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Effects of exposure of wheat plants to metronidazole

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Research article

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Abstract
2-weeks-old wheat plants were exposed to metronidazole by transferring to Hoagland solutions containing between 10 and 100 mg antibiotic per liter. Plants evaluation after 7 days of treatment revealed a moderate inhibition of their growth at high concentrations of metronidazole, whose probability of appearing in natural environments is low. Biochemical parameters, such as the activity of soluble peroxidases, and the concentration of soluble proteins and glucose in the roots of the plants, didn't show wide variations in the metronidazole exposed plants compared to the non-exposed ones. Also, leaf chlorophylls and carotenoids concentrations didn't differ markedly in the exposed plants compared to the unexposed ones, although a redistribution of the pigments in the leaf was noticed. Thus, we concluded that wheat plants didn't experience a major stress, probably due to the limited absorption and activation of metronidazole.

Keywords: wheat plants, metronidazole, peroxidase activity, glucose and protein concentration, photosynthetic pigments

1. INTRODUCTION

Metronidazole (2-methyl-5-nitroimidazole-1-ethanol; MNZ), a synthetic derivative of nitroimidazole, is used to combat infections caused by anaerobic and microaerophilic bacteria, as well as parasitic protozoa, in humans and animals [1]. Being in fact a prodrug, metronidazole enters the cells of the targeted microorganisms without requiring the intervention of any transporter, and is activated by the partial reduction of the nitro group in its structure, at low oxygen pressure. The obtained product (*i.e.* a nitroimidazole radical anion, a nitrosoimidazole or a hydroxylaminimidazole) depends on the number of accepted electrons and the enzymatic redox system involved [2]. The reactive intermediates formed attack cellular targets such as DNA, proteins and the thiol pool; the antibiotic efficiency of metronidazole is therefore based on more than one mechanism, and antimicrobial resistance occurs less often than in other cases [2]. Neurological side-effects of metronidazole are rare and reversible with drug discontinuation [3, 4, 5], while hepatotoxicity appears at high doses or long term treatment, the drug being generally well-tolerated and having a low cost. However, metronidazole "is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in experimental animals" [6]; despite these warnings, the human consumption of metronidazole has not decreased. Although veterinary use of metronidazole is restricted in some countries, it is still widely used in humans due to its high efficacy [1].

Metronidazole is frequently detected in wastewater treatment plant effluents and surface waters, usually at concentrations in the $ng.L^{-1}$ or even low $\mu g \cdot L^{-1}$ range (*e.g.* from 0.4 $ng.L^{-1}$ to 1834 $ng.L^{-1}$ [7]), and it has also been detected in sediments and tissue samples from the fish living in contaminated water bodies [8].

Unlike other antibiotics, metronidazole is very persistent in nature [9] due to its high solubility in water (1 g/100 mL at 20°C; [10]), and low biodegradability [11]. In wastewater treatment plants, emerging recalcitrant pollutants, among which metronidazole is a part, are to a small extent or not at all degraded by biological methods. Literature data pointed out that, in activated sludge, metronidazole

negatively impacted the physiology of bacteria involved in processes like nitrification, with an IC_{50} value of 43 mg·L^{-1} [12]. Metronidazole also inhibits methanogenic archaea used to dispose of sewage sludge through biogas production [13]. New technologies and protocols are needed to remove pharmaceuticals that escape the current wastewater treatment procedures. Experiments with photosynthetic aquatic microorganisms [9, 14] have shown their capacity to extract metronidazole from water, at low concentration in the micromolar range, by adsorption onto biomass. However, higher concentrations of metronidazole become toxic for the microorganisms.

Studies on the effects of metronidazole on aquatic life often concerned the microorganisms in the phytoplankton, that are of crucial importance for these ecosystems [9, 11, 14]. Land plants can also be exposed to metronidazole as a result of crops irrigation with contaminated water or fertilization with manure from animals that have been administered the antibiotic. A study on the effects of single-dose application, on soil, at 50 mg·L^{-1} and 100 mg·L^{-1}, respectively, revealed the concentration-dependent toxicity of metronidazole on the relative growth rate of soybean plants [15].

