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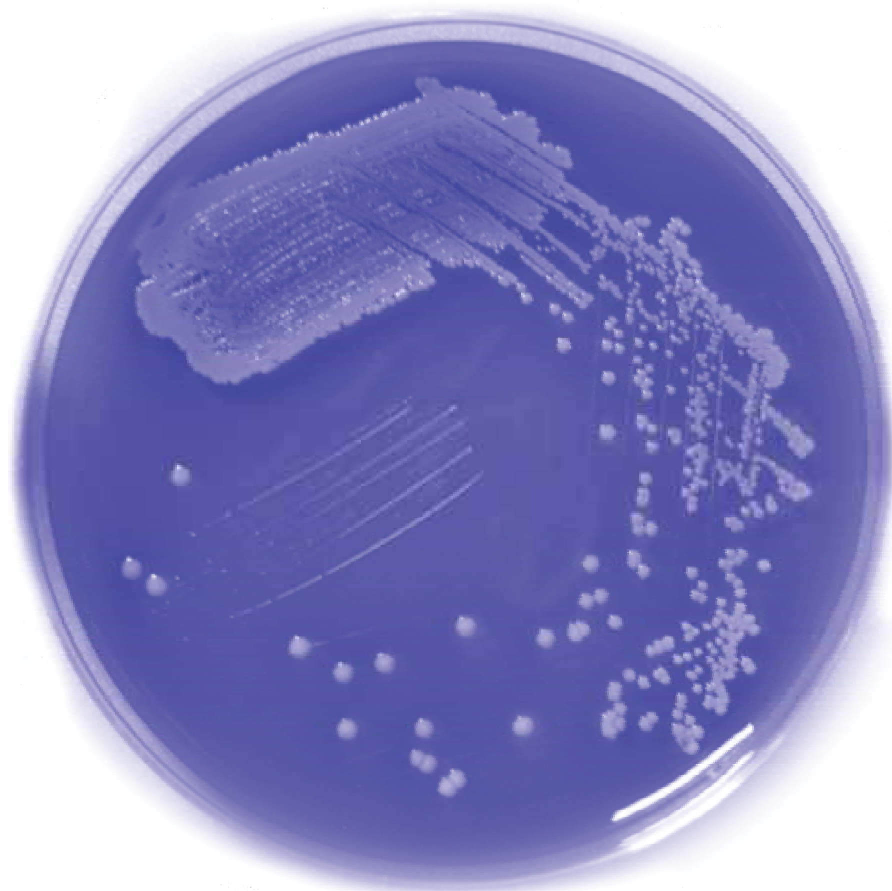

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All authors are responsible for submitting manuscripts in comprehensible US or UK English and ensuring scientific accuracy.

Original pictures on front cover: *Staphylococcus aureus* on Columbia Blood Agar Media © Rahela Carpa

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Bio-chemical analysis of *Datura stramonium* extract

Rahela Carpa[1,2,✉], Dana-Victoria Dumitru[1], Ramona Flavia Burtescu[3], Maria Cornelia Maior[4], Cristina Dobrotă[1,4] and Neli-Kinga Olah[3,5]

SUMMARY. *Datura stramonium* is an annual plant which belongs to the *Solanaceae* family. *Datura stramonium* is also a toxic plant, with toxicity given by the presence of alkaloids hyoscyamine, atropine and scopolamine.This study performs phytochemical and antibacterial analysis of ethanolic extracts from *Datura stramonium.* Fresh plants have been used in order to obtain *Datura stramonium* tincture. The quality index was 1:5, the concentration of ethanol being 90%. Based on this tincture a qualitative and quantitative phytochemical analysis was performed through thin layer chromatography and high performance liquid chromatography. By thin-layer chromatography the qualitative alkaloids, such as atropine and scopolamine from *Datura stramonium* extract, have been identified. By high performance liquid chromatography with reversed phase the alkaloids quantity from *Datura stramonium* herbs was assessed and that is 1.7 mg/mL. The plant extracts were tested on Gram negative bacteria *Escherichia coli* and on Gram positive bacteria *Staphylococcus aureus*. Both tested strains showed resistance but for *E. coli* a higher inhibition was observed at all samples containing *Datura* extract.

Key words: atropine, bacterial susceptibility, *Datura stramonium*, High Performance Liquid Chromatography with reversed phase, scopolamine, thin-layer chromatography.

Introduction

Datura stramonium is an annual plant which belongs to *Solanaceae* family and emits an unpleasant odor because of the presence of tropane alkaloids (Kumar,

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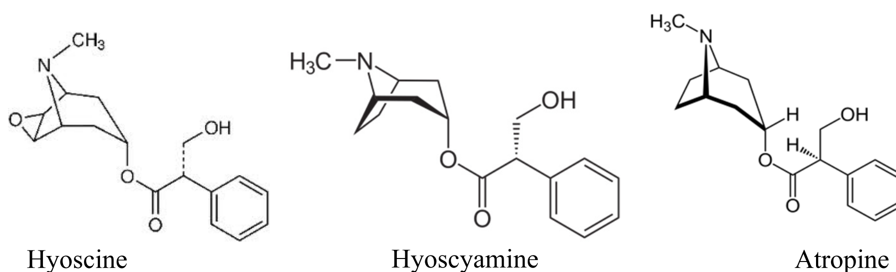
2009). *Datura stramonium* is a toxic plant, also the toxicity is given by the presence of alkaloids: hyoscyamine, atropine and scopolamine. Iranbakhsh *et al*., (2006) determined the quantities of scopolamine and atropine in different stages of growth. The largest amount of scopolamine is found in leaves in the vegetative period, and the smallest amount is found in the root in the vegetative stage. In addition the greatest quantity of atropine is found in the petiole in the vegetative period, and the smallest quantity is found in the seed (Sreenivasa *et al*., 2012). The total concentration of alkaloids in *Datura stramonium* leaves is 0.2 – 0.5%. More than 70 alkaloids were identified in different parts of the plant, but the main alkaloids are: hyoscyamine, atropine, scopolamine (Fig. 1) (Schmelzer and Gurib – Fakin, 2008; Mann, 2008). Hyoscyamine is a major component when the plant is highly developed. Besides this, the plant also contains scopolamine, atropine, tropine, belladonnine, leucine, glutamic acid, enzymes, citric acid, malic acid, etheric oil, mineral salts, etc.



Hyoscine Hyoscyamine Atropine

Figure 1. Chemical structure of tropane alkaloids.

Atropine is a tropane alkaloid, isomer of hyoscyamine. It is found in different concentrations in *Hyoscyamus niger*, *Datura stramonium*, *Brugmansia suaveolens, Atropa bella-donna, Duboisia myoporoides*. The molecular formula is $C_{17}H_{23}NO$. Atropine has two major actions. One of the actions affects the central nervous system (CNS) and provokes an airway stimulation. On the other hand this alkaloid depresses the smooth muscle and the secretory glands which are innervated by the parasympathetic nerves. The central action of atropine is sedative (in paralysis, agitation, Parkinson disease). Peripheral action of atropine refers especially to the secretion of the respiratory glands, bronchial muscles, heart, gastro-intestinal tract, urinary tract (Kasture and Wandodkar, 2008). When it is administrated orally the atropine is absorbed well in the organism and it is eliminated rapidly, the excretion is complete in approximately 24 hours. The normal dose should be 0.4 mg/day by the oral tract or parenteral. If the dose is more than 3 mg it can provoke mental and behavioral changes.

