ACUMULATION PROPERTIES AND FOLIAR LIMB EFFECTS OF TETRACYCLINE AND METABOLITES IN *LACTUCA SATIVA* L., *ERUCA SATIVA* L. AND *SPINACIA OLERACEA* L.

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Abstract

The main goal of this study is to study the tetracycline and metabolites (TCS) accumulation properties in three vegetable plants leafs (lettuce, arugula and spinach). Residues of TCS, used in veterinary and human medicine, end up in crops due to the agricultural farmlands where manure application is used. The studied vegetables were grown hydroponically in greenhouses, using controlled nutrient solution containing 21.00 μg·L^{-1} TCS (quantity from the literature). Mature plants leafs were chemically characterized (water and cations), TCS measured using ELISA screening method (enzyme-linked immunosorbent assay) and foliar limb in terms of anatomical structure studied. Tetracycline was accumulated in the studied vegetables plants leafs in low concentrations (10-15 μg·kg-1), the accumulation rate depending on the plant characteristics. Normal aspect of foliar limb structure was highlighted in leaves of the studied plants, so the TCS accumulation does not lead to leaf structure alterations.

Key words: Tetracycline, ELISA, *Lactuca sativa, Eruca sativa, Spinacia oleracea*

INTRODUCTION

The antibiotics reach the ground from the manure, either while the animals are grazing or originated from those farms whom treat the animals because 30-80% of antibiotics through the intestinal animal tract (Halling-Sørensen et al., 2002; Sarmah et al., 2006; Michelini et al., 2013). It can affect mitochondria and chloroplast (Wang et al., 2015). Wastewater is more used to irrigate crop in many countries and the research has examined the antibiotics occurrence in soil which was irrigated with wastewater and their plant accumulation is less studied (Min et al., 2014). On the other hand, the use of antibiotics as pesticides (eg. oxytetracycline and sulfonamides) in crop could cause soil residues (McManus et al., 2002; Baran et al. 2011). Soil is the most contaminated environment with antibiotics.

Eating contaminated food derived from plants could lead to exposure to humans and animals antibiotics (Grote et al., 2007; Min et al., 2014). The concentrations of sulfonamides found in soil are around 200 μg kg^{-1} (Boxall et al., 2002; Baran et al. 2011), but can reach up to 200 mg L^{-1} in manure (Kumar, 2005).

A relationship between a pollutant and internal structure of vegetative organs is not always obvious. The incidence and severity of changes in leaf structure caused by pollution is closely related to other variables, such as the age of the plant, pollutant concentration, foliar absorption, the nature of the pollutant, the size of the intercellular space, environmental factors - temperature, humidity, light etc. (Dickinson, 2000). The uptake of pharmaceuticals in plants has been reported previously in particular in crops (Boxall et al., 2006; Ferro et al., 2010).

Along with leaves, root structure is closely related to the purchase of water plants and soil nutrients (Lynch, 1995). In this regard have been reported significant decreases in the carrot (*Daucus carota* L.) root length, after treatment with 1000 g L^{-1} and salad (*Lactuca sativa*) and alfalfa (*Medicago sativa* L.), at concentrations of 10.000 µg L^{-1} (Hillis et al., 2011). Baran et al. (2011) mentioned that sulfonamides have a low toxicity to higher organisms such as vertebrates and higher than for microorganisms, algae and certain plants.

In a study on wheat (*Triticum aestivum* L.) hydroponic culture (Li et al., 2011 a) it was suggested that oxytetracycline had a risk to crop growth through photosynthesis inhibition, requiring further confirmation with soil tests. In hydroponic culture too, Michelini et al. (2012) showed that sulfadimethoxine altered willow (*Salix fragilis* L.) root morphology. A year later, Michelini et al. (2013) reported that sulfonamides modified barley plant roots morphology and physiology. They observed that leaves ultrastructure was similar in plants grown in all the experimental conditions, with numerous chloroplasts in the mesophyll tissue, without evident differences between the control lot and the treated groups because their cells was not directly contact with antibiotics. The aerial organs suffer only the antibiotic effects moved to the shoots from the roots, where a high of polluted concentration can be retained (Rascio et al., 2008; Isleyen et al., 2012).

Li et all. (2011 b) found four quinolones, tetracycline and sulfamethoxazole were detected in >94% of the samples in soil from vegetable farmlands. The highest antibiotic concentrations were observed mainly in vegetable farmlands affiliated with livestock farms. Chlortetracycline, sulfameter and quinolones exceed the eco-toxic effect trigger value (100 µg/kg) (Li et al., 2011).