Not only the plant itself, but also the microorganisms in the rhisosphere may be negatively impacted by antibiotic exposure. For example, soil concentrations of 0.5 mg metronidazole·g^{-1} led to a 10-fold reduction of the protozoa density in the rhisophere of soybean plants [16]. The importance of beneficial soil microbiota as a plant growth promoter is becoming more and more evident, as is the need to find the best ways to take advantage of this [17]. In recent years, disruption of the structure and function of soil microbial communities as well as the potential transfer of antibiotic resistance genes are among the negative effects of soil antibiotic pollution and, at the same time, issues of real concern regarding animal and human health [18].

Consequences of the interaction between plants and antibiotics are complex and diverse. For sensitive plants, antibiotics can be toxic, preventing their growth and development and disrupting the food chains in ecosystems. Tolerant plants can accumulate antibiotics or products of their degradation, which they can transfer to higher trophic levels; however, such plants could be used to remediate polluted waters

and soils. The aim of our study, the results of which will be presented below, was to evaluate some biochemical effects of the exposure of young wheat plants to different concentrations of metronidazole within the growth media.

2. MATERIALS AND METHODS

2.1. Materials

Metronidazole (99.7%) was purchased from local pharmacies. All other solutions used in the experiments were prepared with analytical grade reagents. Wheat caryopses (*Triticum aestivum* L.) were obtained from the Faculty of Agronomy of the University of Craiova, Department of Agricultural and Forestry Technologies.

2.2. Plant growth assay and exposure to metronidazole

Before being used in experiments, wheat caryopses were surface-sterilized by immersion in a 5% NaClO solution for 10 min, then rinsed thoroughly with tap water and distilled water, respectively. For germination, the caryopses were placed in Φ 90 mm Petri dishes between two layers of filter paper moistened with distilled water. 24 hours after the initiation of imbibition, uniformly germinated caryopses were transplanted onto liquid culture media consisting half strength Hoagland's solutions [19], 20 caryopses per 500 mL medium. The plantlets were kept under natural light conditions in the laboratory, for 14 days. Afterwards, the young plants were exposed to metronidazole by transferring them on half strength Hoagland's solutions that were supplemented with the necessary volumes of a metronidazole stock solution (1000 $mg \cdot L^{-1}$), in order to reach the concentrations indicated in Table 1. Plants unexposed to metronidazole were considered as controls. All the plants were further maintained in the laboratory for another 7 days, at 16/8 hours photoperiod and temperature of 25/20 °C.

After 7 days of exposure to metronidazole under the above-mentioned conditions, the plants were removed from the growth media

and their roots were washed with tap water and distilled water, then blotted with filter paper. The length of the roots and shoots was measured and registered, in order to calculate the mean values and standard deviation for each experimental variant.

Table 1. The significance of the experimental variants

Experimental variant	Metronidazole stock solution/ mL	Final volume/ mL	Metronidazole concentration within the growth media/	
			$mg \cdot L^{-1}$	$\mu mol \cdot L^{-1}$
1(CONTROL)	0	500	0	0
2	5	500	10	58
3	10	500	20	116
4	25	500	50	298
5	50	500	100	596

2.3. Biochemical assays

Root samples having masses of ~0.07 g were collected from each of the experimental variants. The samples were ground with quartz sand and homogenized with 3 mL of 20 $mmol \cdot L^{-1}$ acetate buffer solution (CH3COOH-CH3COONa, pH 5) containing 10 $\mu mol \cdot L^{-1}$ Ca^{2+}. The obtained homogenates were centrifuged at 12500 rpm for 10 min at 4°C, using a Sigma 2-16 K centrifuge. The supernatants were collected and tested for the activity of total soluble peroxidases [20] and concentration of soluble proteins [21]. Glucose concentration in the extracts was quantified by the coupled enzymatic reaction of glucose oxidase and peroxidase, with 4-aminoantipyrine as a chromogenic substrate.

Acetone extracts were obtained from plant leaf samples, in which the content of photosynthetic pigments (chlorophylls and carotenoids) was evaluated by spectrophotometric methods. For this purpose, from each experimental variant, apical and basal segments of the leaves were collected separately and weighed. Each leaf sample was ground and the acetone-soluble components were extracted (extraction ratio 0.01 g/1 mL pure acetone). The obtained extracts were centrifuged and the supernatants were used to determine the concentration of chlorophyll *a*, chlorophyll *b* and total carotenoids [22].

