

Phytotoxic Effect of Antibiotic Residues on Forage Seeds

Eluk, D.¹; Frank, R.¹; Nagel O. G.¹; Molina, M. P.²; Althaus, R. L.^{1*}

¹Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Kreder 2805, (3080) Esperanza, Argentina.

* Fax number: 54-3496-426304.

²Instituto de Ciencia y Tecnología Animal, Universidad Politécnica de Valencia, Camino de Vera S/N, (46072) Valencia España.

*Corresponding author email id: ralthaus@fcv.unl.edu.ar

Abstract – In veterinary medicine, antibiotics are used for the treatment of infectious diseases. Some of these antibiotics are not completely metabolized and can be eliminated through the milk, urine and/or feces. In this way, antibiotic residues can reach the soil and affect the development of seeds and plants. The aim of this work was to study the effect of different concentrations of five antibiotics (enrofloxacin, kanamycin, oxytetracycline, penicillin and tylosin) on the germination frequency and root elongation of seeds of three forage species (alfalfa (*Medicago sativa*), melilotus (*Melilotus albus*) and white clover (*Trifolium repens*)). The results showed that the antibiotic concentrations allowed for the dairy industry (maximum residue limits) were detrimental to the germination and growth of these seeds: 0.10 mg/L of enrofloxacin affected the three species studied, whereas 0.10 mg/L of oxytetracycline affected *M. sativa* and *T. repens*. Due to the negative effect of antibiotic residues on forage seeds, the whey contaminated with these residues should be treated before being discarded into the soil.

Keywords – Antibiotics, Forage Seeds, Germination Frequency, Root Elongation.

I. INTRODUCTION

In veterinary medicine, antibiotics are frequently used for the treatment of infectious diseases such as enteritis, hoof diseases, mastitis, metritis and pneumonia [1]-[2]. Some of these antibiotics are not completely metabolized and can be eliminated through the milk, urine and/or cattle manure [5]-[9]. Thus, these bioactive molecules can reach the soil and affect both the composition of the microbiota [10]-[13] and the development of plants [14]-[16]. In addition, antibiotic residues can reach the rivers adjacent to livestock farms [17]-[21] or cross the soil, reaching the groundwater [22]-[25]. This problem can be exacerbated in the case of small cheese industries that use landfarming to treat whey contaminated with antibiotics [26]-[27]. This bioremediation technique requires the use of large land plots, presents slow degradation rates, and needs long periods of stabilization to complete the treatment. So, the impact of landfarming on the environment must be carefully evaluated for each case [28].

Among the various methodologies used to evaluate the environmental impact of antibiotics, some authors have proposed to carry out phytotoxicity studies on seed germination and plant growth. For example, [16] analyzed the effect of six antibiotics (chlortetracycline, tetracycline, tylosin, sulfamethoxazole, sulfamethazine and trimethoprim) on the growth of rice (*Oryza sativa* L.), cucumber (*Cucumis sativus* L.) and endive (*Cichorium endivia*) and found that 0.10 mg/L of sulfamethoxazole is

harmful for the development of rice plants. In addition, [21] indicated that 20 mg/L of chlortetracycline produces yellowing, growth retardation and reduction of the leaf expansion in *Arabidopsis thaliana*. Similarly, [29] analyzed the effect of ten antibiotics (amoxicillin, chlortetracycline, levofloxacin, lincomycin, oxytetracycline, sulfamethazine, sulfamethoxazole, tetracycline, trimethoprim and tylosin) on the germination and development of lettuce (*Lactuca sativa*), alfalfa (*Medicago sativa*) and carrots (*Daucus carota*). These authors indicated a wide range of dangerous antibiotic concentrations, ranging from 3.9 µg/L levofloxacin for *L. sativa* to 10000 µg/L trimethoprim for *M. sativa* and *D. carota*, and emphasized that levofloxacin, chlortetracycline and tetracycline are the compounds with highest toxicity and that amoxicillin and trimethoprim are those with lowest toxicity. Reference [30] also evaluated the phytotoxic effects of some veterinary antibiotics (chloramphenicol, erythromycin, norfloxacin, sulfamethazine and tetracycline) on the seed germination and root elongation of lettuce (*L. sativa*), tomato (*Solanum lycopersicum*), carrots (*D. carota*) and cucumber (*C. sativus* L.). Their results showed that these compounds significantly inhibit the root elongation ($p < 0.05$) of the four species studied, highlighting that tetracycline was associated with the highest level of toxicity and that lettuce was the most sensitive crop. Reference [31] studied the effect of five antibiotics (enrofloxacin, kanamycin, oxytetracycline, penicillin and tylosin) on the germination frequency and root elongation of five crops and found phytotoxic effects of 0.10 mg/L enrofloxacin on sorghum (*Sorghum bicolor*), 0.15 mg/L kanamycin on corn (*Zea mays*) and soybean (*Glycine max*), 0.004 mg/L penicillin on corn (*Z. mays*), soybean (*G. max*) and sorghum (*S. bicolor*) and 0.05 mg/L tylosin on soybean (*G. max*).