Petruș –Vancea et al. (2013) studied the structure of some vegetative species from the Crisuri Rivers basin, in order to identify a possible accumulation of pharmaceuticals. The vegetative plants anatomy data were corroborated with chemical data: ELISA tests, HPLC and for non-steroid inflammatory were analyzed by GS-MS method in gas-chromatographic lab. The authors concluded that the pollution in Crişuri Rivers Basin was not so high as to affect the vegetative organs anatomy.

Min et al. (2014) determined the various antibiotics distribution in different environmental matrices in a delta region. Samples were taken from sites where either domestic wastewater or fishpond water was used for irrigation. The mentioned authors reported that fishpond water irrigated soils had higher antibiotics concentrations than wastewater-irrigated soils (Min et al., 2014). They observed the antibiotics accumulation in the different crops parts ((Li et al., 2011 a; Min et al., 2014), albeit the human annual exposure to antibiotics through the consumption of edible crops is low (1.10 to 7950 µg/y).

The main purpose of the present study is to evaluate the TCS accumulation properties of three often alimentary used vegetables (*Lactuca sativa*, *Eruca sativa,* and *Spinacia oleracea)* according to the plants chemical characteristics (water and minerals) and the TCS accumulation effect on the foliar limb anatomical structure.

MATERIALS AND METHODS

Plants hydroponic culture

The three cultivated vegetable plants *Lactuca sativa* (Buttercrunch Lettuce type), *Eruca sativa* (Arugulla Roquette Bellezia type) and *Spinacia oleracea* (Semi-Savoy type) were grown from seeds on germinating support, for 12- to 18 days and transplants in the hydroponic system for 30 days to reach maturity in greenhouse, in spring time, at 16-18^{O}C. Nutrient film technique (NFT), with continuously re-circulate thin layer of nutrient solution, were used to provide nutrients and oxygen.

The three plants were chosen due to them similar grown necessities, same development season and roughly the same maturation period. For Lettuce and Spinach were recorded accidental TCS accumulation rates in plants cultivated on soil enriched with manure (Fodor et al., 2013).

The hydroponic system was used, to ensure uniform development conditions for the studied plants and to minimize the affecting accumulation factors limiting them as possible to the plant chemical characteristics, metabolism and physiology, this way providing the foundation for the TCS accumulation properties and effects study. A typical nutrient formula has been taken from Howard Resh formula (Kessler et al., 2006).

Table 1

Nutrient formula

Ca: 180-200 ppm	Mg: 40 ppm	K: 210 ppm
P: 50 ppm	Ammonium N: 15 ppm	Nitrate N: 165 ppm
Fe: 4 ppm	Mn: 0.5 ppm	Cu: 0.1 ppm
Zn: 0.1 ppm	B: 0.5 ppm	Mo: 0.05 ppm

To study the vegetables TCS accumulation properties the nutrient solution contained 21.00
$\mu g \cdot L^{-1}$ TCS, amount found to be accumulated in the soil farms where manure is administered along the time (Fodor et al., 2013 a, b). Some plants were cultivated in nutrient solution without TCS to be source of control leaves for the limb studies.

Sampling techniques

The studied vegetables leafs samples were taken from the mature plants in the thirtieth day of development. For each vegetable type were taken 50 samples to perform chemical analysis and 10 samples to perform foliar limb anatomy studies.

Chemical analysis

Mature plants leaf were chemically characterized measuring humidity, using gravimetric method and cations content using Atomic Absorption method after the samples mineralization. The mineralization of the samples followed the standard method for trace metals determination. The method comprised add of nitric acid (HNO_3 65%). The buffer solutions comprised certificated materials routinely used for this kind of procedures.
TCS accumulation was measured using ELISA screening method using R-Biopharm Ridascreen® Tetracycline (Art. No. R 3501) test kit. This method was proved to be a suitable analytical technique with specificity, sensitivity and simplicity (Fodor et al., 2013 a). This method was used to monitories the TCS accumulation in agricultural farmland soil and in roots and lefs of vegetables (Fodor et al., 2013 a, b).

All reagents used are contained in the test kit. The used method was the one described in the kit with little modification. Rida ® C18 column was used for sample purification. The HPLC grade distillate water was used. A spectrophotometer ELISA READER was used measuring the absorbance at 450 nm. For each sample determination were carried out 3 measuring and the average calculated.