A Varian Cary 50 UV-Vis spectrophotometer was used to do the spectrophotometric measurements. The analyzed biochemical parameters were expressed in appropriated units, and reported to the fresh weight (FW) of the corresponding sample. All calculations and graphs were performed with Microsoft Excel 2013 software.

3. RESULTS AND DISCUSSION

3.1. Peroxidase activity

Peroxidases are enzymes that catalyze the reduction of hydrogen peroxide, using various substrates *in vivo* (aromatic amines, phenols, etc.). Certain peroxidases have a great specificity regarding the reducing substrate, while others, often called non-specific, show a broad specificity on the electron donor. In the roots of wheat plants unexposed to metronidazole (control), the total activity of soluble peroxidases was 194.11 $U \cdot g_{FW}^{-1}$. Peroxidase activity increased compared to the control in the plants exposed to metronidazole, showing the maximum value at 20 $mg \cdot L^{-1}$ (+18% *vs*. control) (Figure 1).

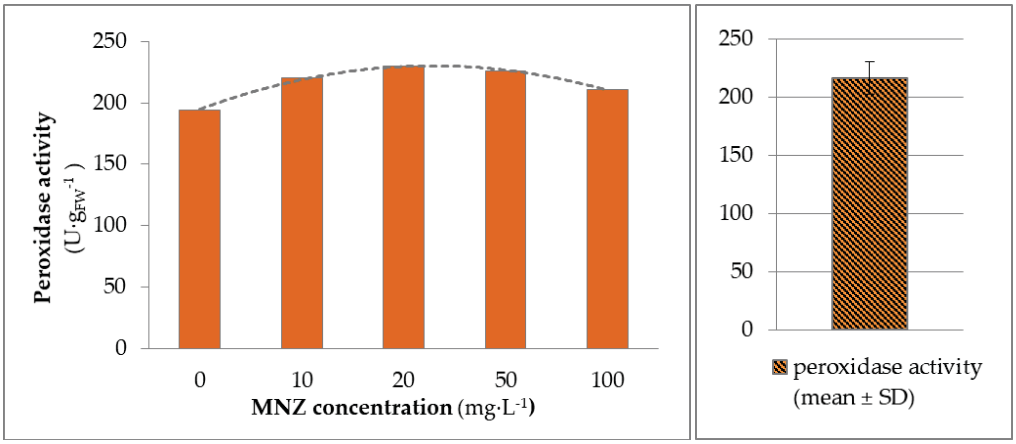

Figure 1. Peroxidase activity in the roots of wheat plants exposed to metronidazole: *left* - the value of peroxidase activity for each of the experimental variants (r=0.11); *right* - the average value of peroxidase activity, considering all the experimental variants

Considering all the experimental variants in this study, the average value of the peroxidase activity was 216.12±14.02 $U \cdot g_{FW}^{-1}$.

3.2. Total soluble protein concentration

In the roots of wheat plants that weren't exposed to metronidazole, the total soluble protein concentration was of 1.71 $mg \cdot g_{FW}^{-1}$. Lower values of protein concentration (by 17% and 10%, respectively, compared to control value) were measured in plants grown on media with 20 $mg \cdot L^{-1}$ and 50 $mg \cdot L^{-1}$ metronidazole, respectively (Figure 2). Considering all the experimental variants taken in the study, the average value of the protein concentration in the roots was 1.61±0.13 $mg \cdot g_{FW}^{-1}$.

