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Comparative study on some effects of exposure of wheat plants to three different antibiotics

Research article

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Abstract
Germinated wheat caryopses were planted on agar solidified nutrient solutions that were supplemented with three antibiotics (ampicillin, tetracycline and chloramphenicol, in separate experiments), each of them at two different concentrations: 4 mg.L^{-1} and 20 mg.L^{-1}, respectively, alongside controls unexposed to antibiotics. Seven days after the initiation of the experiment, the young wheat plants were harvested and the length of their root and shoot was measured. Leaf samples were collected and used for the extraction and assay of chlorophylls and carotenoids content. The obtained results have shown that ampicillin exposure didn't affect the analyzed parameters, while the exposure to tetracycline or chloramphenicol was followed by a marked, dose-dependent, inhibition of plant growth rate, compared to control plants. Lower contents of chlorophylls and carotenoids were also observed in the plants that were exposed to tetracycline and chloramphenicol, compared to unexposed ones. We concluded that besides the amount of antibiotic, its structure and mechanism of action influences phytotoxicity. Thus, on a toxicity scale inferred from the obtained results, chloramphenicol ranks first, followed by tetracycline and ampicillin.

Keywords: wheat seedlings, ampicillin, tetracycline, chloramphenicol, growth rate, photosynthetic pigments.

1. INTRODUCTION

Antimicrobial therapy saves lives, but overuse of antibiotics and poor waste management have led to contamination of ecosystems, disruption of ecological balances and promotion of antibiotic resistance. Antibiotics are used not only for therapeutic purposes, but also in aquaculture and animal husbandry to promote growth and avoid bacterial diseases. Nowadays, antibiotics are considered emerging pollutants [1].

Water and soil pollution with antibiotics is a threat to plants, both directly and through their microbiome; plants can also transfer antibiotics to higher trophic levels in the ecosystems.

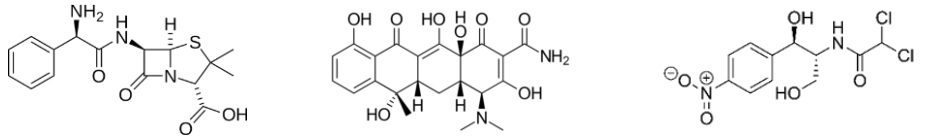
The experimental study, whose results will be presented below, pursued the following objectives:

- to cultivate wheat seedlings for 7 days, on solid nutrient solutions that were supplemented with antibiotics (ampicillin, tetracycline or chloramphenicol; Figure 1) at concentrations 4 and 20 $mg \cdot L^{-1}$, alongside untreated control.
- to analyze the plants growth data and the concentration of the photosynthetic pigments in theirs leaves;
- to correlate the obtained results with the level of plants' exposure to each antibiotic;
- to compare the observed effects of the antibiotics on plants, based on their mechanism of action.

2. MATERIALS AND METHODS

2.1. Biological material and experimental conditions

Wheat grains (*Triticum aestivum* L.) were purchased from a local market. Before sowing, the seeds were surface sterilized by immersion for 5 min in a 5% NaClO solution, then washed repeatedly with distilled water. For germination, seeds were placed between two filter paper discs moistened with distilled water, in 90 mm diameter Petri dishes that were closed and kept at 4°C in the dark for 24 h.



a. Ampicillin (AMP) [2] **b.** Tetracycline (TET) [3] **c.** Chloramphenicol (CHL) [4]

Figure 1. Structural formulas of the antibiotics used in the study

For each of the tested antibiotics, pharmaceutical-grade substances were used to prepare stock solutions of 0.2 mg·mL^{-1}, as it follows: ampicillin sodium salt ($C_{16}H_{18}N_3O_4SNa$), tetracycline hydrochloride ($C_{22}H_{24}N_2O_8 \cdot HCl$), and chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$). The stock solutions were adequately diluted to obtain concentrations of 4 and 20 mg· L^{-1} respectively, in Hoagland solutions with 0.5% agar. The obtained mixtures were stirred for homogenization, poured into 50 mL Petri dishes and allowed to cool to room temperature. Then, the germinated caryopses were planted on the solidified solutions supplemented with antibiotics, 20 in each Petri dish. Similarly, control samples were prepared without added antibiotic (Table 1).

Table 1. Significance of the experimental variants.