Atropine biosynthesis begins with the amino acid L-phenylalanine. Through transamination the– NH_2 group is transferred and it forms pyruvic acid who in turn is reduced to phenyl lactic acid. Through a series of reaction tropine and litorin is formed which undergoes a rearrangement initiated by the enzyme *p450* forming hyoscyamine aldehyde. Through a dehydrogenation reaction hyoscyamine is formed and through racemization passes into atropine (Behçet, 2014). Atropine is cleaved by the atropinesterase enzyme to the tropic acid in the roots of the plant, *Datura stramonium.*

Hyoscyamine (daturine) is an ester of tropic acid with atropine, being an amino alcohol. It is a secondary metabolite also found in the leaves of *Datura stramonium*. The molecular formula is $C_{17}H_{23}NO_3$. After the plant is dried, the structural composition is modified and hyoscyamine goes through the levogirous isomer called atropine. Hyoscyamine is an antagonist of muscarinic acetylcholine receptors having 98% of the anticholinergic power of atropine.

The place and the hyoscyamine biosynthesis in the plant is similar to atropine (Roberts and Wink, 2013). Hyoscyamine is used in gastrointestinal disorders, spasms, ulcers, pancreatitis, cholics and cystitis. Also it is used in heart problems as well as controlling Parkinson’s disease symptoms (Schmelzer and Gurib – Fakin, 2008).

Scopolamine (hyoscine) is a toxic alkaloid met at species of the *Solanaceae* family respectively in *Hyoscyamus niger, Brugmansia suaveolens, Datura stramonium, Duboisia myoporoides, Atropa bella-donna.* Scopolamine takes part of the tropane alkaloids class, alkaloids which contain nitrogen in heterocycle and are synthesized from amino. The molecular formula is $C_{17}H_{21}NO_4$. Scopolamine is a hallucinogenic substance if is administrated in high doses, which can even lead to coma or body death (Sweta and Lakshimi, 2015). It has medical properties, frequent are used for motion sickness, it is administrated after surgery because it prevents nausea and vomiting, also it is used as a sedative. Recently it has been shown to be useful as a detoxifier because it has significantly reduced the desire for the use of heroin and its effects (Liu *et al*., 2013). It is used in intestinal cramps (Alvarez and Marconi, 2011), it has pharmacological properties on the CNS, on the gastrointestinal system, in shortness of breath, and it is also administered to people suffering from Parkinson’s disease (Yang *et al*., 2014). Scopolamine takes part in the class of anesthetic drugs, adjuvants, antiemetics, gastrointestinal and anti-vertigo. The pathway of penetration into the body is transdermal.

The scopolamine biosynthesis can be obtained in 3 steps: a) Beginning from the ornithine amino acid in the presence of the enzyme ornithine decarboxylase is obtained putrescine which in the presence of the enzyme putrescine N-methyl transferase result the N – methyl putrescine. Through a spontaneous reaction tropinone is formed, which in the presence of the tropinone reductase 1 enzyme leads to the obtaining of tropine. b) Starting from shikimic acid, phenylalanine, phenyl pyruvic acid and tropic

acid are obtained. c) The products from step a and b (tropine and tropic acid) leads to the obtaining of hyoscyamine which is transformed in scopolamine by hyoscyamine 6 β hydroxylase enzyme (Facchini, 2001).

The place where biosynthesis begins can be determined through locating alkaloid enzyme of synthesis (Indra, 2012). Thus, the *h6h* enzyme which converts hyoscyamine into scopolamine and *pmt* enzyme which initiates the transformation of putrescine into N-methyl putrescine are found in the pericycle of the root. The pericycle is a thin layer which is found in between the endoderm and the phloem (Dubrovsky and Rost, 2012) and scopolamine is synthesized in the pericycle being translocated through the xylem to aerial parts where it accumulates in the vacuoles (Chandra, 2012; Roberts, 2007; Hashimoto *et al*., 1991). The mechanism of alkaloids translocation from the root to the aerial parts is controvertible (Kanegae *et al.,* 1994). Scopolamine translocation towards aerial parts of plants is realized with the help of the xylem. More precisely from pericycle the scopolamine passes into xylem. Scopolamine will be translocated with sap xylem to aerial parts of plant (Kandoth *et al*., 2010). An important factor on which scopolamine depends it is light. Studies made on root crops of *Scopolia carniolica* indicated the fact that the roots kept in darkness strongly stimulates the synthesis of scopolamine. In this article it was realized qualitative and quantitative chemical analysis of the ethanolic extract of *Datura stramonium* by two methods: Thin layer chromatography (CSS) and High Performance Liquid Chromatography with reversed phase (HPLC). Than was determined antimicrobial activity of the *Datura* extract both on *Gram positive* and *Gram negative* bacteria.

Materials and Methods

1. Extraction protocol from *Datura stramonium*. For *Datura stramonium* fresh plants and blossoming herb were used. The extract passes through a series of stages, beginning with the grinding of the used part, up to obtaining the final extract. If all parameters correspond then the entire quantity of the grinded plants is weighed and then the pharmacopoeia quality index is established. The quality index was 1:5, the concentration of ethanol being 90% because it is used the fresh herb. The herb of *Datura stramonium* along with the alcohol mixture were stirred, cleaned and left to macerate for five days in the concentration of ethanol established. During this time the density was determined. It was then filtered and after the analysis it was sealed. *Datura stramonium* extract was obtained from PlantExtract in Rădaia, Cluj County and for compounds analyze from extract of *Datura stramonium* the German Pharmacopoeia was used (HAB, 2011).

2. Identification of the compounds in the extract of *Datura stramonium* by thin-layer chromatography (TLC). Thin layer chromatography is a qualitative method which consists in dividing into three equal areas on the chromatographic plate

and resemble of the compounds in the mixture with specific standards or by identifying compounds based on the values of retention factor. Thin layer chromatography can also be a quantitative method by correlating the concentration with the peak area. In order to analyze the compounds of the *Datura stramonium* extract by TLC a silica gel plate was used with a fluorescent indicator of 254 nm, with a thickness of 0.25 mm and 7x13 cm dimensions. The migration distance is 100 mm, and the eluent is a mixture of ammonia, purified water and acetone (3:7:90, v/v). From the extract, 20 µl were applied.

As standards were used scopolamine hydrobromide (5 mg/mL), atropine sulfate (15 mg/mL) dissolved in 10 mL of methanol and then applied in volumes of 10 µl. The plate was dried at 100-105 ºC and was sprayed with diluted Draggendorf reagent and finally with sulfuric acid 0.05 mol/l until the red and red-orange bands appear from yellow to brown. The chromatogram is visualized in visible light.

3. Alkaloid quantification, namely the atropine and scopolamine content in the *Datura stramonium* ethanolic extract using High Performance Liquid Chromatography with reversed phase (HPLC). High performance liquid chromatography is an analytical method whereby the substances in a mixture are identified, separated and dosed. HPLC is an instrument comprising the solvent (constitutes the mobile phase, is used to transport the substance to be analyzed), the pump (continuously pumps the solvent), injector (introduces the sample into the system), chromatographic column (contains the stationary phase), detector (sends the signals to the software of the computer which posts a chromatogram). In this study we used the reversed-phase HPLC (stationary phase is nonpolar and the mobile phase is polar). HPLC protocol was used according to the protocol used by (HAB, 2011).

Sample: At 1 g tincture 1 mL of water and 0.5 mL ammonia are added and the mixture is passed through a chromatographic column with 14 mm internal diameter, filled with 2.5 g granulated Kieselgur. After 15 minutes it is twice eluted with 15 mL ethylic acid. The united eluates are dried. The residue is retaken with 3.0 mL mixture of 65 volumes trifluoracetic acid and 35 volumes acetonitrile. The sample is filtered through 0.45 µm filter. As standard 0.1 g atropine and scopolamine are used, dissolved in 10 mL methanol. The injection volume was 10 µl, 15 µl, 20 µl, 25 µl, 30 µl of sample and standard.