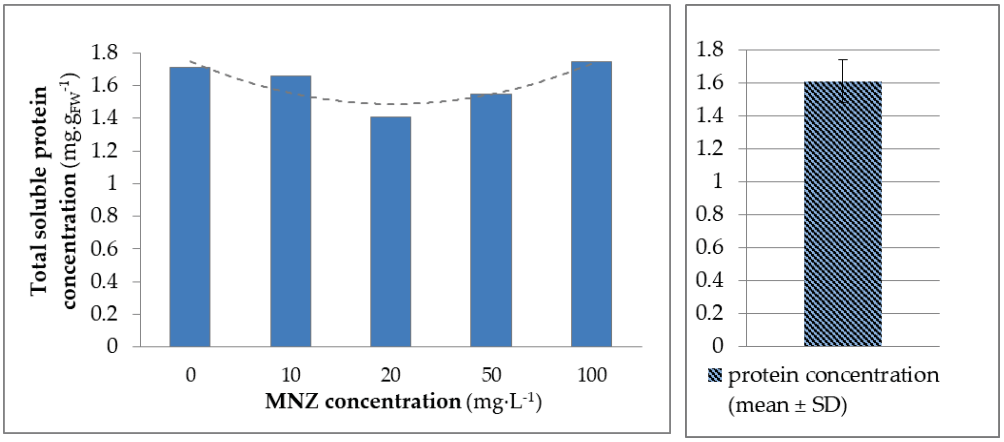

Figure 2. Soluble proteins concentration in the roots of wheat plants exposed to metronidazole; *left* - the values of protein concentrations for each of the experimental variants (r=0.28); *right* - the average value of protein concentration, considering all the experimental variants

3.3. Glucose concentration

In the roots of wheat plants unexposed to metronidazole, glucose concentration was 4.12 $mg \cdot g_{FW}^{-1}$. Glucose concentration decreased compared to control in the plants exposed to metronidazole, with the lowest value observed at 20 $mg \cdot L^{-1}$ (20% lower than the control; Figure 3), but increased thereafter, reaching approximately the control value at

the highest concentration of metronidazole tested. The average glucose concentration for the five experimental variants was 3.83±0.35 mg.g_{FW}^{-1}.

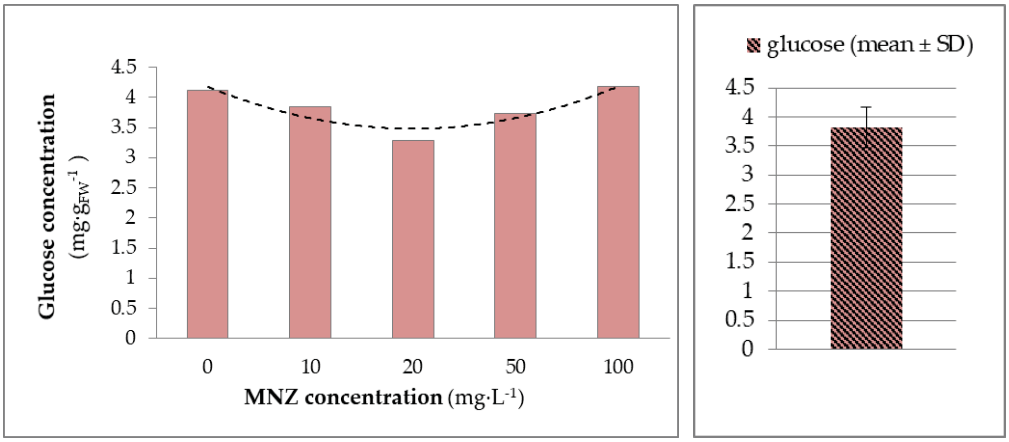

Figure 3. Glucose concentration in the roots of wheat plants exposed to metronidazole: *left* - the values glucose concentration for each of the experimental variants (r=0.33); *right* - the average value of glucose concentration, considering all experimental variants

Pearson correlation coefficient (*r*) calculations have shown that the analyzed biochemical parameters are not linearly correlated with the concentration of metronidazole in plant's grow media. Among the analyzed biochemical parameters in plant roots, it can be seen that the variation of peroxidase activity is negatively correlated with both protein concentration (r=-0.77) and glucose concentration (r=-0.81). Glucose concentration and soluble protein concentration were well positively correlated (r=0.98). However, considering that the range of variation of the exogenous concentration of metronidazole was from 10 mg·L^{-1} to 100 mg·L^{-1} (a 10-fold increase from the lowest to the highest tested concentration), the less extensive variations of the analyzed biochemical parameters do not appear to be correlated with or determined by the exogenous concentration of metronidazole.

Most likely, metronidazole bioavailability for plants is low; a small amount of the antibiotic is taken up by the plants, and plant cells cannot reduce the nitro group to activate metronidazole, in order to exert a phytotoxic effect similar to its antibacterial action.