Experimental variant	$C_{,antibiotic}$/ mg·L^{-1}	Final volume/ mL	Antibiotic quantity	
			mg·probe^{-1}	μg ·plant^{-1}
1 (CONTROL)	0	50	0	0
2	4	50	0.2	10
3	20	50	1	50

The plants were kept under natural lighting conditions (photoperiod of 16/8 hours), and temperature of 25/20°C, in the laboratory, and their height was daily monitored from d3 to d7 after planting, in order to calculate their stem growth rate. At d7, leaf samples were collected for the extraction and analysis of the photosynthetic pigments, then the seedlings were harvested, their roots were washed with distilled water and blotted with absorbent paper. The length of the roots and aerial parts of the plants was measured, and the mean values and standard deviations of the data were calculated. For each of the

experimental variants, the obtained data were plotted *vs*. the antibiotic concentration to which they were exposed.

2.2. Sample preparation for biochemical assay

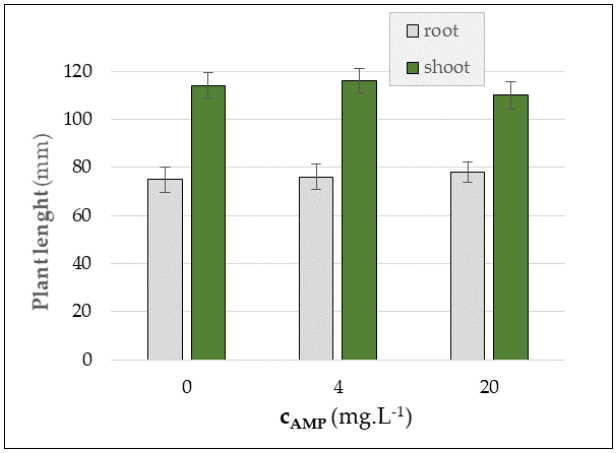
For chlorophylls and carotenoids extraction, samples of 0.02 g of fresh leaf tissue were ground in a mortar with pestle, and 4 mL of 95% ethanol were added to each of them. The obtained homogenates were kept for 1 hour at room temperature, in the dark, and stirred from time to time, then were centrifuged for 10 min at 8000 rpm and 4°C, in a Sigma 3-16 K refrigerated centrifuge. The clear supernatants obtained were decanted into clean test tubes and allowed to equilibrate at room temperature. For data acquisition and processing, a Varian Cary 50 spectrophotometer, equipped with the Scan Application Software, Version 3.00, was used. Leaf extracts were put in glass cuvettes with the optical path of 10 mm, and theirs absorption spectra from 350 to 750 nm were recorded at the scanning speed of 300 $nm \cdot min^{-1}$. Calculation of the pigments' concentration in the ethanolic extracts, as $\mu g \cdot mL^{-1}$, was performed using the equations of Lichtenthaler [5].

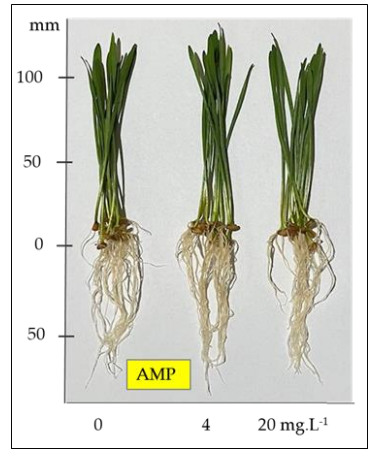
The obtained data were reported to the samples' fresh weight (FW), the presented results being mean values ± standard deviations of three measurements. All the calculation and graphs were done with the Microsoft Excel software, the 2013 version.