Foliar limb anatomical structure were studied in transverse sections to fresh plant (Andrei and Paraschivoiu, 2003). Sections were colored with 'Red

Congo' 3%. For each experimental types were examined every 30 sections per sample. The preparations were examined under the optical microscope Leitz brand, Webster M, with eye-10X and 20X - 40X objectives. The images were photographed with a Nikon 3100 digital camera adapted to the microscope.

RESULTS AND DISCUSSIONS

Chemical leafs characterisation

The obtained data (table 2) were compared and found in goog agreement with the data from National Nutrient Database for Standard Reference, Release 27, of the United States Department of Agriculture, Agricultural Research Service (http://fnic.nal.usda.gov/food-composition/vitamins-and-minerals).

Table 2

Chemical characterisation of the sampled lefs

Parameter /100 g leaf	***Lactuca sativa* L.**	***Eruca sativa* L.**	***Spinacia oleracea* L.**
Water (mg)	96.20	92.30	92.20
Ca (mg)	37.80	156.60	134.50
Fe (mg)	0.52	1.30	3.40
Mg (mg)	26.00	45.30	85.20
K (mg)	326.20	350.40	460.30
Zn (mg)	0.25	0.45	0.72

TCS accumulation

The results obtained for the TCS screening in the three usual herbaceous vegetables leafs, cultivated in nutrient solution containing 21.00 $\mu g \cdot L^{-1}$ TCS, and are presented in table 3.

Table 3

TCS values in the sampled lefs

TCS Value $\mu g \cdot Kg^{-1}$	***Lactuca sativa* L.**	***Eruca sativa* L.**	***Spinacia oleracea* L.**
Minimum	12.23	22.09	18.30
Maximum	19.90	28.40	24.80
Average	15.04	25.55	20.85

The obtained accumulated TCS average values have the same order as were noticed for lattice (13.05 $\mu g \cdot Kg^{-1}$) and spinach (15.31 $\mu g \cdot Kg^{-1}$) cultivated in soil containing TCS (derived from added manure; 21,54 $\mu g \cdot Kg^{-1}$ TCS) (Fodor et al., 2013). Unlike, the cultivated vegetables on soil the differences between the minimum and maximum determinate TCS quantities were around 85%, in the hydroponic culture the differences were about 15%, therefore to evaluate the TCS accumulation properties of the vegetables the hydroponic technique is necessary. Therefore if the TCS pollution in the case of soil cultivated plants can be appreciate as accidental in the hydroponic culture the TCS accumulation is systematically registered.

The TCS quantitative accumulation ratio can be determined by the each species characteristics, environment conditions, stage of development and growing period. The studied vegetables were cultivated in the same hydroponic system and conditions, the leaf samples were performed in the same maturity period (30 days), to minimize the affecting accumulation factors, limiting them as possible to the plant chemical characteristics, metabolism and physiology.

From the perspective of the chemical caracteristics of the three vegetables, it can be notice that the greather TCS accumulation is recorded for the arugula (*Eruca sativa)* with the grater content of calcium. Probably the TCS accumulation mechanism involves the formation of chelating compounds.

Foliar limb anatomy

Because we have found no differences between the structures of leaves from seedlings tested group and that of the control one (cultivated in nutrient solutions without TCS), we present below only tested leaf structure (figure 2-4).

To arugula leaves from lot with TCS (figure 2 A-C) we have not identified anomalies in terms of anatomical structure. Upper (figure 2 A) and lower epidermis and fundamental parenchyma (figure 2 B) from the nervure had normal aspects. The conformation of the xylem and the phloem were normal (figure 2 C).

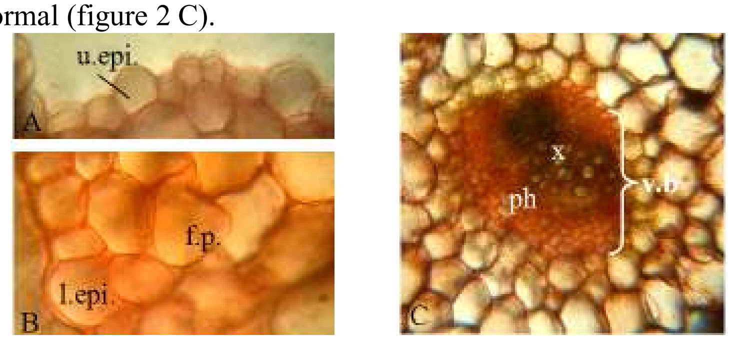

Fig. 2 Arugula (*Eruca sativa* L.) leaf anatomy, growth on tested lot (with TCS): A – upper epidermis (200X); B – lower epidermis (200X);
C – vascular bundle (200X) (f.p. – fundamental parenchyma; l.epi. – lower epidermis; ph – phloem; u.epi. – upper epidermis; v.b. – vascular bundle;
x – xylem).