3.4. Photosynthetic pigments

For the analysis of photosynthetic pigments, we have collected samples from both the lower half of the leaf (base) and the upper half of the leaf (apex), considering the uneven distribution of pigments in the leaf and possible differential effects of the exposure to metronidazole.

Chlorophylls. Higher plants have two types of chlorophylls, with specific distribution in chloroplast photosystems. Chlorophyll *a* is mainly found in the reaction centers of the photosystems, and together with chlorophyll *b* is part of the peripheral antenna (the light harvesting complexes) [22]. Data presented in Figure 4 and Figure 5 have shown that chlorophylls concentrations didn't vary markedly from one experimental variant to another.

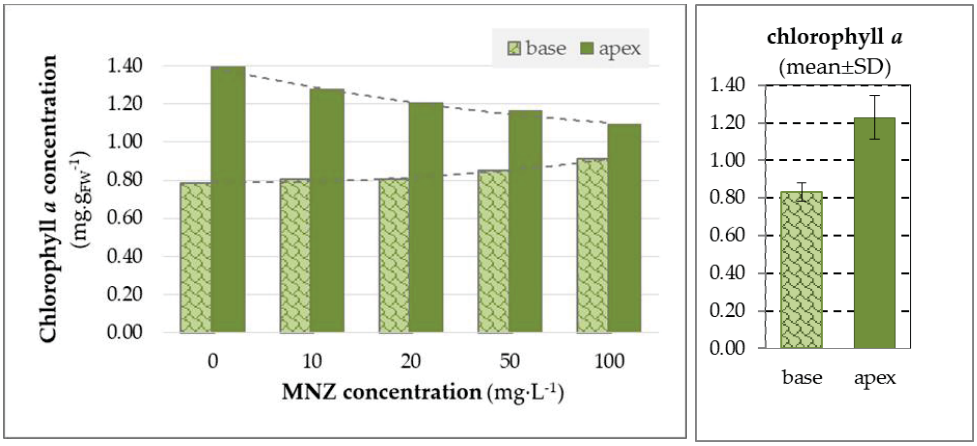

Figure 4. Chlorophyll *a* concentration in the leaves of wheat plants exposed to metronidazole: *left* - the values of chlorophyll *a* concentration for each of the experimental variants (r_{apex}=0.99 r_{base}=-0.88); *right* - the average value of chlorophyll *a* concentration, considering all experimental variants

In wheat plants unexposed to metronidazole, chlorophyll *a* concentration was 0.79 mg·g_{FW}^{-1} at the leaf base and 1.40 mg·g_{FW}^{-1} at the leaf apex. The average value of chlorophyll *a* concentration for the five variants (control and plants exposed to metronidazole) was 0.83±0.05 mg.g_{FW}^{-1} in the basal region of the leaves, and 1.02±0.12 mg.g_{FW}^{-1} in the apical region (Figure 4). Chlorophyll *b* concentration in control plants was 0.29 mg·$g_{(FW)}^{-1}$ at the leaf base and 0.48 mg·$g_{(FW)}^{-1}$ at the leaf apex. The

average value of chlorophyll *b* concentration for the five variants was 0.30±0.02 mg.g_{FW}^{-1} in the basal region and 0.43±0.04 mg.g_{FW}^{-1} in the apical region of the leaf (Figure 5). It should be noted the decreasing trend of chlorophyll concentration in the apical zone of the leaves as the exogenous concentration of metronidazole increased. Thus, at 100 mg·L^{-1}, chlorophyll *a* concentration was 22% lower than in control plants, a similar variation being observed for chlorophyll *b*. At the same time, chlorophylls concentration increased in the basal segments of the leaves, by 16% for chlorophyll *a* and 15% for chlorophyll *b*, respectively, at the highest metronidazole tested concentration. These data are suggestive of a redistribution of chlorophylls, from the apical to the basal region of the leaf. Moreover, in some plants exposed to high concentrations of metronidazole, the tips of the leaves were wilted.