3. RESULTS AND DISCUSSION

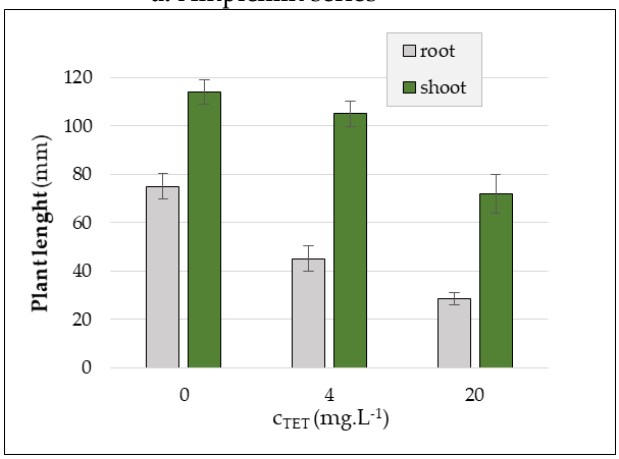
3.1. Growth parameters of wheat plants

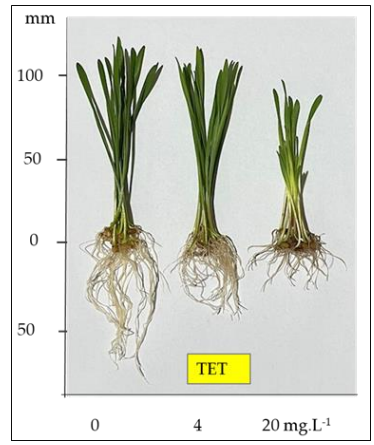
Data on the plants height and the length of their roots are presented in Figure 2 for each of the experimental series, along with photographs of the plants that were taken 7 days after the initiation of the experiment.



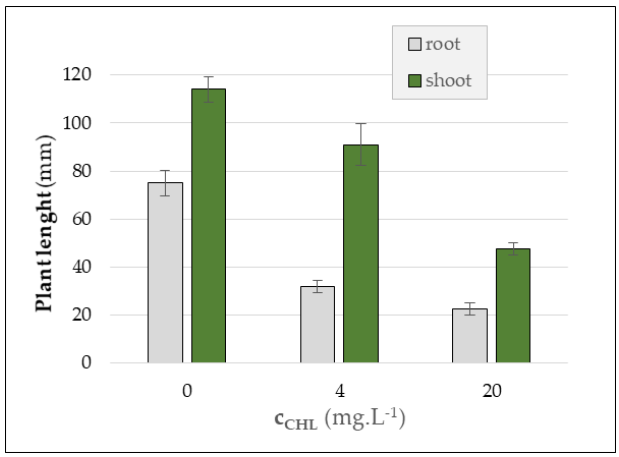


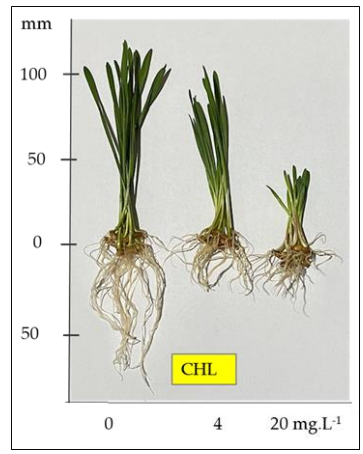
a. Ampicillin series





b. Tetracycline series





c. Chloramphenicol series

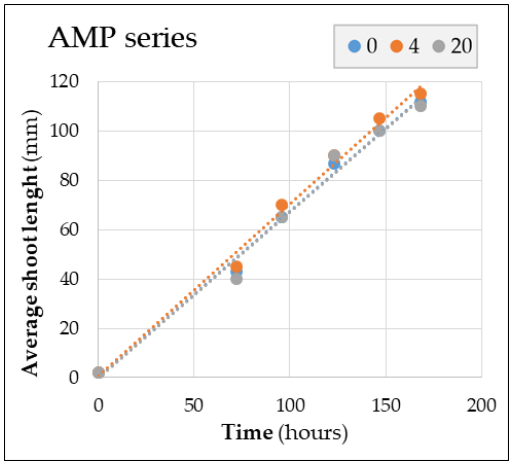
Figure 2. Growth parameters of the wheat plants

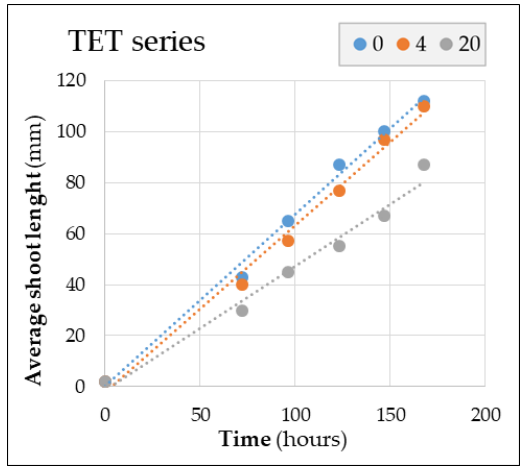
Wheat plants grown on nutrient media without antibiotics (control) had mean root and stem length values of 75±5 mm and 114±5

mm, respectively. Growth parameters of the plants exposed to ampicillin didn't markedly differ from control plants (Figure 2a), while a dose dependent inhibition of shoot and root growth was observed in the plants exposed to tetracycline and chloramphenicol (Figures 2b, and c.)