The purpose of this work was to study the phytotoxicity of five antibiotics (enrofloxacin, kanamycin, oxytetracycline, penicillin and tylosin) on the germination frequency and root elongation of three forage species frequently cultivated in Argentina: alfalfa (*Medicago sativa*), melilotus (*Melilotus albus*) and white clover (*Trifolium repens*).

II. MATERIALS

A. Antibiotics

Representative drugs were selected from five groups of antibiotics: enrofloxacin (quinolones), kanamycin (aminoglycosides), oxytetracycline (tetracyclines), penicillin (beta-lactams) and tylosin (macrolides). To

evaluate the possible phytotoxic effect of whey containing antibiotic residues deproteinized whey was used. To this end whey was treated at 120°C for 20 minutes, followed by a removal of the precipitated proteins by filtration. Subsequently antibiotic aliquots at concentrations equivalent to their acceptable maximum residue limits (MRLs) in milk [32]-[33] were added as minimum study values (MRL_{enrofloxacin}: 0.10 mg/L, MRL_{oxytetracycline}: 0.10 mg/L, MRL_{kanamycin}: 0.15 mg/L, MRL_{penicillin}: 0.004 mg/L, MRL_{tylosin}: 0.05 mg/L). Based on these levels, solutions containing the following concentrations of antibiotics were prepared using a logarithmic scale: C₀= 0 MRL, C₁= 1 MRL, C₂= 10 MRLs, C₃= 100 MRLs and C₄= 1000 MRLs.

B. Forage Seeds

Seeds from the following forage species were provided by the Department of Intensive Cultivation of Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, (Argentina): alfalfa (*Medicago sativa*), melilotus (*Melilotus albus*) and white clover (*Trifolium repens*).

C. Phytotoxicity Studies

The standard protocol to perform toxicity tests on terrestrial plants was followed [4]-[29]-[34]. Seeds from the chosen species were stored for one month at 4°C. Subsequently, 900 experimental units of each species were selected according to the toxicity tests in the protocol for terrestrial plants [35]. For each seed and antibiotic concentration, five replicates were made in Petri plates (100 x 15 mm) using 25 Petri plates/antibiotic and seed. On each plate, 10 seeds were placed on a Whatman cellulose filter. Subsequently, 10 ml of each of the deproteinized whey solutions containing different levels of antibiotics (C₀, C₁, C₂, C₃ and C₄) was added.

The plates were covered with Parafilm M (Sigma-Aldrich) to avoid drying and incubated for seven days at 25°C in a chamber. According to the protocol for the control of germination, the criterion of acceptability was set at 70%.

III. METHODS

I. Relative Germination Frequency.

For each seed and antibiotic concentration, relative germination frequencies were calculated at 12-hour intervals and over a period of seven days (14 controls). For this, the following Logistic Regression Model (LRM) of

the statistical software Stat Graphics® Centurion was used:

$$Y_{ijk} = L_{ijk} \left[\frac{P_{ijk}}{1 - P_{ijk}} \right] = \gamma_0 + \gamma_1 t_i + \sum \gamma_2 W_j + \varepsilon_{ijk} \quad (1)$$

Where: Y_{ijk} = L_{ijk} : Logistic function= $\ln [P_{ijk}/(1 - P_{ijk})]$; P_{ijk} : Seed germination frequency (1- P_{ijk}): Absence of germination frequency; γ_0 , γ_1 , γ_2 : Coefficients estimated by the logistic model; t_i : Effect of the germination time ($i=14$); W_j : Effect of antibiotic concentration in terms of dummy variables (W_0 : C₁=0, C₂=0, C₃=0 and C₄=0; W_1 : C₁=1, C₂=0, C₃=0 and C₄=0; W_2 : C₁=0, C₂=1, C₃=0 and C₄=0; W_3 : C₁=0, C₂=0, C₃=1 and C₄=0; W_4 : C₁=0, C₂=0, C₃=0 and C₄=1) and ε_{ijk} : Residual error.

