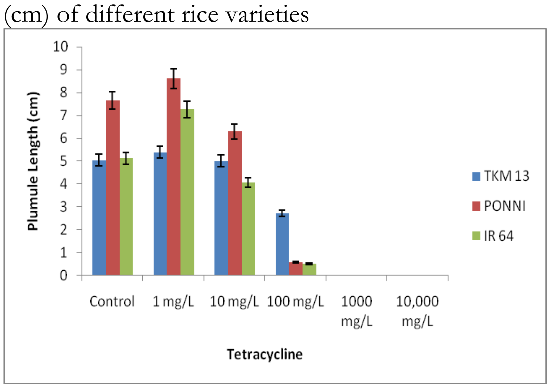
Germination test :

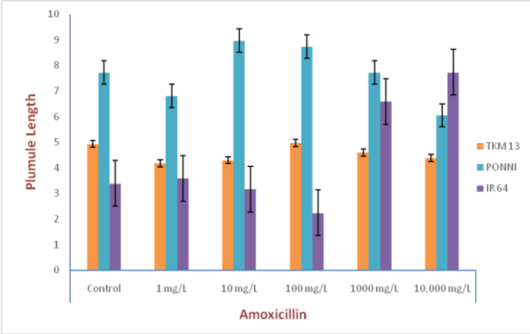
None of the antibiotics caused a significant decrease in seed germination for the three indigenous rice varieties (Tables 1,2,3). Effect of antibiotics on seed germination of three types of rice varieties at the concentration of EC50 0.2 > 10,000 mg/L (Table 4). The ANOVA results showed that there was an effect of antibiotic on all the characters studied at 100 mg/ L (the median value) at p≥0.01.

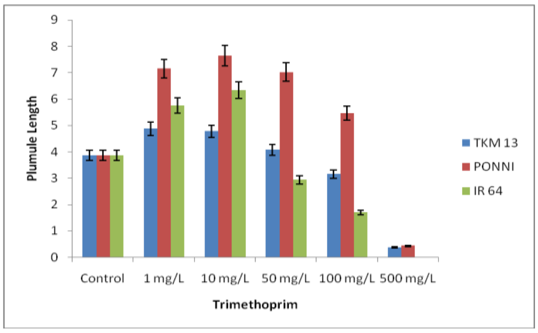
Plumule Length:

The plumule length gradually decreased with the increasing concentrations of all the three antibiotics (Fig. 1). No emergence of the plumule was seen at very high concentrations of tetracycline (1,000 and 10,000 mg/L) and trimethoprim (100 mg/L and 500 mg/L) concentrations. Although, there was no effect of Amoxicillin on the germination energy, the plumule length of the germinating seeds was affected. At 100 mg/L of tetracycline concentration, the plumule lengths were measured to be 2.73 cm (TKM 13), 0.57 cm (Ponni) and 0.51 cm (IR 64); with 100 mg/L of Amoxicillin, the plumule lengths were measured to be 4.97 cm (TKM 13), 8.73 cm (Ponni) and 2.24 cm (IR 64); and at 100 mg/L of trimethoprim, the plumule lengths were 3.15 cm (TKM 13), 5.46 cm (Ponni) and 1.7 cm (IR 64). However at very low concentrations of the antibiotic (1 mg/L), there was an increase in the plumule length. This could be due to osmopriming effect as it is a low antibiotic concentration. When subjected to one -way ANOVA, the results remained Significant (p≥0.01).

Figure 1. Effect of 3 antibiotics on plumule length (cm) of different rice varieties