The HPLC column was silica gel C18, 125 x 4 mm x 4 µm and the mobile phase was represented by A = acetonitrile; B = phosphate buffer pH= 3.5 (15:85 v/v). The injection debit was 0.6 mL/min. The detection is spectrophotometrically performed at 210 nm and the retention time for scopolamine is about 3.5 minutes and for atropine is about 7.6 minutes.

4. Disk paper method for testing the bacteria susceptibility to *Datura stramonium* extract. The susceptibility of the tested bacteria (*Staphylococcus aureus* ATCC 25923, Gram positive and *Escherichia coli* ATCC 25922, Gram negative) to the plant extract was determined using a paper disc diffusion assay on Nutrient Agar plates

(Atlas, 2010), following the method described by Carpa *et al*., (2014). Bacterial suspensions were adjusted to 0.5 McFarland turbidity (1-2 x 10^6 cfu mL^{-1}) and spread evenly over the entire surface of the agar plates using a sterile cotton swab. The plates were allowed to air-dry for approximately 10 minutes before the paper disc (6 mm) was placed on the agar plate. Each extract test was replicated three times. The plates were incubated at 37ºC for 24 hours. For each microorganism tested, zones of inhibition of growth were examined, and the diameter of each zone was recorded.

Results and discussions

1. Qualitative analysis by thin layer chromatography for *Datura stramonium* extract. The silica gel plates were cut according to the dimensions in the protocol. In the meantime the eluent was prepared, composed of a mixture of ammonia, purified water and acetone. On the chromatograph plate 20 µl of *Datura stramonium* extract were applied. As standard a mixture of scopolamine hydrobromide and atropine sulfate was used, out of which 10 µl were applied. After the migration of compounds the plate was pulverized with Draggendorf reactive and sulfuric acid until the bands appear. The chromatogram was observed in visible light (Fig. 2).

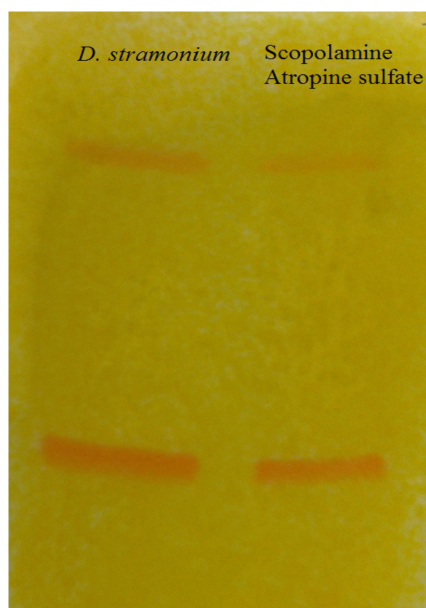


Figure 2. Chromatogram of *Datura stramonium* extract plate in visible light

The compounds in the extract were qualitatively identified using thin layer chromatography. The standards chromatogram shows, in the superior third, an orange belt for atropine sulfate and in the median third an orange belt for scopolamine hydrobromide. The sample chromatogram shows belts off the standards, which resemble the belts form the standards chromatogram as regards position, size and color (Fig. 2). The presence of tropane atropine and scopolamine alkaloids is highlighted in the *Datura* extract while the intensity of the belts suggests that atropine is found in a larger quantity than scopolamine. By using eluent the alkaloids in the extract were successfully drawn out.

2. Quantitative analysis of alkaloids in *Datura stramonium* extract by high-performance liquid chromatography (HPLC). The quantities and areas obtained for atropine and scopolamine standards are shown in Table 1. Based on the areas obtained, one calibration curve was obtained for atropine and one for scopolamine, representing the peak area dependent on concentration.

Table 1.

Standard quantity and areas

Atropine, scopolamine quantity (mg/mL)	Atropine area	Scopolamine area
0.1	1058283	5103550
0.15	1587478	7855325
0.20	2096786	10507100
0.25	2545708	12758875
0.30	3174879	15310650

Afterwards the equation of a straight line was established, where x represents the concentration [mg/mL], while y = the area of the peak form the sample. Using the equation, the quantity of compounds in the *Datura stramonium* ethanolic extract was calculated in the moment when the area of the sample pick was reached. The aria of the pick for atropine and scopolamine will be taken for the chromatogram of *Datura* extract.

In Fig. 3 is presented the chromatogram of atropine standard.

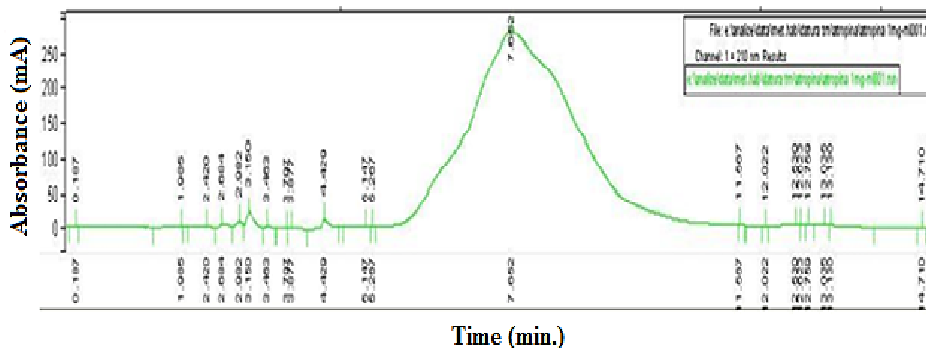


Figure 3. Chromatogram for atropine standard 1 mg/mL at 210 nm.

Fig. 4 shows the absorption spectrum of atropine standard from its chromatogram.

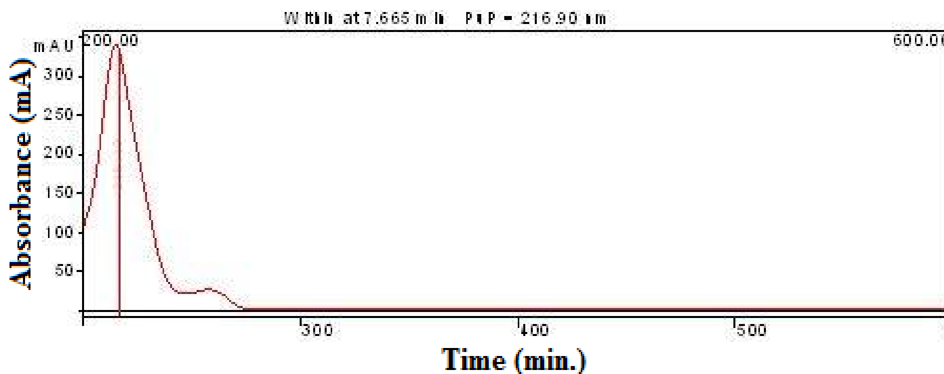


Figure 4. Atropine spectrum in atropine standard chromatogram at 210 nm

From the atropine standard chromatogram can be marked out that atropine will come out in the sample around minute 7.665. Besides atropine scopolamine was identified. The scopolamine standard chromatogram is shown in Fig. 5.

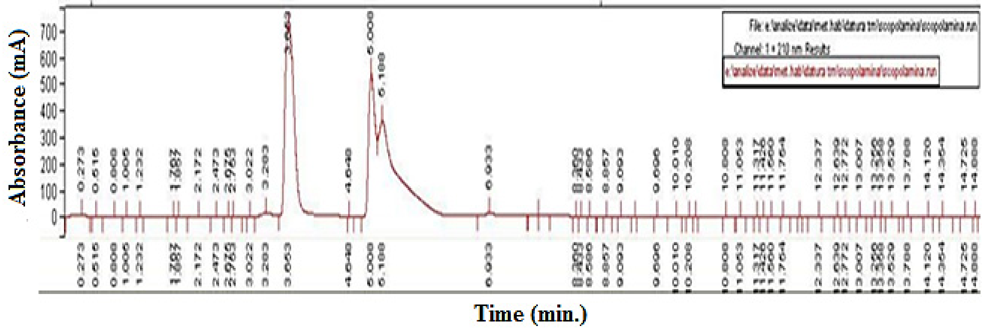


Figure 5. Scopolamine standard chromatogram 1 mg/mL at 210 nm

Based on the chromatogram was established the spectrum of scopolamine alkaloid represented in Fig. 6.