II. Root Elongation.

Seven days after the start of the experiment, the lengths of each root were measured in duplicate. Measurements were made from the tip of the radicle to the hypocotyl, using a Vernier caliper with a precision sensitivity of 0.05 mm. Analysis of Variance (ANOVA, Stat Graphics® Centurion) was used to evaluate the effect of the different concentrations of antibiotics on root elongation. Then, the Tukey test was applied to analyze significant differences in each antibiotic concentration.

IV. RESULTS AND DISCUSSION

I. Relative Germination Frequency.

Table 1 shows the statistical results (“ χ^2 ” and “p” value) of the application of the logistic regression model for the different concentrations of antibiotics that significantly affected the germination frequencies of the seeds ($p < 0.05$). In that sense, enrofloxacin affected *Medicago sativa* and *Trifolium repens*, whereas kanamycin inhibited *M. sativa* and *Melilotus albus*. Oxytetracycline negatively affected the germination of the three forage species, whereas penicillin and tylosin were the most harmless ones.

Table 2 presents the significant effect ($p < 0.05$) of the time on the germination frequencies (γ_1 positive coefficients) and the action of the different concentrations of antibiotics on the seeds (γ_2 negative coefficients). The phytotoxicity of antibiotics was evidenced by the increase in the magnitude of the γ_2 coefficients. Thus, the effect of enrofloxacin on *T. repens* was stronger ($\gamma_2 = -2.4049$) than that of kanamycin on *M. albus* ($\gamma_2 = -0.5021$).

Table 1: Statistical parameters of the effects of time and concentration of antibiotics on the germination frequency of seeds calculated using the logistic regression model

Antibiotic	Seeds	Time		Antibiotic concentration	
		χ^2 valor	P valor	χ^2 valor	p-valor
Enrofloxacin	<i>Medicago sativa</i>	433	0.0001	112	0.0001
	<i>Trifolium repens</i>	250	0.0001	65.0	0.0001
Kanamycin	<i>Medicago sativa</i>	19.2	0.0001	12.9	0.0003
	<i>Melilotus albus</i>	778	0.0001	6.24	0.0124
Oxytetracycline	<i>Medicago sativa</i>	252	0.0001	12.0	0.014
	<i>Melilotus albus</i>	777	0.0001	301	0.0001
	<i>Trifolium repens</i>	338	0.0001	5.53	0.0192

Table 2: Logistic equations relating the germination frequency of seeds forage with time and concentrations of antibiotics

Antibiotic	Seeds	$L = \gamma_0 + \gamma_1 t + \sum \gamma_2 W$			Cr%	
		γ_0	γ_1	γ_2 Concentration		
Enrofloxacin	<i>Medicago sativa</i>	-2.0385	0.914	- 1.2529	C ₁ =C ₂ =C ₃ =C ₄	82
	<i>Trifolium repens</i>	-3.9283	1.1798	- 2.4049	C ₁ =C ₂ =C ₃ =C ₄	78
Kanamycin	<i>Medicago sativa</i>	-1.2559	1.3593	- 1.3379	C ₄	75
	<i>Melilotus albus</i>	-1.7020	1.6749	- 0.5021	C ₁ =C ₂ =C ₃ =C ₄	80
Oxytetracycline	<i>Medicago sativa</i>	-0.6633	0.9637	- 0.9481	C ₂ =C ₃ =C ₄	70
	<i>Melilotus albus</i>	-1.7802	0.7856	- 1.4885	C ₁ =C ₂ =C ₃ =C ₄	79
	<i>Trifolium repens</i>	-3.6733	2.0077	- 0.9481	C ₁ =C ₂ =C ₃ =C ₄	90

$\gamma_0, \gamma_1, \gamma_2$ = Coefficients estimated by the model. t = Test time. Cr% = Percentage relative agreement.