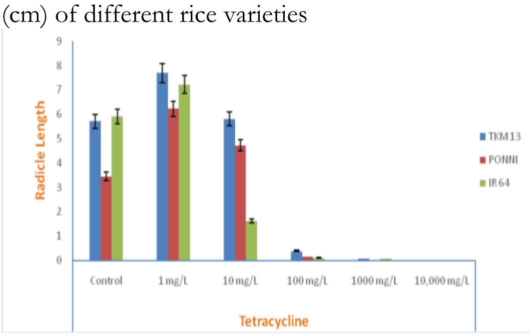


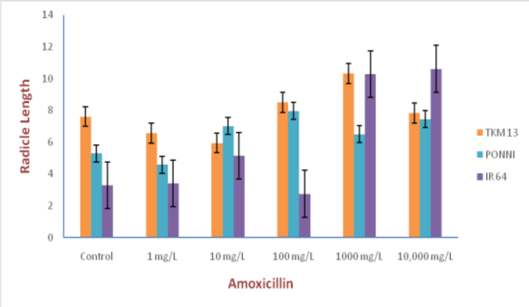


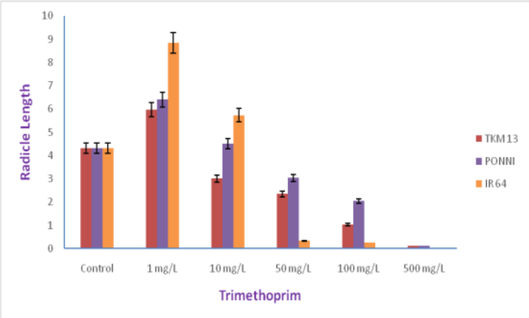
Radicle Length:

Similarly, the antibiotics had an effect on the radicle length of the growing seedlings (Fig. 2). In TKM 13, the radicle length at 100 mg/L varied from 0.39 cm in tetracycline, 1.03 cm in trimethoprim to 8.49 cm in amoxicillin. In Ponni, the average radicle length was measured to be 0.15 cm (Tetracycline), 2.04 cm (Trimethoprim) and 7.96 cm (Amoxicillin). In IR 64, the average radicle length was found to be 0.11 cm (Tetracycline), 0.26 cm with trimethoprim and 2.74 cm with 100 mg/L of Amoxicillin. At 1 mg/L, due to the effect of osmopriming, there was a slight increase in the radicle length. The results remained significant (p≥0.01) when subjected to one –way ANOVA.

Figure 2. Effect of 3 antibiotics on radicle length (cm) of different rice varieties







Seeding Vigour Index (SVI):

Tetracycline and Trimethoprim showed significant effects on the rice seedlings. They had an enhancing effect on the seeds at 1 mg/L of the antibiotic. At 100 mg/L of tetracycline, the seedling vigour index was calculated to be 312 for TMK 13, 72 for Ponni and 61.4 for IR 64. With 100 mg/L of Trimethoprim, the SVI was 418 in TKM 13, 750 in Ponni and 196 in IR 64. In contrast, Amoxicillin did not show significant changes for the values obtained in SVI.

EC50:

The effects of the antibiotics were compared using EC50 values for the most sensitive end points. Root length was the most sensitive end point for all three of the tested species exposed to Tetracycline. Tetracycline showed a consistent inhibitory response, affecting the Root and Shoot lengths of the three rice varieties. IR64 was most sensitive to Tetracycline with EC50 affecting Root Length at 3.522 and Shoot Length at 11.082. Amoxicillin did not induce measurable effect on the rice seeds. However, it resulted in significant decrease on the Root Lengths of Ponni (EC = 0.342) and Shoot Length of TKM13 (EC = 13.705). Trimethoprim also showed significant decrease to the Root Length of TKM13 with an EC50 value of 0.201. Ranking of the geometric mean of EC50's for the most sensitive end point for the three varieties of seeds to a given antibiotic indicated an order of most to the least affecting - Tetracycline > Trimethoprim > Amoxicillin.

Responses to antibiotics were conducted using EC50 values for the most sensitive end point (Table 4). Based on regression analysis, only tetracycline produced an EC50 < 110 mg/L. Interestingly, tetracycline was the only compound that showed a consistent phytotoxic response for all three rice seeds. Of the 3 tested compounds, amoxicillin did not induce measureable phytotoxic effects on any plant seeds. Amoxicillin was the only antibiotic not to produce a significant adverse effect level in any species up to the maximum concentration tested of 10,000 mg/L. Ranking of the geometric mean of EC50 for the most sensitive end point for the three types of seeds to a given antibiotic indicated an order of most to least phytotoxic of tetracycline > trimethoprim > amoxicillin. As a whole, the range of phytotoxicity of the antibiotics was large, with EC50s ranging from 0.2 to > 10,000 mg/L. Among the tetracyclines, within a plant seeds, potency was moderate, and phytotoxic responses to tetracycline and trimethoprim were similar. Similar trends have been found in aquatic plants exposed to tetracyclines (Brain *et al.*, 2008). The relative similarity in threshold values for a given class of pharmaceutical is to be expected because these compounds, despite structural differences, behave similarly and elicited similar responses in the three types of rice tested.

