Influence of Antibiotics on Copper Uptake by Plants

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Use of antibiotics in agriculture may cause serious problems, because their presence generates antibiotic resistant bacteria that can reach the human body through various ways, directly, by ingestion of food or indirectly through environmental contamination. In this way, the antibiotics represent a risk for aquatic organisms or even human health. One of the sources of environmental contamination with antibiotics is the fact that about 75% of the ingested antibiotics by the animals pass unaltered through the digestive tract and the animal manure may contain both antibiotics and antibiotic resistant bacteria. But there could be a beneficial aspect of the presence of antibiotics in very small amounts in the environment- that one that antibiotics may contribute to the enrichment in copper in some plants and in this way in animal feed, making it also the soils polluted with copper to be used in crops. This paper presents the influence of traces of tetracycline on copper uptake capacity by bean plants grown on contaminated soil with tetracycline and the possibility of using manure contaminated with antibiotics to enrich with copper the forage used for feeding the livestock.

Keywords: copper uptake, antibiotics, contamination, decontamination

Since early 1900s, antibiotics have been used in infectious diseases, the most of them being used to treat infections in humans and animals, but important quantities of them are also used in animal feed. Therefore, the antibiotics are often placed in the feeding at sub-therapeutic levels as growth promoters. Among the antibiotics most commonly used as growth promoters (from 1 to 100 mg per head per day) are those from tetracycline antibiotic group (chlortetracycline, aureomycin, oxytetracycline), and macrolide (erythromycin). The fate of these antibiotics in soils depends on sorption and desorption processes and leaching, that are strongly influenced by the reactions between the antibiotics and the soil. Dominant way for the emergence of antibiotics in the environment seems to be spreading manure containing antibiotics on land.

The issue of antibiotics residues in food, especially in meat, honey and milk was studied for many years, due of the real risk of becoming resistant to antibiotics. Limited data are available regarding the impact of antibiotics on plant grown on soil fertilized with manure containing antibiotics. In 2005, Kumar *et all*. [1] reported that green onion, cabbage, and corn plants can take up small amounts of chlortetracycline from soil (2 to 17 ng/g in fresh tissue) which had been amended with swine manure; uptake of the antibiotic tylosin was not observed in these cases.

Animal antibiotics may be administered either by injecting them directly or by mixing them into feed and water. Studies on the metabolism of antibiotics in animals have shown that most antibiotics fed to animals are poorly absorbed in the animal gut, so that 90% of some antibiotics may be excreted as the parent compound [2]. These chemicals are excreted in urine and faeces, which in turn end up in manure and once in the environment, they can disrupt biogeochemical cycles or become pollutants. That's why the antibiotics resistance that can result from the wide-scale use of antibacterial in animals became a real problem.

The most common antibiotics present in manure are tetracycline, oxytetracycline and chlortetracycline, tyrosine, sulfamethazine, amprolium, monensin, virginiamycin, penicillin, and nicarbazin, their concentration values ranging from trace to greater than 216 mg/L of slurry manure [3]. Generally, these antibiotics remain stable (in their initial state) during manure storage and end up in agricultural fields on manure applications [4]. The main contribution of repeated fertilizing with liquid manure is the building up of persistent antibiotic residues and their accumulation in the environment [5].

Although there are also clear restrictions on the use of raw manure (fresh, dried or composted) in organic farming due to the concern of bacterial contamination (*Salmonella* and *E. coli*) there are no guidelines on the presence of contaminants such as antibiotics in manure [6] .

There are studies that show that the adsorption of antibiotics, as tetracycline, on soil and sediments strongly depended on the *p*H and on the presence of some metallic cations, such as copper, because tetracycline possesses a great tendency to form complexes. Thus, it was demonstrated that the bivallent copper facilitated the adsorption of tetracycline on soil and sediments at low pH (pH<5), possibly due to the complexation of this cation and copper adsorption on soil and sediments [7].

Copper as trace element is needed for proper development and function in plants, and it is an essential mineral required by the body for bone and connective tissue production, and for coding specific enzymes that range in function from eliminating free radicals to producing melanin. The food naturally contains copper, an adult ingesting about 1 mg of copper every day [8]. A deficiency of copper in human body can lead to various diseases (osteoporosis, joint pain, lowered immunity, etc.), but over-consumption of copper is toxic, leading in long term to depression, schizophrenia, hypertension, senility, and insomnia.

Signs of deficiency of copper in the livestock diet there are: coat colour changes (red to yellow; black to brown),

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Set number (pot number)	Spiking copper, mg/kg	Tetracycline, mg/kg
1 (1.1; 1.2; 1.3)	0	0
2 (2.1; 2.2; 2.3)	30	0
3 (3.1; 3.2; 3.3)	0	0.25
4 (4.1; 4.2; 4.3)	0	0.5
5 (5.1; 5.2; 5.3)	30	0.25
6 (6.1; 6.2; 6.3)	30	0.5

Table 1
ADDITIONAL COPPER AND TETRACYCLINE CONCENTRATION IN SOIL

ill thrift, infertility and skeletal abnormalities in young stock. Most copper deficiency in ruminants is associated with the presence of copper antagonists, e.g. molybdenum, sulph and iron in the diet that bind to copper in the rumen thus preventing the absorption of copper, as well as with the soil ingestion by cattle [9].