C₁ = 1 MRL, C₂ = 10 MRL, C₃ = 100 MRL, C₄ = 1000 MRL.

MRL_{enrofloxacin}: 0.10 mg/L, MRL_{oxytetracycline}: 0.10 mg/L, MRL_{kanamycin}: 0.15 mg/L, MRL_{penicillin}: 0.004 mg/L, MRL_{tylosin}: 0.05 mg/L

Fig. 1 shows that low concentrations of enrofloxacin (0.10 mg/L) negatively affected the germination of *M. sativa* and *T. repens*. In contrast, higher concentrations of kanamycin (150 mg/L) were required to inhibit the germination of *M. albus* and *M. sativa* (Fig. 2). Fig. 3 shows that low concentrations of oxytetracycline (0.10 mg/L) affected *M. albus* and *T. repens*, whereas higher concentrations (1.0 mg/L) were necessary to inhibit *M. sativa* germination.

Fig. 4 shows photos illustrating the inhibitory effect of the antibiotics on the germination frequency of forage seeds. These show that a low concentration of enrofloxacin (0.1 mg/L) affected *M. sativa* (a: treated group and b: control group). A decrease in the number of germinated seeds compared to the control group (without antibiotic) was observed on day 2 (a: 2 vs. b: 4), day 4 (a: 6 vs. b: 8) and day 6 (a: 9 vs. b: 10) of the experiment.

The phytotoxic action of low concentrations of enrofloxacin (0.10 mg/L) on *M. sativa* and *T. repens* observed in this study is in agreement with that reported by [31], who evaluated the effect of this quinolone on the germination frequency of sunflower (*Helianthus annuus*). Whereas in the present study, penicillin and tylosin had no effect on any of the three forage species studied, these

authors noted that low concentrations of penicillin (0.004 mg/L) had a phytotoxic effect on *G. max* and *S. bicolor* and that 0.05 mg/L of tylosin had a phytotoxic effect on *S. bicolor*. Also, [16] indicated that a high concentration of tylosin (300 mg/L) affects the germination of rice (*O. sativa* L.) and cucumber (*C. sativus* L.). However, [29] observed no phytotoxic effect of tylosin (10 mg/L) on the germination of alfalfa (*M. sativa*), carrot (*D. carota*) or lettuce (*L. sativa*).

In summary, it can be established that concentrations of enrofloxacin (0.10 mg/L) and oxytetracycline (0.10 mg/L) equivalent to their MRLs cause phytotoxic effects on the germination frequency of the forage seeds studied.

II. Root Elongation.

Table 3 shows the mean values, standard deviations and statistical parameters ("F" and "p" values) calculated with ANOVA for the antibiotics that presented phytotoxic effect ($p < 0.05$) on the forage seeds studied. It also shows the significant differences obtained with the Tukey Test. This table shows that enrofloxacin and oxytetracycline significantly affected ($p < 0.05$) the three forage species studied, whereas kanamycin inhibited *M. sativa* and *M. albus*, and that tylosin only affected *M. albus*. Penicillin was innocuous to all the forage seeds studied.

Table 3: Effect of antibiotic levels on whey on root lengths of forage seed measured at the seven day