3.2. Analysis of the plants' growth rate

The average values of plant height, recorded daily from the 3rd day after planting, were graphically represented as a function of time, expressed in hours (Figure 3).





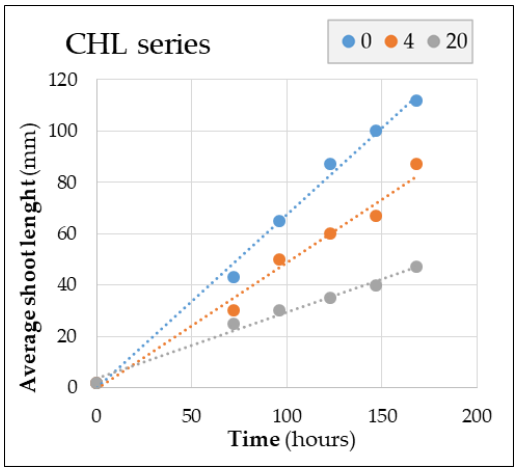


Figure 3.
Linear regression of the data on plant height (mm) *vs*. time (hours), at each concentration of the tested antibiotics (0, 4 and 20 mg.L^{-1}, respectively). Slope values are mentioned in Table 2.

From the linear regressions of the data series obtained for each antibiotic, at each of the tested concentration, and for the control plants, growth rates were inferred *as slopes of the linear regressions* (Table 2).

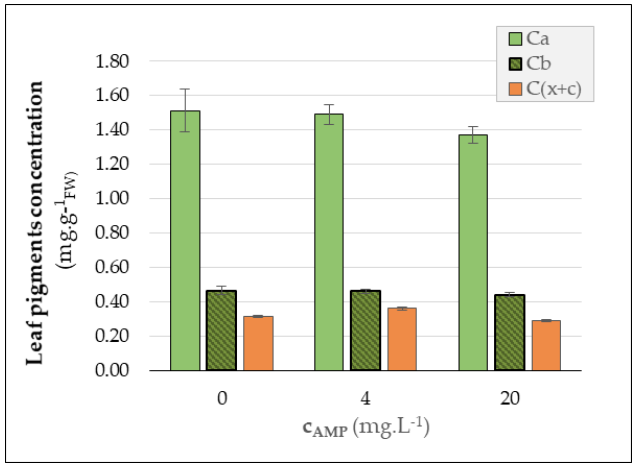
Table 2. Slope values for linear regressions of plant height data *vs*. time

AMP ($mg \cdot L^{-1}$)	**slope** ($mm \cdot h^{-1}$)	**TET** ($mg \cdot L^{-1}$)	**slope** ($mm \cdot h^{-1}$)	**CHL** ($mg \cdot L^{-1}$)	**slope** ($mm \cdot h^{-1}$)
0	0.6744	0	0.6744	0	0.6744
4	0.6754	4	0.6543	4	0.4907
20	0.6972	20	0.486	20	0.2579

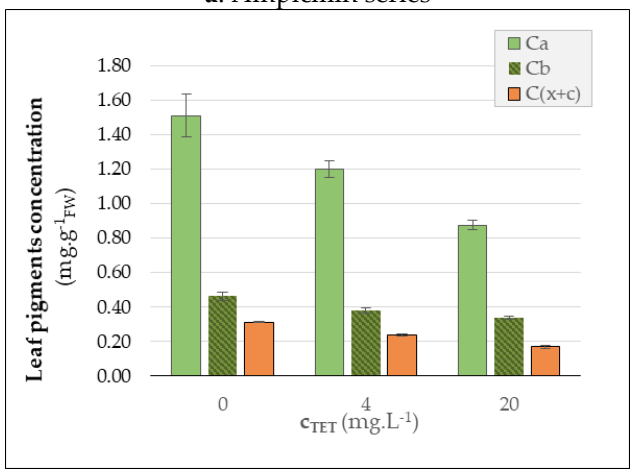
These results highlighted the differences between the tested antibiotics in terms of their impact on plant growth rate. Since the r^2 values for the linear regressions of the data were greater than 0.9, it follows that, during the monitoring, the plants height increased at a constant rate, specific to each of the experimental variants.