On the other hand, a high content of copper becomes toxic. Commission Regulation (EC) 1334/2003 of 25 July 2003 provides controls on the use of copper–based additives in animal feed. The presence of copper in feedstuff is needed, but maximum content of copper for ruminating cattle of the complete feedstuff is 35 mg/kg (88% dry matter), which equates to 40 mg/kg of total diet dry matter [10].

The degree of remaining pharmaceutical bioactivity of antibiotics in environment, depends on the antibiotic; recent studies [11] concluded that β-lactams and florfenicol antibiotics remain bioactive in soils, while ciprofloxacin, neomycin, and tetracycline are neutralized. The antibiotic resistance to tetracycline in soil seems to be uncertain.

The risk of groundwater contamination with tetracycline is unlikely to occur due to the strong adsorption of tetracycline – copper complex on soil and sediments. It was hypothesized that although tetracycline might be detected in surface water, they may not occur in groundwater [12]. Furthermore, the strong binding of copper-tetracycline complex on the soil reduces the hazard of copper leaching in groundwater.

A potential risk is represented by the genes resistant to tetracycline present in the manure and that might be distributed and preserved in the broader environment without antibiotic selective pressure [13]. Studies regarding the tetracycline resistance [14] showed that *Escherichia coli* strains that are resistant to tetracycline had higher mobility than the sensitive strains in saturated porous media.

This paper presents the impact of the presence of tetracycline on the copper uptake performed on leaves of bean plants (*Phaseolus vulgaris*), the plants being cultivated on controlled contaminated soil with both copper and tetracycline.

Experimental part

Materials

The bean seeds and the soil used for this study were provided by the market. The seeds were of the type intended for consumption (not for seeding). Copper standard solution 1000 mg/L ($Cu(NO_3)_2$ in HNO3 0.5 mol/L) was purchased from Merck. Tetracycline, intended for human use, was purchased from the drugstore.

Sample preparation

2 sets of 100 bean seeds (previous selected) were used in the experiments. In order to remove any potential fungus or pathogen bacteria that may interfere with the experiment, the bean seeds were rinsed with tape water and than three times with sterile water. To asses the efficiency of the previous operation, the water resulting from the rinsing procedure was inoculated on yeast agar and the total number of germs and fungus was counted. Neither pathogens, nor fungus were found.

Before the experiments, the germination rate was investigated using flat trays of about 30 cm square and 5 cm deep, the bottom of each tray being covered with a cotton wool moisturized with bidistilled water. The germination rate, representing the total number of germinated seeds was in the range of 63- 67%.

The bean seeds, previously rinsed three times with sterile water, were placed on the moist cotton wool, spacing them as evenly as possible. After 3-7 days, preserving all the time the moisture in the tray, the germinated seeds were counted.

Six sets of 3 pots each were filled with 400 g of garden soil and subsequently three bean seeds were cultivated in every pot. The soil was spiked with copper solution and with tetracycline as shown in table 1, and was watered daily with bi-distilled water. Any leaching from the pot was prevented.

Leaves samples preparation were achieved as follows: fresh bean leaves were collected and thoroughly washed with tape water, followed by distilled water and dried in oven at 80°C temperature for 24 h (until the dry weight was constant). The dried leaves were smashed and powdered, 0.2-0.3 g of each powdered sample being digested with a mixture of 3 mL of nitric acid 65 % and 2 mL of hydrogen peroxide 30% in DAP-60K Teflon reaction vessels of Berghof speed-wave MWS-2 microwave pressure digestion system. Before closing the vessel, the mixture was stirred thoroughly with a clean glass bar and then waited at least 20 min. The heating of the Teflon vessels of the microwave oven was achieved with a temperature program set in three steps, optimized for the samples under study (table 2).

After the end of the temperature program, the digestion vessels were cooled down to room temperature waiting about 30 min before opening the vessels cover. The resulting clear sample solution is transferred into a 50 mL measuring flask and completed with bidistilled water. A blank-sample was also performed.

In order to prevent any contamination, all stages of sample preparation and analysis were carried out in a clean environment. All the sample containers and glassware were cleaned with nitric acid 20% for 24 h and then rinsed thoroughly with bidistilled water prior use.

Analytical Method for Copper Analysis

Copper was determined by flame atomic absorption spectrometry using a spectrometer VARIAN SpectrAA – 250 Plus equipped with hollow cathode lamps for Cu. The absorbance measurements were performed using the spectral lines of Cu at 324.8nm. Peak height absorbance measurements were used as analytical signal.

Table 2
THE MICROWAVE OVEN PROGRAM OPTIMIZED FOR BEAN LEAVES DIGESTION

Step	1	2	3
T [°C]	150	190	100
Power [%]	80	90	40
Time [min]	7	10	10

The analytical parameters of the spectrometer were optimized for Cu analysis, and the calibration curve presented a good linearity in the range of 0-2.0μg/L (R^2 = 0.9994). The detection and quantification limits were set, obtaining values of 0.0265mg/L and respectively 0.0383mg/L, with a calculated measurement uncertainty of 9.10%.