Antibiotic	Seeds	Concentration (MRL)					Valor "F"	Valor "p"
		0	MRL	10MRL	100MRL	1000MRL		
Enrofloxacin	<i>Medicago sativa</i>	5,1 _a ± 0,2	3,5 _b ± 0,3	3,8 _b ± 0,2	4,5 _b ± 0,2	4,2 _b ± 0,2	18,67	0,0001
	<i>Melilotus albus</i>	7,2 _a ± 0,2	5,0 _b ± 0,3	3,0 _c ± 0,3	2,9 _c ± 0,4	3,4 _c ± 0,3	45,98	0,0001
	<i>Trifolium repens</i>	4,7 _a ± 0,3	1,7 _b ± 0,4	1,3 _b ± 0,4	0,9 _b ± 0,3	1,8 _b ± 0,3	22,76	0,0001
Kanamycin	<i>Medicago sativa</i>	5,4 _a ± 0,2	5,1 _a ± 0,2	4,7 _a ± 0,2	4,4 _a ± 0,2	3,4 _b ± 0,2	17,59	0,0001
	<i>Melilotus albus</i>	5,9 _a ± 0,4	6,1 _a ± 0,4	5,7 _a ± 0,4	5,2 _a ± 0,4	2,9 _b ± 0,5	6,84	0,0001
Oxytetracycline	<i>Medicago sativa</i>	2,8 _a ± 0,1	2,6 _a ± 0,1	2,2 _b ± 0,1	1,0 _c ± 0,1	1,1 _c ± 0,1	52,33	0,0001
	<i>Melilotus albus</i>	4,4 _a ± 0,2	4,3 _a ± 0,2	3,1 _b ± 0,2	2,2 _c ± 0,2	1,7 _c ± 0,3	30,27	0,0001
	<i>Trifolium repens</i>	4,0 _a ± 0,3	3,6 _{a,b} ± 0,2	2,9 _b ± 0,2	1,2 _c ± 0,2	1,1 _c ± 0,2	28,80	0,0001
Tylosin	<i>Melilotus albus</i>	5,3 _a ± 0,3	5,4 _a ± 0,2	4,8 _a ± 0,3	4,3 _{a,b} ± 0,3	3,3 _b ± 0,3	10,09	0,0001

a, b, c: Different subscripts for values of the same row indicate significant differences at a level of $p < 0.05$.

MRL_{enrofloxacin}: 0.10 mg/L, MRL_{oxytetracycline}: 0.10 mg/L, MRL_{kanamycin}: 0.15 mg/L, MRL_{penicillin}: 0.004 mg/L, MRL_{tylosin}: 0.05 mg/L

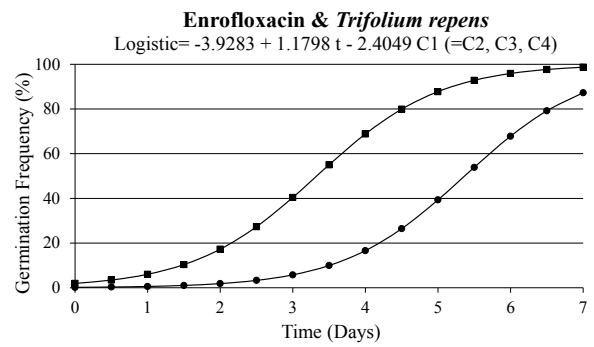
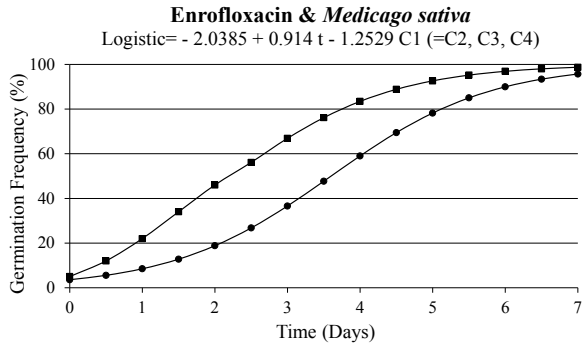


Fig. 1. Effect of different concentrations of enrofloxacin on the germination frequency of seeds tested (■: control; ●: C₁= 0.10 mg/L, C₂= 1.0 mg/L, C₃= 10 mg/L y C₄= 100 mg/L)

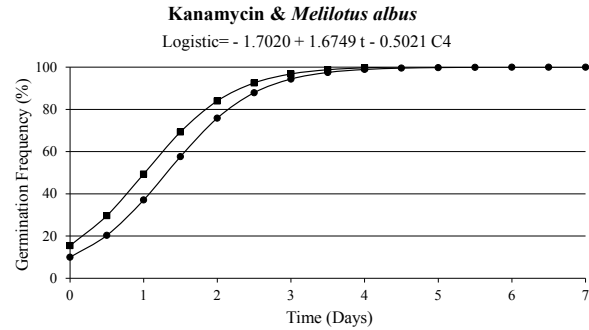
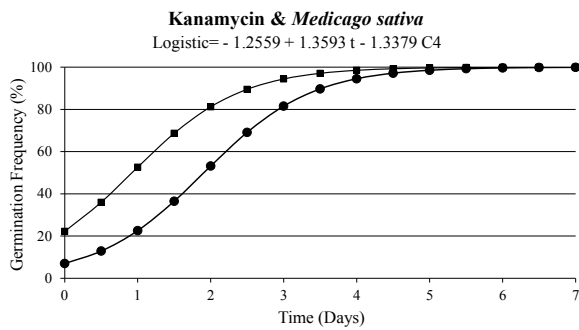