3.3. Photosynthetic pigments: chlorophylls and carotenoids

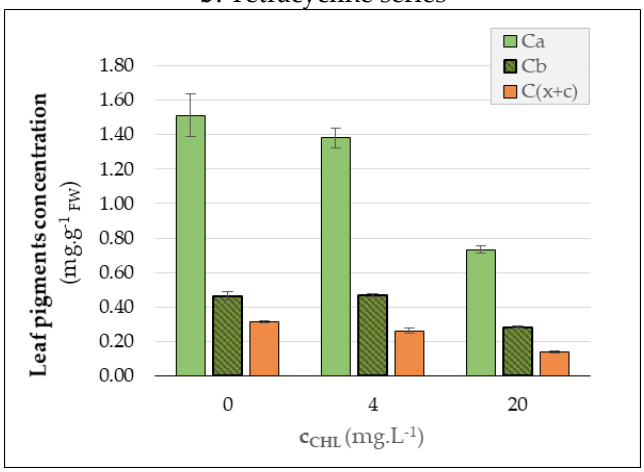
In the leaves of wheat plants that weren't exposed to antibiotics, chlorophyll *a* concentration was of 1.51±0.12 $mg \cdot g_{(FW)^{-1}}$, chlorophyll *b* concentration was of 0.46±0.025 $mg \cdot g_{(FW)^{-1}}$, and total carotenoids concentration was of 0.31±0.04 $mg \cdot g_{(FW)^{-1}}$ (Figure 4). Compared to control, no significant differences in chlorophylls contents were observed in the leaves of the plants exposed to ampicillin at 4 $mg \cdot L^{-1}$, while carotenoids content increased with about 15%. Wheat plants exposed to 20 $mg \cdot L^{-1}$ ampicillin had lower concentrations of the analyzed pigments compared to control, but the differences didn't exceed 10%. Thus, the concentrations of leaf pigments didn't differ markedly from one variant to another, the average value for the three experimental variants of the AMP series being of 1.46±0.0.076 $mg \cdot g_{(FW)^{-1}}$ for chlorophyll *a*, 0.45±0.012 $mg \cdot g_{(FW)^{-1}}$ for chlorophyll b and 0.32±0.035 for the total carotenoids (Figure 4a).



a. Ampicillin series



b. Tetracycline series



c. Chloramphenicol series

Figure 4. Leaf pigments concentrations in the plants exposed to antibiotics; Ca-chlorophyll a, Cb-chlorophyll b, C(x+c)-total carotenoids

Following the exposure to tetracycline at 4 mg·L^{-1}, the content of photosynthetic pigments in the leaves of wheat plants was about 20% lower compared to the control, irrespective of the concerned pigment. (Figure 4b). In the leaves of the wheat plants grown on media supplemented with tetracycline at 20 mg·L^{-1}, chlorophyll a and total carotenoids concentrations were up to 40% lower than in control plants, while chlorophyll b concentration was lower by only 27%. A similar downward trend of the pigments content with the increase of antibiotic concentration was observed in the plants exposed to chloramphenicol, the effect being lower than in TET series at 4 mg.L^{-1} (about 90% of control values) but higher at 20 mg·L^{-1} (about 40-50% of control values) (Figure 4c).

3.4. Same amounts but different outcomes; what are the targets of antibiotics in plant cells? Comparison of the effects of three antibiotics on wheat seedlings

The consequences of the exposure of wheat seedlings to the three antibiotics were more or less different from one to another, which could be caused by: the stability of each antibiotic under the experimental conditions, its uptake/absorption by plants, and the presence of sites of interaction with the antibiotic in the plants.

Ampicillin (a semisynthetic amino penicillin; Figure 1a) is a β-lactam antibiotic that targets bacterial cell wall synthesis. By binding to the so called penicillin binding proteins (PBS), β-lactam antibiotics inhibit the transpeptidation step in bacterial cell wall synthesis [6].

Studies on the degradation of β-lactam antibiotics have shown that their hydrolysis in surface water occurred over several weeks, the resulting products having low microbial activity because of the β-lactam ring hydration [7]. Thus, it is to be expected that, during the presented experiment, the degradation of the β-lactam ring did not affect the activity of ampicillin in a major way. Either or not this was the case in conditions of our experiment, it must be taken into account that the toxic effects of drugs can be caused not only by the drugs themselves, but also by their metabolites [8].

A study on the capacity of two edible plants (carrot and lettuce) to uptake antibiotics from irrigation water have shown that the plants

exposed to concentrations of 0.1 to 15 $mg \cdot L^{-1}$ absorbed the antibiotics from water, their mean concentrations in plant samples being of 27.1 $ng \cdot g^{-1}$ for amoxicillin and 20.2 $ng \cdot g^{-1}$ for tetracycline; when consumed, theses quantities could promote antibiotic resistance [9]. Thus, it can be concluded that antibiotics were available to plants in our experiments as well.