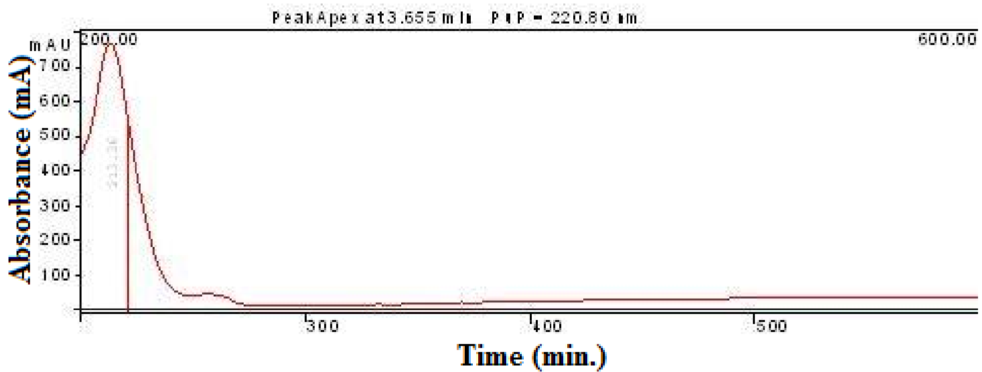


Figure 6. Scopolamine spectrum in the scopolamine standard chromatogram at 210 nm.

Based on the chromatogram we can assess that scopolamine will come out into the sample around minute 3.655. Fig. 7 shows the overlapped chromatograms of the standards (atropine and scopolamine) as well as the ethanolic extract from *Datura stramonium*.

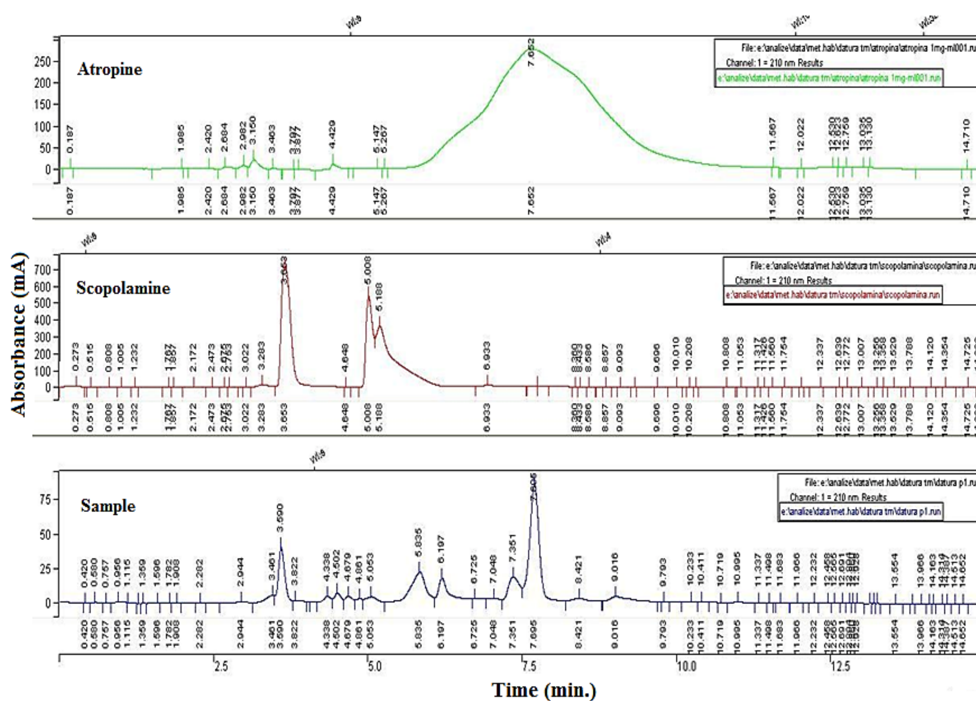


Figure 7. Standards chromatograms and chromatogram of the extract form leafs of *Datura stramonium*

The absorption spectrums of atropine and scopolamine in the sample were also recorded. In Fig. 8 is shown the absorption spectrum of atropine in the ethanolic extract of the herb.

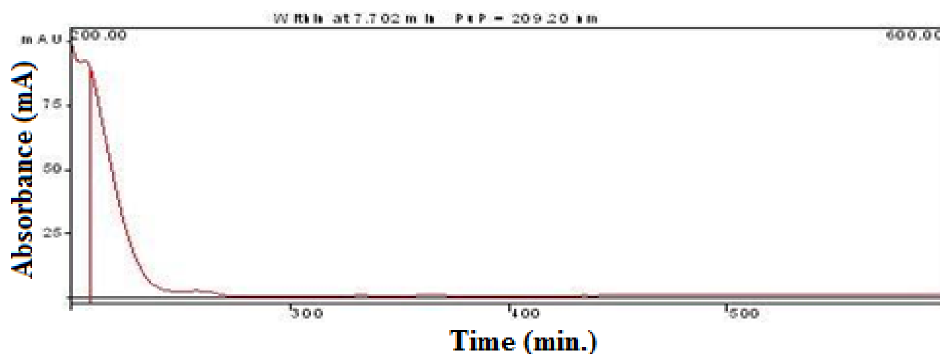


Figure 8. Atropine spectrum in the ethanolic extract of *Datura stramonium*

The absorption spectrum of scopolamine form *Datura stramonium* extract is depicted in Fig. 9.

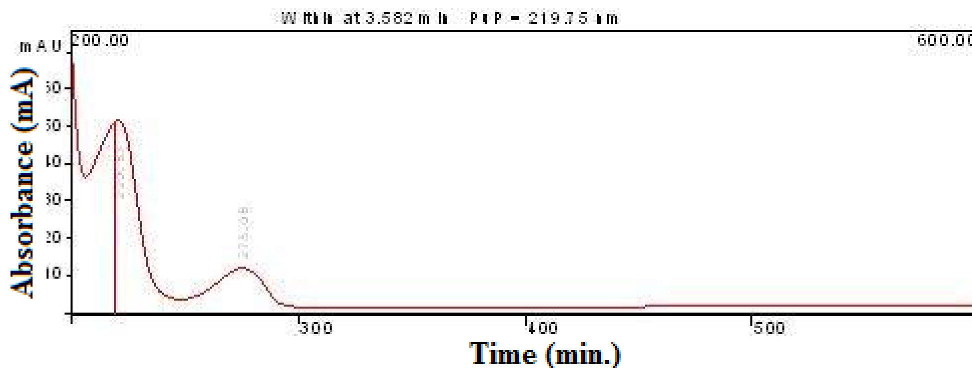


Figure 9. Scopolamine spectrum in the ethanolic extract of *Datura stramonium*

In the ethanolic extract of *Datura stramonium* can be observed an absorption pick for atropine at minute 7.659, and for scopolamine at minute 3.590. These results, compared to the absorption spectrums of standards (atropine and scopolamine) show that the substances are correctly identified. Using the equations from the standard curves of standards and according to the pink area form the extract the alkaloids quantity in an mL of sample was calculated. Based on these areas were calculated the alkaloid concentrations in *Datura stramonium* (Table 2).

Table 2.

The alkaloids quantity in the sample

90% ethanolic extract of *Datura stramonium*	**Pick area in the extract at 10 nm**	**Quantity (mg/mL)**
Atropine	11319234 (at minute 7.695)	1.13 mg/mL
Scopolamine	2906174 (at minute 3.590)	0.57 mg/mL

Both by CSS and HPLC analyses it was observed that atropine is found in a larger quantity in the ethanolic extract of *Datura stramonium*. The other peaks in the chromatogram are represented by other unidentified compounds.