The effect of TCS on lettuce leaf structure was absent, in our experimental conditions. At the epidermis and its formations (figure 3 A, B) level and at the nervure and vascular bundle (figure 3 C) were not identified histo-anatomical changes.

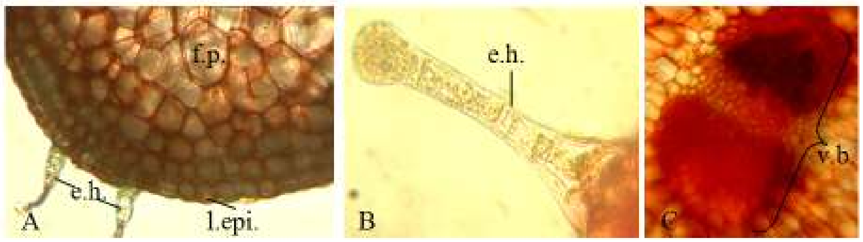

Fig. 3 Lettuce (*Lactuca sativa* L.) leaf anatomy, growth on tested lot (with TCS): A – lower epidermis (200X); B epidermal hair (400x); C – vascular bundle (200X) (e.h. – epidermal hair; f.p. – fundamental parenchyma; l.epi. – lower epidermis; v.b. – vascular bundle).

Normal aspect of leaf structure was highlighted in leaves of spinach plants treated with TCS (figure 4 A-C). Cells shape, cells integrity, lack of large intercellular spaces demonstrate that on these tissues were not acting polluter or toxic factors.

When chemical pollutants concentrations and the exposure are high this can highlight changes in anatomical structure. In this case may appear damaged tissue, abnormal cell sizes. In polluted forest communities was identified a xylem reduction caused by reduced cambial activity (Dickinson, 2000).

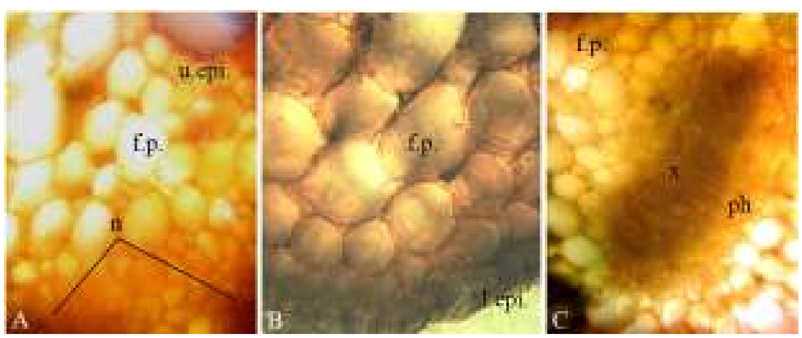

Fig. 4 Spinach (*Spinacia oleracea* L.) leaf anatomy, growth on tested lot (with TCS): A – upper epidermis (200X); B – lower epidermis (200X); C – vascular bundle (200X) (f.p. – fundamental parenchyma; l.epi. – lower epidermis; n – nervure; ph – phloem; u.epi. – upper epidermis; x – xylem).

From the structural point of view, in tested plant leafs did not notice the difference between plants grown in nutrient solution containing TCS and those cultivated in nutrient solution without TCS. Possibly, in an ultrastructure study some changes could be made out.

CONCLUSIONS

The hydroponic system could provide the ideal support for the cultivation of plants subject to research of residual accumulations and its effects due to the minimization of the affecting accumulation factors (limiting them to the plant chemical characteristics, metabolism and physiology).

TCS were systematically find to be accumulated in leafs of *Lactuca sativa* L., *Eruca sativa* L., and *Spinacia oleracea* L. cultivated in hydroponic medium (NFT technique), in nutrient solutions containing residues of TCS (21.00 μg $\cdot L^{-1}$), in low concentrations (15-26 μg $\cdot Kg^{-1}$). The TCS obtained values (minimum, maximum and average) are close in value, showing the fact that the accumulation is not accidental (as was shown in studies on plants cultivated on soil poluted with TCS coming from manure administration). It was registered that the greather TCS accumulation is recorded for *Eruca sativa* L., which is richer in calcium. Probably the TCS accumulation mechanism involves the formation of chelating compounds.

Normal aspect of foliar limb structure was highlighted in leaves of the three studied plants treated with solution containing tetracycline, meaning that this antibiotic was not sufficiently acted or was in too low concentrations to induce histo-anatomical change.

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