The soil *p*H was measured using a digital MV-870 pracitronic pH-meter with glass electrode and saturated calomel.

Results and discussions

In the present experiments, as test material were chosen the bean plants because they develop and grow relatively quickly, and all stages of development, such as germination, leaf rising, flowering, fruiting, can be easily observed. Also, bean seeds are of great importance due to their high food value: rich in protein (25-28%) and vitamins, very nutritious and very cheap. Bean stalks left after threshing (haulms), along with beans, are also high in protein and represent a good feed especially for sheep.

Because between antibiotics tetracycline was frequently detected in the environment, it was chosen for this study a controlled soil contamination with tetracycline and with copper. In order to obtain a soil contaminated with cooper, the soil from the experimental pots was spiked with 30 mg copper/kg soil (table 1).

Thus, the set number 2 was enriched with 30 mg/kg cooper (without tetracycline), the sets 3 and 4 were only with tetracycline (0.25 and 0.5 mg/kg, respectively); the sets 5 and 6 were enriched with both copper (30 mg/kg) and tetracycline (0.25 or 0.5 mg/kg). After each addition of the two components, the soil in each pot was thoroughly mixed.

Bean plants have sprung up to 5-6 days after sowing.

The plants were then developed equally, without major differences in terms of appearance. After 50 days from sowing, the leaves of bean plants were harvested and the copper content was analyzed as mentioned**.**

Figure 1 presents the individual values of copper content for bean leaves from each pot, as well as the *p*H of soil, and the average copper content of three pots for each set..

Analyzing the copper content from different sets presented in table 1, it is observed that copper uptake by bean plants was different from one set to another, as shown in figure 1.

It can be seen that the plants grown on the soil contaminated only with copper (set 2), as well as the plants grown on the soil contaminated only with low content of tetracycline (set 3) uptakes more copper then the blank (set 1) but in almost equal amounts. It is interesting that the plants grown on the soil spiked with high content of tetracycline (set 4) uptake less copper than those from the set 3 with low content of tetracycline and also from the blank. The highest impact on the copper uptake has been observed for the set 5, spiked with 30 mg/kg copper and with low level of tetracycline (0.25 mg/kg). At the same level of enrichment in cooper but with high tetracycline level (0.5 mg/kg) the copper uptake is lower. That means that the high content of tetracycline in soil (0.5 mg/kg) has a negative impact on copper absorption (3.408 mg/kg versus 7.483 mg/kg, as well as 7.565 mg/kg versus 17.172 mg/kg).

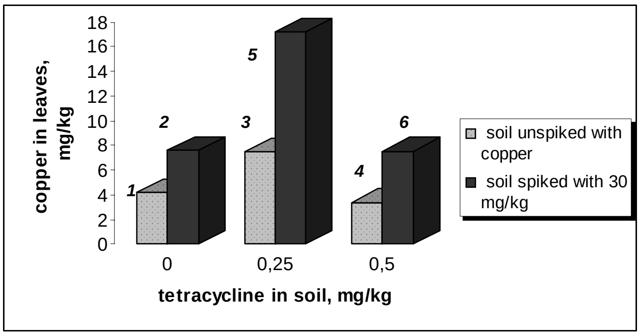

Fig. 1. Copper content in bean leaves

The ratio between copper content in set 3 and set 4 is about 2.1957, similar ratio being found for set 5 and 6, respectively 2.2699.

This results lead to conclusion that there is a synergic effect of tetracycline on copper uptake in been plants that may be used to obtain plants with higher content of copper (up to 40 mg/kg as dry mass), appropriate as feedstuff for ruminating species, in different development stages. So, two different polluted environments (the manure containing tetracycline residues and the soil contaminated with copper) can merge and may represent an appropriate substrate for forages plants.

The fertilization of soils contaminated with copper with manure containing tetracycline in traces may have the following benefits: the manure disposal and elimination, decontamination of soil polluted with copper, obtaining forage with appropriate content of copper. The leaching of copper in groundwater is limited by sequestration of copper in plants and in the copper-tetracycline complex.

The most important requirement that this forage has to meet is the lack of antibiotic. This condition may be acquired by thermal conditioning and light exposure because the tetracycline is light and temperature sensitive.

Conclusions

The present study shows that trace level of tetracycline seems to enhance the copper uptake, comparing with the blank, on the soil with normal level of copper, as well as on the contaminated soil. A high level of tetracycline, more than 0.250 mg/kg in soil results on a lower uptake, due to the strong sorption of tetracycline on soil.

No significant differences in the plant development and growth were noticed regardless of the concentration of tetracycline and copper in soil.

If no other undesired elements occur in the final product, the use of the manure containing tetracycline in traces on soil contaminated with copper may represent a sustainable treatment method to rehabilitate the soils contaminated with copper in order to obtain forages with an adequate content of copper, as well as to decontaminate the soil. Thus, spreading manure provides nutrients for crops, and is also a means of eliminating unwanted waste. As a result, copper supplementation and overdosing in feed are avoided.

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