The 3 compounds, hyoscyamine, atropine and scopolamine, present a special medical importance, being anticholinergic, antimuscarinic, in competition with the acetylcholine neurotransmitter present in the central and peripheral nervous system.

In case atropine or scopolamine are bound to muscarinic receptors in place of acetylcholine, the cleavage mechanism is inhibited (Akal *et al.,* 2014). Blocking of muscarinic receptors by scopolamine triggers increasing the cardiac frequency, which makes it useful in bradycardia, at urinary retention and overactive bladder. In small doses they have a sedative effect, but in large doses they have negative effects on the organism, causing agitation, disorientation, hallucinations, delirium, mental confusion and insomnia (Schmelzer and Gurib – Fakin, 2008).

3. Testing bacteria sensibility to *Datura stramonium* extract. The test strains (*Staphylococcus aureus* for Gram-positive bacteria and *Escherichia coli* for Gram-negative bacteria) were inoculated on Petri dishes with Nutrient Agar (Atlas, 2010), in aseptic conditions. At the inoculation of the test microorganism 1 mL of bacterial suspension, spread on all the surface of the medium, was used, and the surplus of culture was eliminated. After the drying of the inoculated media 6 mm paper disks were applied (Fig. 10).

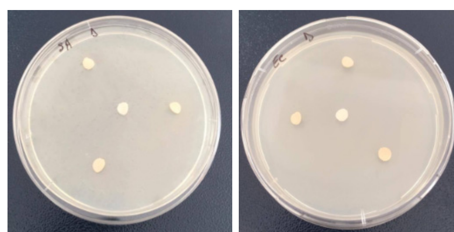


Figure 10. Applying paper disks on the NA culture medium inoculated with *Staphylococcus aureus* and *Escherichia coli* (0 moment).

The Petri dishes were incubated 18-24 h. After 24h incubation the results were assessed by measuring the emerged inhibition zone, in millimeters (Fig. 11), at *Staphylococcus aureus* and *Escherichia coli* strains. In Fig. 13 it is visible that at Gram-positive strain (*Staphylococcus aureus*) the sensibility is very high at all the tested samples, but also at Gram-negative strain (*E. coli*) the sensibility is very high at all tested samples.

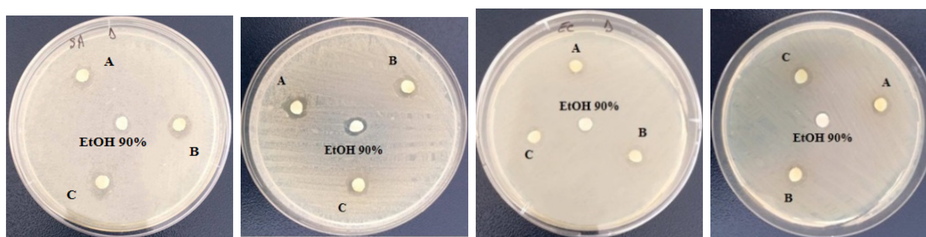


Figure 11. Assessing the sensibility *Staphylococcus aureus* and *Escherichia coli* with paper disks with *Datura stramonium* extract at 24 incubation hours.

Antibacterial and antifungal activity of plant extracts depends on the solvent used (ethanol, methanol, water, chloroform, benzene, petroleum ether), It was proved that if methanol is used as solvent a large alkaloid concentration is extracted and a small concentration of iridoids, flavonoids, saponins, sterols, tannins. Likewise, if the petroleum ether is used as extraction solvent, a very large alkaloids quantity and a smaller flavonoids concentration are obtained (Sreenivasa *et al*., 2012). The mathanolic leaves extracts showed antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Rhizopus stolonifer* (Sharma and Sharma, 2013). At a higher concentration of ethanolic stramonium extract antimicrobial activity against *Klebsiella pneumoniae*, *Fusarium sp*. and *Aspergillus niger* was observed (Reddy, 2009). The aqueous extracts from raw *Datura stramonium* leafs showed inhibition on *Bacillus*, *E. coli* and *Klebsiella* and a high sensibility on *S. aureus* and *Sarcina* (Shobha *et al*., 2014). The raw extract of *Datura* leaves, using ethyl acetate as solvent displayed a good inhibition against *S. aureus* resistant to methicillin, the strain isolated from festering samples of skin infections. (Venkanna *et al*., 2013).

Conclusions

Datura stramonium is a plant which belongs to *Solanaceae* family and contains alkaloids as atropine, hyoscyamine and scopolamine, considered anticholinergics, which are biosynthesized in root, transported through xylem to the aerial part and stored in vacuoles. In order to obtain ethanolic *Datura* extract fresh herb is used and a 90% ethanol concentration.

By CSS alkaloids as atropine and scopolamine were qualitatively identified at the *Datura* extract.

By HPLC the alkaloids quantity in the extract was assessed: 1.7 mg/mL (out of which 1.13 for atropine and 0.57 for scopolamine).

For testing the sensibility of *E. coli* and *S. aureus* the paper disks method, with 6 mm paper disks impregnated with 40 µl extract, was used. Both tested strains showed resistance but at *E. coli* a higher inhibition was observed at all the samples containing *Datura* extract. Not only the identified compounds (atropine and scopolamine) are responsible of the antimicrobial activity, but all the secondary metabolites present in the extract.

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Influence of salt content on enzymatic activities and halophytes distribution in Cojocna zone, Romania

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SUMMARY. Salty soil samples from three zones of Cojocna (Cluj county, Romania) have been investigated physico-chemically and enzymologically. Rizosphere of some halophytes were investigated in order to assess saline stress on bacterial enzyme activities and halophytes adaptation mechanisms to enhanced salt content. Several physico-chemical parameters of salty soil were determinated: pH, conductivity and different mineral ion contents. Changeful bacterial enzymatic activities and mineral ion composition were detected according to soil salt content. Reduced values of all enzymatic activities were detected. Based on the studied enzyme activities the enzymatic indicators of soil quality (EISQ) were calculated. The EISQ values were low, between EISQ = 0.201 and 0.236. In the sudied salty area, only a few halophyte species were observed and identified, the most common are: *Salicornia herbacea, Puccinellia distans*, *Artemisia santonica, Limonium gmelinii* and *Salsola soda.* All three salty zones were classified as sodic and saline-sodic soils.

Key words: Cojocna, enzymatic activities, halophyte, salty soil.

Introduction

Soil enzymes have a very active contribution in soils, being involved in biogeochemical cycles, energy metabolism, pollution removal and other main biogeochemical processes of soil ecosystem (Caldwell, 2005). Soil microorganisms,

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along with their activities, have a fundamental contribution to the biotic community and the ecosystem functioning in natural terrestrial habitats. All types of soil contain a bulk of enzymes which triggers the soil productivity, physical and chemical properties, and health. They play a main role in the decomposition of organic matter (Sinsabaugh *et al.*, 1991). The amount of enzymes varies according to the type, constituents, organic matter content and biota of soil and heir biological functions. It is known that microorganisms contribute to soil enzyme composition, but this composition is completed by animals and plants. Soil enzyme activity constitutes a main indicator for soil quality, especially regarding different types of pollution (Yang *et al.*, 2016). High-salinity in soil, sometimes caused by saline groundwater, impedes the germination and growth of plants (Zhang *et al.*, 2015; Guo and Liu, 2014). Beside high salinity, lack of macronutrients also characterize the saline soils and causes their limitation.