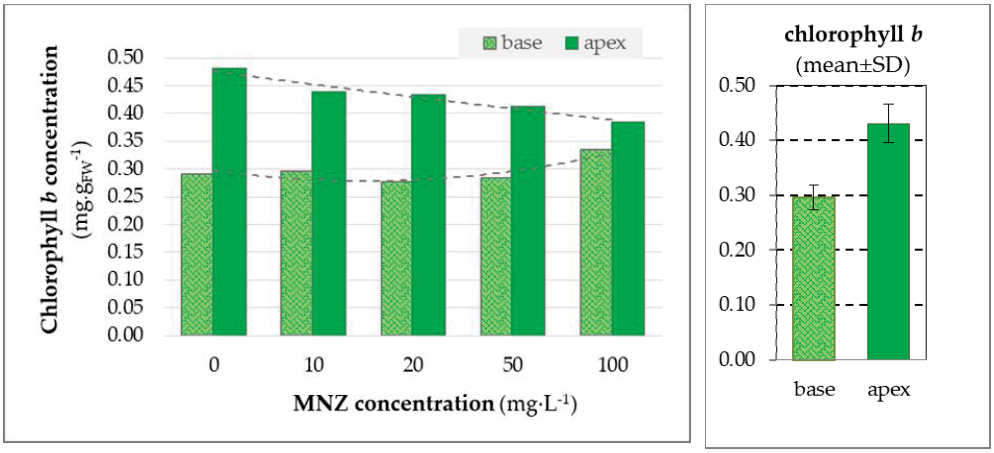

Figure 5. Chlorophyll *b* concentration in the leaves of wheat plants exposed to metronidazole: *left* - the values of chlorophyll *b* concentration for each of the experimental variants (r_{apex}=0.78 r_{base}=-0.93); *right* - the average value of chlorophyll *b* concentration, considering all experimental variants

Carotenoid pigments are classified into two groups: carotenes (*c*), which have an exclusively hydrocarbon structure, and xanthophylls (*x*), which contain one or more oxygen atoms. In higher plants, carotenoids fulfill many roles: they absorb light energy for use in photosynthesis and protect chlorophyll from photodamage [23], being also important for phytohormones' synthesis and signaling [24].

Data on total carotenoids concentration (*x+c*) are presented in Figure 6. The average value of total carotenoids concentration for the five experimental variants (control and plants exposed to metronidazole) was 0.20±0.01 mg.g_{FW}^{-1} in the basal region of the leaves, and 0.27±0.024 mg.g_{FW}^{-1} in the apical region. In the plants unexposed to metronidazole, carotenoids concentration was 0.19 mg·g_{FW}^{-1} at the leaf base and 0.30 mg·g_{FW}^{-1} at the leaf apex. It should be noted the decreasing trend of carotenoids concentration in the apical zone of the leaves, as the exogenous concentration of metronidazole increased. At 100 mg·L^{-1} metronidazole, the total carotenoids concentration in the apical region of the leaf was 20% lower than in the plants unexposed to metronidazole. In the basal region of the leaves, carotenoids concentration varied in opposite direction compared to the apical one, increasing with the increase of exogenous metronidazole concentration: by 14.5% at the highest metronidazole tested concentration.

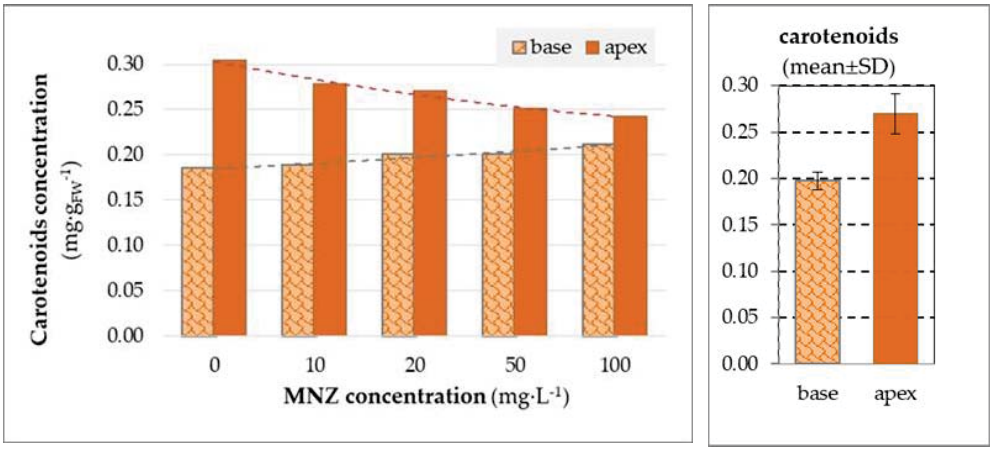

Figure 6. Carotenoids concentration in the leaves of wheat plants exposed to metronidazole: *left* - the values of carotenoids concentration for each of the experimental variants (r_{apex}=-0.89 r_{base}=0.92); *right* - the average value of carotenoids concentration, considering all experimental variants