Fig. 2. Effect of different concentrations of kanamycin on the germination frequency of seeds tested (■: control; ●: C₄= 150 mg/L)

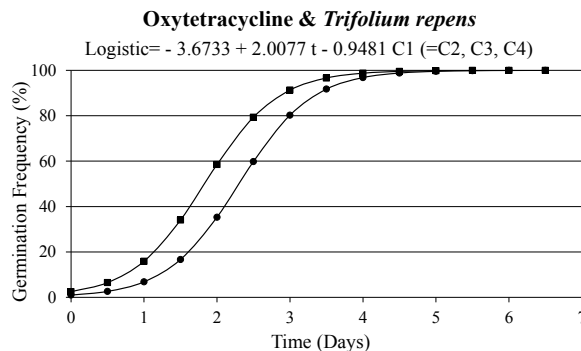
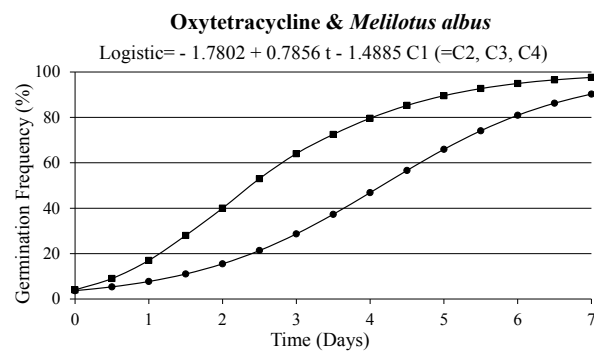
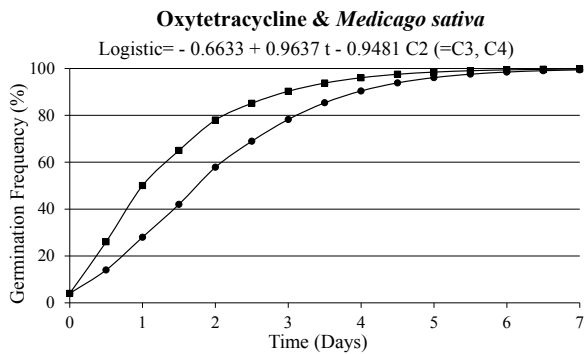
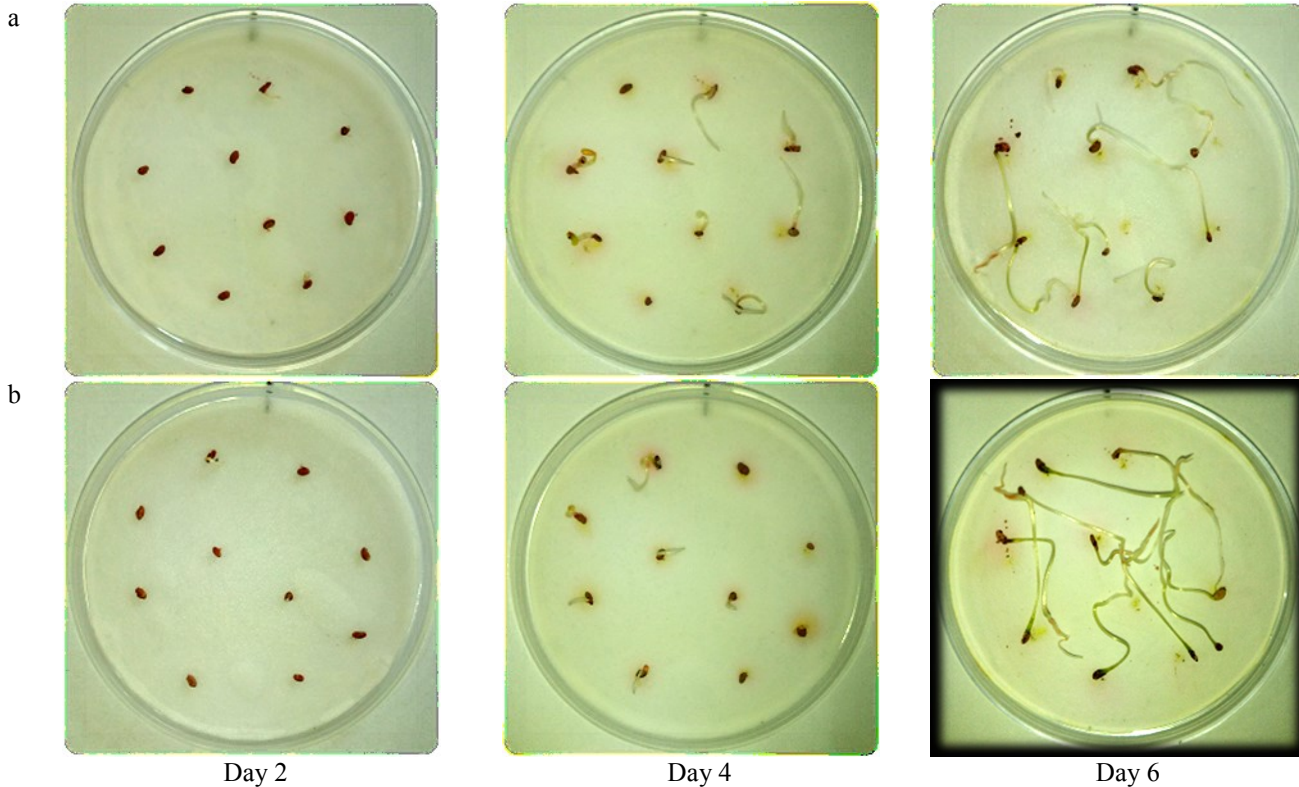