Soil enzymes catalyze the organic matter decomposition, and their activity is in tight relation with the physical and chemical properties of soil (Kussainova *et al.*, 2013), structural composition of microbiota (Nielsen *et al.*, 2014) or with the vegetation (Mierzwa-Hersztek *et al.*, 2016). Both the composition of microbiota and the enzymatic activity are main qualitative indicators of soil quality, but the generation and functioning mechanisms of soil bacterial community are yet to be fully understood (Xun *et al.*, 2015).

This study aims to assess the salty soil enzymatic activity, the micro- and macro elements in it, which underlie the functioning of saline soil ecosystems. Such parameters have been prior used to assess the status and productivity of soil in natural ecosystems.

Materials and methods

Study site and soil sampling. The field site is located in the Transylvanian Plateau, East of Cluj-Napoca city at an elevation about 330 m. Cojocna zone is representative for the salty areas in the Transylvanian Plateau. The climate is temperate-continental. The prevalent halophytes are *Salicornia herbacea, Puccinellia distans*, *Artemisia santonica, Limonium gmelinii* and *Salsola soda*, with a coverage of approximately 50% in Cojocna areas.

Soil samples were collected in 2016 from Cojocna. The three collection sites were: 1) N46.74304 E23.84265, 2) N46.74319 E23.84290 and 3) N46.74328 E23.84295. The soil samples were passed in a portable storage box and transported into the laboratory. The soil samples used for measuring enzymatic activity and physico-chemical properties were air-dried and then temporarily stored at 4 ºC, for further analyses.

Physical and chemical properties of soils. After removing the grass and other external objects, soil samples were air-dried, ground and sieved (<2 mm) prior to determination of available nutrients and soil characteristics. The salinity, electrical conductivity (EC), pH and redox potential (Eh) of the soils were potentiometrically

measured in aqueous fraction (1:5) (ISO- 10390). Mineral N (NO_3^-N and NH_4-N), available phosphorus (PO_4), available potassium (K), sodium (Na) and other micro- and macro-elements of the soil were determined by ion chromatography (Ion Chromatograph IC 1500 DIONEX).

In order to determine the elements above were used: Ultra-pure water (0.055 µS/cm; 18.2 MΩ/cm), free of oxidants, purified using the Ultra Clear TWF UV system (SG GmbH, Germany); Elution solution for cations: 20 mM methanesulfonic acid (99.0%) (Fluka, Germany); Eluent solution for anions: 4.5 mM Na_2CO_3/0.8 mM $NaHCO_3$ (Dionex, USA). The analyze procedure followed strictly the conditions mentioned in the IC 1500 DIONEX Chromatograph Operating Manual as well as the specific standard procedures (US-EPA 1993; ASTM 1999; Jackson, 2000; US-EPA 2007).

Enzymatic activities in soil samples. Activities of the following four enzymes in soil were measured: phosphatase, catalase, actual and potential dehydrogenase (Alef and Nannipieri, 1995; Carpa *et al*., 2014).

Dehydrogenase activity (actual and potential) was determined after 24 h incubation of the soil samples at 37 ºC with TTC solution, and expressed by the amount of the formed 2,3,5-triphenylformazan (mg formazan/g soil). Enzymatic activity of dehydrogenases was determined by spectrophotometry using an Able Jasco V530 spectrophotometer at 440 nm wavelength.

Phosphatase activity was determined after 24 h incubation of the soil samples at 37 ºC with phenyl phosphate disodic solution, and it is expressed in mg phenol/g soil. Phosphatase activity was determined by using an Able Jasco V530 spectrophotometer at 620 nm wavelength.

Catalase activity was determined after 1 h incubation of the soil samples at room temperature with H_2O_2 solution. The residual H_2O_2 is determined by titration with $KMnO_4$ in the presence of H_2SO_4. Catalase activity was expressed in mg split H_2O_2/g soil.

The analytical data serves as the base for calculating the enzymatic indicator of the soil quality (EISQ) (Muntean *et al*., 1996).

Results and discussions

Halophytes in Cojocna zone. The vegetation growing in salty zones may be very diverse in some part of world, while in other regions they are dominated by a few halophytic species (Isacch *et al*., 2006). Halophytes, the vegetation of saline habitats, are a specialized plant group, characterized by the possession of great osmotic tolerance (Chaudhary *et al*., 2015). *Salicornia herbacea, Puccinellia distans*, *Artemisia santonica, Limonium gmelinii* and *Salsola soda* are dominant species in the salty zone of Cojocna (Fig. 1).

Figure 1. Saline soil in Cojocna zone. *Limonium gmelinii* and *Artemisia santonica*

Physico-chemical analyses of soil samples. The categories in which soils affected by salt are divided are as follows: saline soils, saline-sodic soils and sodic soils (Table 1) (Brady and Weil, 2002; Eynard *et al*., 2006). This division takes into account the electrical conductivity (EC), exchangeable sodium percentage (ESP) and sodium adsorption ratio (SAR).

Table 1.

Classification of salt-affected soils, according to the electrical conductivity (EC), exchangeable sodium percentage (ESP) and sodium adsorption ratio (SAR) values (Eynard *et al*., 2006).

Type of soil	EC (dS/m)	ESP (%)	SAR ($mmol^{0.5}/L^{0.5}$)
Non saline and non sodic soil	< 4	< 15	< 13
Saline soil	> 4	< 15	< 13
Saline-sodic soil	> 4	> 15	> 13
Sodic soil	< 4	> 15	> 13

The analyzed soils according to EC, ESP and SAR are presented in the following table (Table 2). Sodium adsorption ratio (SAR) was calculated online (http://turf.okstate.edu/water-quality/sar-calculator) and for ESP using the formula (http://www.iadclexicon.org/exchangeable-sodium-percentage-esp/):

$$EPS = \frac{[100(-0.0126 + 0.01475 \times SAR)]}{[1 + (-0.0126 + 0.01475 \times SAR)]}$$

Table 2.

Classification of analyzed soils, according to to the electrical conductivity (EC), exchangeable sodium percentage (ESP) and sodium adsorption ratio (SAR) values

Sample	EC (dS/m)	ESP (%)	SAR ($mmol^{0.5}/L^{0.5}$)	Type of soil
1	0.927	36.06649	39.1	Sodic soil
2	3.140	48.42104	64.5	Sodic soil
3	4.450	54.63361	82.5	Saline-sodic soil

Physico-chemical parameters detected in salty soil from Cojocna are presented in Table 3.

Table 3.

Physico-chemical parameters of salty soils from Cojocna

Sample	pH	Conductivity (µS/cm)	Na	Cl	NH_4	NO_3 (mg/kg)	K	Mg	Ca	SO_4
1	8.84	927	951.6	912.6	0.0	18.3	59.0	5.4	35.0	89.4
2	9.21	3140	2240.8	3566.7	0.2	26.2	188.0	24.0	51.4	105.7
3	9.09	4450	3785.2	6049.4	0.4	53.4	247.7	40.8	92.2	229.0

Sodium chlorides and sulphates, calcium, kallium and magnesium are the most commonly present soluble salts. Nitrates also may be present or not. Sodium and chloride are the principal ions encountered in highly saline soils, but the present calcium and magnesium serve as nutritional elements for plants.

Saline soils have poor macronutrient availability and high soil salinity (Zhang *et al*., 2015). The high content of Na and Cl in all three samples indicates the presence of salt (NaCl) in soil, from salt deposits in the geological substrate (Fig. 2a). The high salt content along with alkaline pH affects plant growth by inhibiting nutrient absorption (K, Mg, Ca) at the root level (Fig. 2b,d). The vast majority of nutrients are absorbed by plants in a neutral, slightly alkaline pH environment.