Although the concentrations of chlorophylls and carotenoids didn't vary widely from one experimental variant to another, a clear tendency of redistribution was observed. One could notice the opposite trend of pigment concentration variation in the two analyzed zones of the leaf: as the concentration of metronidazole increased, pigments'

concentration decreased in the apical segments and increased in the basal ones. Certain values of Pearson's *r* for pigments concentration variation *vs.* metronidazole concentration approach to 1.

3.5. Biometric data on wheat plant

Data on root and shoot lenght of the plants grown in this experiment are presented in Figure 7. Apart from a 10% increase in root length compared to control plants, observed at 10 mg·L^{-1} metronidazole, the overall trend was of gradual growth inhibition, up to 20% at the maximum concentration tested.

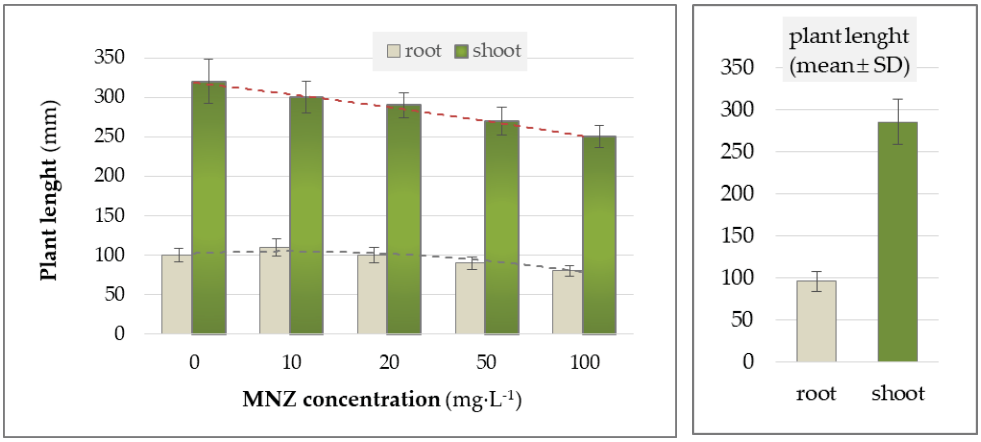

Figure 7. Growth parameters of wheat plants exposed to metronidazole: *left* - root and shoot length for each of the experimental variants (r_{root}= - 0.91, r_{shoot}= -0.95; *right* - the average values of root and shoot length, considering all experimental variants

4. CONCLUSION

The exposure of wheat seedlings to metronidazole concentration ranging from 10 to 100 mg·L^{-1} was followed by a gradual inhibition of root and shoot growth up to 20 % compared to the unexposed (control) plants.

While the concentration of metronidazole increased tenfold, the biochemical parameters measured in the roots of wheat plants registered a U-shaped variation, with a vertex at about 20 mg·L^{-1}. Peroxidase activity increased at low concentration of metronidazole, then decreased, while

glucose and protein concentration followed the opposite trend. Either negative or positive, the observed variations compared to control didn't exceed 20% and weren't linearly correlated with metronidazole concentration.

Data on photosynthetic pigments concentration have shown their uneven distribution within the leaf, with higher values in the apical zone compared to the basal one. Exposure to increasing concentration of metronidazole was followed by a decrease of pigment concentration in the apical zone, paralleled by an increase in the basal one. This trend was suggestive for a "redistribution" of the pigments in the plants exposed to metronidazole.

These results suggested that the plants didn't experience severe stress, although the concentration of metronidazole within their growth medium was high: either antibiotic uptake by plants was low, or it can't follow an activation mechanism alike in bacterial cells *or both*. However, the wilted tips of the leaves, observed at high metronidazole concentrations, could indicate a dehydration effect or premature senescence caused by metronidazole toxicity.

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