Fig. 3. Effect of different concentrations of oxytetracycline on the germination frequency of seeds tested (■: control; ●: C₁= 0.10 mg/L, C₂= 1.0 mg/L, C₃= 10 mg/L y C₄= 100 mg/L)



Day 2 Day 4 Day 6
 Fig. 4. Effect of 0.10 mg/L of enrofloxacin on the germination frequency of *Medicago sativa* seeds: (a) treated group, (b) control group.

It is observed that 0.10 mg/L enrofloxacin and oxytetracycline (1.0 mg/L) affected the root lengths of the three forage seeds, but that higher concentrations of kanamycin (150 mg/L) were necessary to affect *M. albus* and *M. sativa*, similar to that observed in the germination frequency (Figures 1 and 2). In contrast, 50 mg/L of tylosin inhibited the elongation of *M. albus* roots, but did not impair their germination frequency. This can be attributed to the fact that root elongation represents a more sensitive variable than the germination frequency, according to [4].

Similarly, [31] indicated that a low concentration of enrofloxacin (0.10 mg/L) affects *S. bicolor*, kanamycin (0.15 mg/L) inhibits *G. max* and *Z. mays* and tylosin (0.05 mg/L) affect *G. max*. In contrast, these authors indicated that a low concentration of penicillin (0.004 mg/L) is detrimental to the roots of *G. max*, *S. bicolor* and *Z. mays*.

Reference [29] reported the phytotoxic effect of oxytetracycline on the root elongation of *M. sativa* (10 mg/L), *L. sativa* (4.8 mg/L) and *D. carota* (1.6 mg/L). These authors also mentioned that tylosin (10 mg/L) inhibited the root elongation of *L. sativa* and *M. sativa*.

The effect of low antibiotic concentrations on root elongation can be seen in Fig. 5, where the action of enrofloxacin (0.10 mg/L) on *M. sativa* appears on day 7 of the experiment. This figure shows the significant decrease in root length due to enrofloxacin (Fig. 5a) compared to untreated seeds (Fig. 5b).

In summary, 0.10 mg/L enrofloxacin (MRL) caused a delay in the root elongation of the three forage species studied, with an average decrease of 60% in the length of

T. repens roots, whereas higher concentrations of kanamycin (150 mg/L), oxytetracycline (1.0 mg/L) and tylosin (50 mg/L) were needed to produce effects on the roots of some forage seeds (Table 3). Similar to that observed in the study of germination frequencies, penicillin had no effect on the root elongation of any of the three forage species studied.