The lack of toxicity of ampicillin on wheat seedling, irrespective of the tested concentration, is rather related to the absence of a target for β-lactams in plant cells. Although both bacteria and plant cells have cell walls, their composition and structure are different. In bacteria, the cell wall is composed of peptidoglycan, consisting of long chains of N-acetylmuramic acid and N-acetylglucosamine, which are cross-linked by short peptides [10]. Plant cell wall has a complex architecture, consisting of five major types of polymers: cellulose, hemicellulose, pectin, wall-associated proteins and lignin [11]. The plant cell wall is not directly targeted by β-lactam antibiotics, as it does not have specific interaction sites with them; however, being a dynamic structure it may be involved in the plant's adaptive responses to the stress of antibiotic exposure.

The generic name tetracycline denotes "A subclass of polyketides having an octahydrotetracene-2-carboxamide backbone, substituted with many hydroxy and other groups" [12]. As antibacterial drugs, the tetracyclines comprise three generations of molecules, with improved properties and diversified uses, derived from the basic structure of the first identified representative, of natural origin, synthesized by soil actinomycetes, and which gave the name to this subclass [13]. Bacteriostatic action of tetracycline(s) occurs by interfering with bacterial protein synthesis, which take place on ribosomes.

Chloramphenicol was initially isolated from the soil actynomicete *Streptomyces venezuelae* [14]. It was the first antibiotic produced by synthesis in large quantities, and used on a large scale as the first broad spectrum bacteriostatic antibiotic. Nowadays, chloramphenicol uses were restricted because of its marked toxicity [15]. Among its side effects are bone marrow suppression, aplastic anemia, and the gray baby syndrome; these effects are related to the route of administration. According to the safety standards, in many countries oral medications containing chloramphenicol were banned from use in humans.

However, its use in animal husbandry and aquaculture, as well as the poor treatment of waste waters, facilitate the way of chloramphenicol to soil, where it can interfere with plant growth and the health of soil microbial comunities [16].

The antibiotic action of tetracycline and chloramphenicol is based on the inhibition of bacterial protein synthesis. Translation, that is the final stage of decoding the genetic information, occurs on ribosomes that are complex assemblies of RNA and proteins. Bacterial cells have 70S ribosomes that are made up two subunits, of 30S and 50S, respectively. Tetracycline binds to the 30S ribosomal subunit and prevents the attachment of the aminoacyl-tRNA complex to the ribosome, thus blocking the elongation of the nascent polypeptide chain [17, 18]. The small, lipid soluble molecule of chloramphenicol easily crosses bacterial cell membrane; it binds to the 50S subunit of the bacterial ribosome, and inhibits peptidyl transferase activity, peptide bond formation and thus protein synthesis [18].

Eukaryotic cells have 80S ribosomes, made up of a 40S subunit and a 60S subunit. Thus, the selectivity of antibiotic action is insured by the differences between the PK and EK ribosomes. However, at high concentrations, antibiotics that target bacterial ribosomes may inhibit mitochondrial protein synthesis in EK cells, that results in major side effects, like decrease of ATP synthesis and oxidative stress [19, 20]. In plant cells, both tetracycline and chloramphenicol can inhibit protein synthesis in chloroplasts, whose ribosomes are similar to those of the prokaryotes.

4. CONCLUSION

Wheat plants have grown for 7 days in the laboratory on nutrient media supplemented with antibiotics that belong to 3 different mechanistic classes, at two different concentrations, along with plants that weren't exposed to antibiotics (control). According to the obtained results, AMP didn't affect the analysed parameters of the plants (neither plants' growth nor the photosynthetic pigments' content of their leaves). The absence of specific action sites for β-lactam antibiotics in plant cells could explain the lack of toxicity of ampicillin. On the contrary, the plants exposed to TET

and CHL presented a dose-related inhibition of growth, and also decreased photosynthetic pigments contents. The antibiotic action of both tetracycline and chloramphenicol is based on the inhibition of bacterial protein synthesis. Eukariotic cells mitochondria have their own ribosomes; in plant cells, the chloroplasts have also their own protein synthesis machines, represented by prokaryotic type ribosomes. Thus, organellar protein synthesis in plant cells can be specifically targeted by some antibiotics, like tetracycline and chloramphenicol in our study. Their toxic action on wheat sedlings was dose dependent, while ampicillin exposure had no effect, irrespective the tested concentration.

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