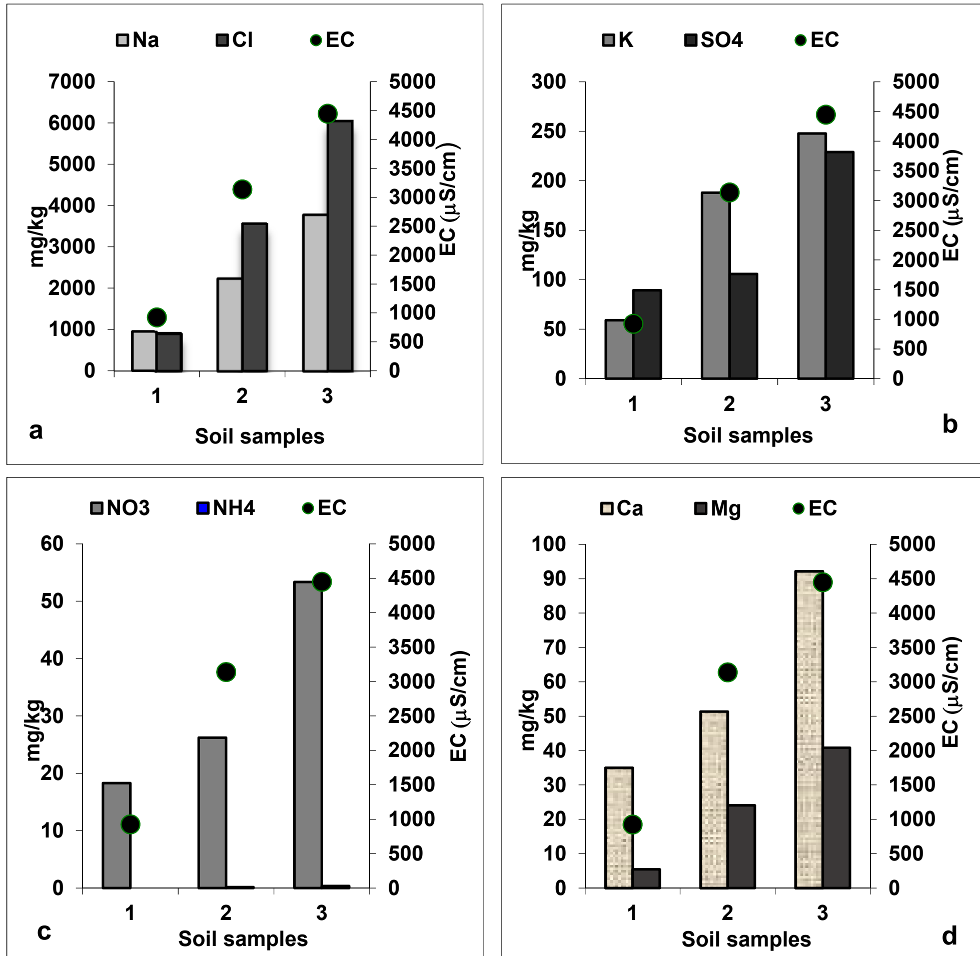

Figure 2. Micro- and macroelements content of soil samples
a=Na, Cl; b=K, PO_4; c=NH_4, NO_2, NO_3; d=Mg, Ca, SO_4.

Available nitrogen is taken up by plant roots in the form of (NO_3^-) and (NH_4^+) (Fig. 2c). The available forms of nitrogen are very water soluble and move rapidly through the soil profile with rainfall and irrigation. Changing and soluble ammonium is found in the upper horizon of soils, typically in small and relatively constant amounts, below 10 mg N-NH_4/kg (Vintila *et al.*, 1984). The nitrate content is less than 60 mg/kg in all analyzed samples, which is a normal value. At persistent amounts of more than 100 mg N-NO_3/kg, the phenomenon of nitrate pollution of the soil, plant and groundwater can occur (Lăcătuşu *et al.*, 2000).

Enzymatic activities in soil. The activity of enzymes is dependent on the physico-chemical properties of the soil. In salinized soils the soil enzyme activity is heavily hindered, which indicate a high level of stress in those soils (Chaudhary *et al*., 2015). This activity is directly related to the active biomass in soil, and both are precise indicators for soil quality (Stepniewska *et al*., 2009). The activities of enzymes in halophytic soil are depicted in Table 4.

Table 4.

Enzymatic activities in soil samples from Cojocna

Samples	Enzymatic activities				EISQ
	Dehidrogenases activities (mg formazan/g soil)		Phosphatase activity (mg fenol/g soil)	Catalase activity (split H_2O_2/g soil)	
	actual	potential			
1	0.301	1.016	0.248	49.98	0.236
2	0.101	0.439	0.249	44.88	0.201
3	0.216	0.338	0.140	46.07	0.204

Actual and potential dehydrogenases activities. The whole amount of oxidative activity performed by the microbial consortia in soil is assessed by dehydrogenase activity, so it represent a reliable indicator of microbial activity in soil (Kussainova *et al.,* 2013).

Dehydrogenase activity is growing by increasing the organic matter in the soil. Actual dehydrogenase activity reaches maximum values of 0.301 mg formazan/g. soil in the Sample 1. The minimum value was 0.101 mg formazan/g. soil recorded for the Sample 2. The potential dehydrogenase activity (PDA) presented much higher values for all zones studied due to the carbon source (glucose) added (Fig. 3).

The dehydrogenase activity is the result of the action of living microorganisms and the proliferation capacity. There is a strong relationship between the number of microorganism in the soils and dehydrogenase activity.

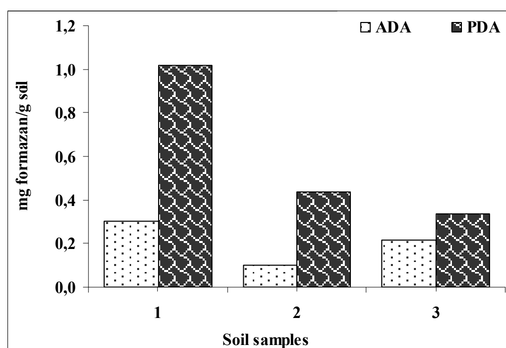

Figure 3. Actual and potential dehydrogenases activities(ADA and PDA) in soil samples from Cojocna

Phosphatase and catalase activities. Plants need free, available phosphate, which is released mainly by phosphatases, from the decomposition of organic matter. These enzymes can be classified in rhizosphere phosphatases and nonrhizosphere soil phosphatases, the first ones having a more intense activity (Chen *et al.,* 2012). The solubility of organic P compound is another factor influencing the activity of these enzymes (Zhu *et al*., 2017). The spatial distribution of phosphatases in soils influences this enzyme activity (Story and Brigmon, 2017). Phosphatase activity from Cojocna salty zones is quite uniform in samples 1 and 2. Slightly lower values came from the Sample 3, populated with *Puccinellia distans*, *Artemisia santonica, Limonium gmelinii* and *Salsola soda*, where the maximum value was 0.14 mg phenol/g. soil (Fig. 4a).

Dehydrogenase and phosphatase are considered enzymes which play key roles in the metabolic functions of bacteria in their habitats (Huang *et al*., 2009). Thus, these enzymes can provide usefull information regarding the efects of environmental changes (Zheng *et al*., 2017).

The intracellular enzyme catalase is present in all aerobic bacteria and most of the facultative anaerobes, but is not present in obligate anaerobes (Alef and Nannipieri, 1995). The number of aerobic microorganisms in soil and fertility of the soil are in conjunction with catalase activity, enzyme activity which indicate the aerobic microbial activity (Trasar-Cepeda *et al*., 1999). Catalase activity is a very stable activity in soil and decreases with depths, similar to the content of organic carbon (Alef and Nannipieri, 1995).

Catalase activity from Cojocna salty zones is quite uniform in all samples. Slightly higher values came from the Sample 1, populated *Artemisia santonica* and *Limonium gmelinii* where the maximum value was 49.98 mg H_2O_2/g. soil (Fig. 4b). Catalase activity is very stable in soil and has a significant corelation with the organic carbon content and the depth of soil (Alef and Nannipieri 1995).