To avoid the environmental impact of these antibiotics, different technological alternatives tending to inactivate these molecules should be analyzed. For example, for the degradation of quinolone residues in water, [36] suggested solar photocatalytic processes. Another alternative for the degradation of antibiotic residues in milk is the use of thermal treatments. In fact, the sterilization process (120°C – 20 min) of milk contaminated with antibiotics causes a significant decrease in the antimicrobial activity of kanamycin [37], penicillin [38]-[39] and tylosin [40], but is not enough to inactivate enrofloxacin [41].

V. CONCLUSIONS

The results presented in this work show that antibiotics cause a phytotoxic effect on forage seeds. In fact, concentrations equivalent to the maximum residue limits (MRLs) established for the dairy industry are harmful to crops. In this sense, 0.10 mg/L enrofloxacin (MRL) affected alfalfa (*Medicago sativa*), melilotus (*Melilotus albus*) and white clover (*Trifolium repens*), whereas 0.10 mg/L oxytetracycline (MRL) affected *M. sativa* and *T. repens*.

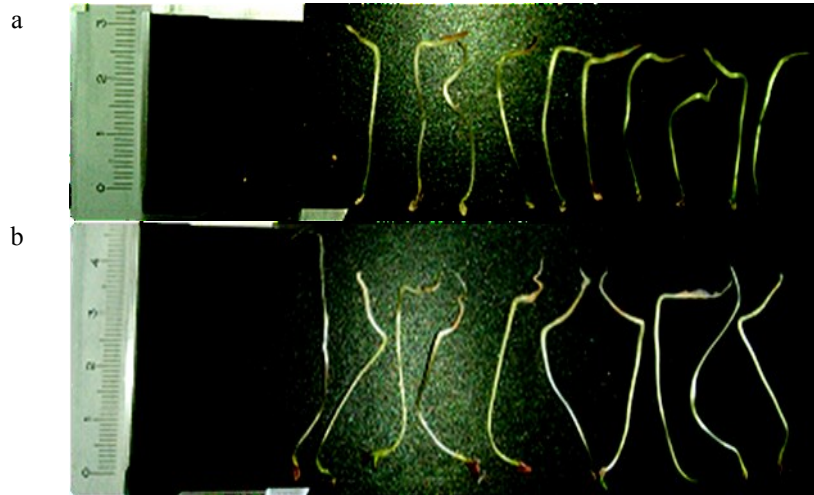


Fig. 5. Phytotoxicity of enrofloxacin (0.10 mg/L) on *Medicago sativa* root elongation after seven days of testing: (a) treated group, (b) control group

With the aim to expand these findings, other antibiotics should be evaluated. On the other hand, to find a suitable biosensor to evaluate the environmental impact of antibiotics, it would be interesting to continue these studies with other biomarkers such as bacteria (e.g. *Vibrio fischeri*), algae (e.g. *Chlorella vulgaris*) and invertebrates (e.g. *Daphnia magna*) or fish (e.g. *Salmo trutta*).

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AUTHOR'S PROFILE



Eluk D. was born in La Paz, Bolivia (1975). In 2002 she graduated in Biotechnology (Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral). She continued her studies and in 2007 she obtained a Master's degree in Food Sciences and Technology (Facultad de Ingeniería Química, Universidad Nacional del Litoral). She is currently a doctoral student in Veterinary Sciences of the Facultad de Ciencias Veterinarias (Universidad Nacional del Litoral).

She is Professor of Physics and Chemistry in the Department of Basic Sciences of the Facultad de Ciencias Veterinarias (Universidad Nacional del Litoral). Among her publications, a recent article stands out: Eluk, D., Nagel, O.G., Zimmermann, J., Molina, M.P., Althaus, R.L. 2016. Effect of antibiotics on the germination and root elongation of Argentine intensive crops. *International Journal of Environmental Research*. 10(4), 471-480.

MSc. Eluk served as a jury in several theses to obtain a Degree in Nutrition at the University of Concepción del Uruguay. Among the prizes obtained by Eluk, is the 1st Prize of the contest "Thesis in 3 minutes" organized in 2015 by the Ministry of Science and Technology (National University of the Litoral), the mention obtained by a paper presented at the XVI Argentine Congress of Health and Environment in 2008 and the Postgraduate Scholarship obtained in 2014 to carry out her PhD studies.