Effect of radicle length and germination :

The results of this study suggest that germination is not a useful end point for plant testing. Germination was only significantly affected by two antibiotics at the highest treatment concentration of 10,000 mg/L of tetracycline and 500 mg/L trimethoprim. Germination did not seem to be affected by the presence of amoxicillin. Fundamentally, this confirms that plant germination is a highly conserved process, with many of the nutrients, carbohydrates, and proteins stored and available for seedling emergence even if cellular processes to convert these compounds to more bioavailable forms are negatively affected (Basset *et al.*, 2002, 2005).

The increased sensitivity of root growth compared with germination to phytotoxic compounds is a common observation in plant studies. With few exceptions, root elongation was the most sensitive end point of the three length measurements for each of the plant species. Compared with germination, root elongation detected antibiotic-induced phytotoxic responses regularly. This result supports the continued use of root elongation as the primary end point in conducting short-duration culture experiments (Kapustka, 1997).

Risk Assessment:

determination of toxicity of antibiotics to plants is beneficial in order to understand the risk of antibiotics to the environment. For example, in this study, tetracycline was determined to be one of the more toxic compounds at environmentally relevant concentrations, with significant effects observed as low as 1,000 mg/L and EC50 ranging from 3.5 to 103 mg/L (Table 4). Another important consideration in assessing the risk of antibiotics to the environment is the specific use of the antibiotic, such as whether it is primarily used in human or veterinary treatment as well as whether exposures result from low-level prophylactic uses or a large pulse after a disease outbreak. Of importance in an agricultural context is how biosolids or manures containing pharmaceuticals are stored and treated before they are applied to a terrestrial system (Haller *et al.*, 2002). For plants, anaerobic digestion of animal manure in holding tanks before application onto agricultural fields might decrease the risk associated with phytotoxicity from land application of manure (Göbel *et al.*, 2005).

Table 1. Effect of 3 antibiotics on Plumule Length (cm) of 3 indigenous Rice Varieties:

Variety	Tetracycline					
	Control	**1 mg/L**	**10 mg/L**	**100 mg/L**	**1000 mg/L**	**10000 mg/L**
TKM 13	5.04±0.49	5.39±0.51	5.01±0.46	2.73±0.37	0±0	0±0
PONNI	7.65±0.49	8.61±0.51	6.29±0.46	0.57±0.37	0.01±0	0±0
IR 64	5.13±0.49	7.27±0.51	4.07±0.46	0.57±0.37	0±0	0±0
	Amoxicillin					
	Control	**1 mg/L**	**10 mg/L**	**100 mg/L**	**1000 mg/L**	**10000 mg/L**
TKM 13	4.93±0.71	4.17±0.57	4.30±0.90	4.97±0.96	4.6±0.53	4.38±0.53
PONNI	7.72±0.71	6.81±0.57	8.97±0.90	8.73±0.96	7.73±0.53	6.05±0.53
IR 64	3.39±0.71	3.59±0.57	3.17±0.90	2.24±0.96	6.59±0.52	7.73±0.53
	Trimethoprim					
	Control	**1 mg/L**	**10 mg/L**	**50mg/L**	**100 mg/L**	**500 mg/L**
TKM 13	3.86±0.44	4.87±0.34	4.77±0.45	4.07±0.61	3.15±0.58	0.37±0.06
PONNI	3.86±0.44	7.15±0.34	7.64±0.45	7.02±0.61	5.46±0.58	0.43±0.06
IR 64	3.86±0.44	5.76±0.34	6.34±0.45	2.93±0.61	1.7±0.58	0±0.06

Values represent mean ±standard error

Table 2. Effect of 3 antibiotics on Radicle Length (cm) of 3 indigenous Rice Varieties:

	Tetracycline					
Variety	**Control**	**1 mg/L**	**10 mg/L**	**100 mg/L**	**1000 mg/L**	**10000 mg/L**
TKM 13	5.71±0.49	7.69±0.51	5.81±0.78	0.39±0.04	0.07±0.01	0±0
PONNI	3.45±0.49	6.23±0.51	4.72±0.78	0.15±0.04	0.03±0.01	0±0
IR 64	5.91±0.49	7.22±0.51	1.63±0.78	0.11±0.04	0.07±0.01	0±0
	Amoxicillin					
	Control	**1 mg/L**	**10 mg/L**	**100 mg/L**	**1000 mg/L**	**10000 mg/L**
TKM 13	7.61±0.79	6.55±0.54	5.95±0.35	8.49±1.03	10.33±0.84	7.83±0.59
PONNI	5.29±0.79	4.58±0.54	7.01±0.35	7.96±1.03	6.50±0.84	7.45±0.59
IR 64	3.28±0.79	3.41±0.54	5.13±0.35	2.74±1.03	10.27±0.84	10.61±0.59
	Trimethoprim					
	Control	**1 mg/L**	**10 mg/L**	**50mg/L**	**100 mg/L**	**500 mg/L**
TKM 13	4.31±0.52	5.97±0.62	3.01±0.54	2.34±0.54	1.03±0.28	0.1±0.01
PONNI	4.31±0.52	6.40±0.62	4.50±0.54	3.04±0.54	2.04±0.28	0.1±0.01
IR 64	4.31±0.52	8.85±0.62	5.73±0.54	0.32±0.54	0.26±0.28	0±0.01

Values represent mean ±standard error

Table 3. Effect of on seedling vigour of *indica* rice seed varieties after 7 days growth

	Tetracycline					
Variety	**Control**	**1 mg/L**	**10 mg/L**	**100 mg/L**	**1000 mg/L**	**10000 mg/L**
TKM 13	1075±50.73	1309±51.19	1082.6±117.19	312±42.09	7±1.42	0±0
PONNI	1110±50.73	1483±51.19	1101±117.19	72±42.09	4±1.42	0±0
IR 64	1104±50.73	1449±51.19	570±117.19	61.4±42.09	7±1.42	0±0
	Amoxicillin					
	Control	**1 mg/L**	**10 mg/L**	**100 mg/L**	**1000 mg/L**	**10000 mg/L**
TKM 13	1254±126.59	1073±0.05	1025±117.98	1413±187.16	1493±71.24	1221±106.27
PONNI	1301±126.59	1139±0.05	1599±117.98	1669±187.16	1423±71.24	1398±106.27
IR 64	672±126.59	699±0.05	831±117.98	498±187.16	1686±71.24	1036.8±106.27
	Trimethoprim					
	Control	**1 mg/L**	**10 mg/L**	**50mg/L**	**100 mg/L**	**500 mg/L**
TKM 13	816.7±90.27	1084.6±68.97	778±91.36	641.3±103.15	418±84.58	46.7±8.57
PONNI	817±90.27	1355±68.97	1214±91.36	1006±103.15	750±84.58	53±8.57
IR 64	817±90.27	1461±68.97	1207±91.36	324±103.15	196±84.58	0±8.57

Values represent mean ±standard error

Table 4. Effect of 3 antibiotics on germination of 3 indigenous rice varieties over 7 days

Antibiotics	**Root Length**			**Shoot Length**		
	IR64	**Ponni**	**TKM13**	**IR64**	**Ponni**	**TKM13**
Amoxicillin	612.433	0.342	36.864	796.956	17587.230	13.705
Tetracycline	3.522	16.963	18.475	11.082	18.469	103.980
Trimethoprim	12.561	120.192	0.201	52.119	142.916	177.082

Conclusion

In this study, the toxicological effect of three antibiotics was observed on the germination of three indigenous rice varieties. Characters such as seedling height, root length and seedling vigour index were collected and recorded. Root length was the most sensitive end point for all three of the tested species exposed to Tetracycline. Tetracycline was found to be the most toxic to seed germination. This was followed by Trimethoprim and Amoxicillin which showed the least phytotoxicity. Considering the environmental levels and fate of these antibiotics in soil, we would expect low toxic effects on plant growth and also a fast recovery from the stress due to the loss and/or binding of the antibiotics onto soil components.

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