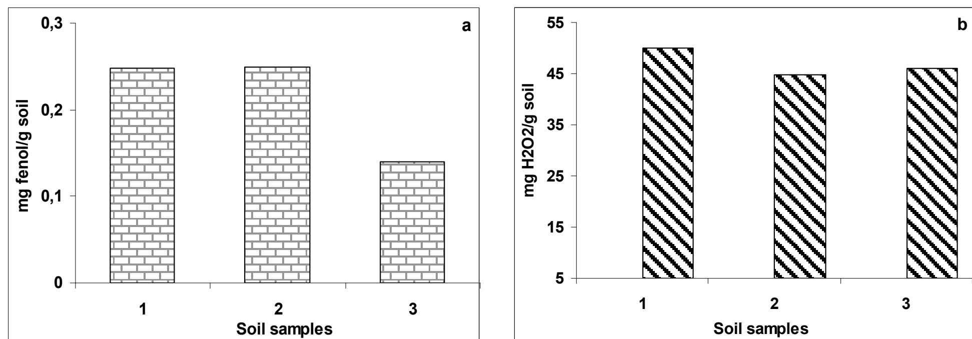

Figure 4. Phosphatase (a) and catalase (b) activities in soil samples from Cojocna

Enzymatic Indicator of Soil Quality (EISQ). Assessing the enzyme activities in soils constitute a research instrument for evaluating the functional diversity of microbiota and the biochemical processes in these habitats. Theoretically, the enzymatic indicator can have values between 0 (when there is no activity in any sample) and 1 (when all the real individual values are equal to the maximal theoretical individual one). The absolute values of enzyme activities in the studied salty soils are presented in Fig. 5. It can be noticed that the Cojocna salty soils have a lower enzymatic indicator. The maximum values were found in Samples 1 (0.236). The quality of these salty soils are lowest, and it is known that the quality of a soil is better the higher EISQ gets.

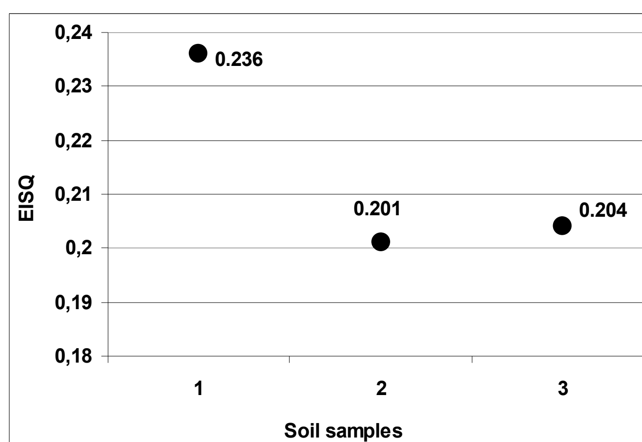

Figure 5. EISQ in soil samples from Cojocna

Generally, the enzyme potential of soil directly or indirectly reflects the activity of microbiota, the influence of different physical, chemical anthropogenic factors and even of intensity of different enzyme activities in the soil. Therefore, the function of an ecosystem can not be understood without the active implication of enzyme processes (Drăgan-Bularda *et al.*, 2004).

Conclusions

Several halophytes were identified on salty soils from Cojocna, the prevalent being: *Salicornia herbacea, Puccinellia distans*, *Artemisia santonica, Limonium gmelinii* and *Salsola soda.*

Soil samples from the rhizosphere of the prevalent halophytes were physico-chemically analysed. The pH ranges between 8.84 and 9.21. Out of all the samples, the one populated with *Puccinellia distans*, *Artemisia santonica, Limonium gmelinii* and *Salsola soda* (sample 3) showed high values of the salts composition. All the soils were included to sodic and saline-sodic soils.

All the studied enzyme activities (actual and potential dehydrogenases, phosphatase, catalase) were detected in salty soil samples from Cojocna. Their intensity varied considerably, according to the location and the halophilic plants from whose rhizosphere the soil was sampled.

Based on the studied enzyme activity the enzymatic indicator of soil quality was calculated, which was low, between 0.201 and 0.236. As can be observed, the enzymatic indicator of soil quality reached low levels, meaning that the soils are not hosting an abundant bacterial population. This is due to the environmental conditions,especially to the high salinity, soils that form an infertile, unfavorable for life environment.

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Development of a bidirectional PCR tool for Tenera oil palm (*Elaeis guineensis* Jacq.) identity

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SUMMARY. Three forms of fruit are distinguished in the Oil palm (*Elaeis guineensis* Jacq.): Dura, Pisifera and Tenera. The most cultivated high yielding oil palm variety, the thin shell Tenera is produced from the cross between the thick shelled Dura and the shell-less Pisifera. Due to the perennial nature of the oil palm, it is impossible to determine the fruit phenotype until it is reproductively mature. Oil palm farmers are therefore faced with the challenge of uncertainty of the kind of fruits and yield output expected until production time. The recent sequencing of the shell gene in the oil palm made it possible to identify Single Nucleotide Polymorphism (SNP) mutations that are now useful in developing molecular markers for fruit form predictions. In this study, Allele Specific PCR (AS-PCR) was used to assess regions of the shell gene containing SNPs for oil palm fruit form discrimination. Three primer pairs (S1, S2 and S3) were designed as common primers while four AS-PCR primers (S20, S22, S32 and S33) were adopted as inner primers. Possible combinations of common and AS primers were investigated in Dura, Pisifera and Tenera genomes. A 300bp fragment was observed to be unique in the Tenera shell gene when S3 was combined with S20 and S32. Other inner primer combinations investigated with S3 generated similar assays in Dura, Pisifera and Tenera. The result from this study detects a marker for the Tenera fruit form of the oil palm.

Keywords: Allele Specific PCR, Dura, Fruit forms, Oil palm, Pisifera, Shell gene, Tenera.

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a diploid, monocotyledonous plant, belonging to the family Arecaceae. It is economically an important tree, as it is the source of palm oil.

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Palm oil is the most traded vegetable oil in the international market (Corley and Tinker, 2003). Oil palm is widely cultivated in the tropical zones which include Malaysia, Indonesia, Nigeria, Ivory Coast, Columbia and Thailand (Wahid *et al*., 2005).

It is the highest producing oil seed crop in the world. Some cultivars produce as much as 5-9 tonnes of oil per hectare per year; three times the yield of coconut and ten times that of soya bean per hectare. In addition, another 0.5 tonnes of kernel oil per hectare per year can also be obtained (Jalani *et al.,* 1997). The large amount of oil produced in the oil palm fruit is a unique biological characteristic of this palm species (Hartley, 2000). The oil palm fruit can be classified into three forms: Dura (thick shelled), Pisifera (shell-less) and Tenera (thin shelled). The Tenera form is the accepted commercial cultivar with higher palm oil content. In conventional breeding practices, the time taken to differentiate between the oil palm fruit forms has been a major limitation. Molecular studies are carried out in oil palm research laboratories to investigate the genetic makeup of this crop in relation to its oil yield character. The fruit shell thickness is an important trait because it defines oil yield of the crop. This important trait is controlled by the SHELL gene (Singh *et al*., 2013).

The recent sequencing of this gene made it possible to identify Single Nucleotide Polymorphism (SNP) mutations that are now useful in developing molecular markers for fruit form predictions. Some molecular markers have been used to investigate the genetic diversity in the oil palm these include: Simple Sequence Repeat (SSR) (Ihase *et al*., 2014), Restriction fragment length polymorphisms (RFLPs) of ribosomal DNA (Singh *et al*., 1993), Random amplified polymorphic DNA (RAPD) (Sathish and Mohankumar 2007). In this study, a traditional SNP method known as the Allele Specific PCR (AS-PCR) was used in assessing the shell gene of the three fruit forms of the oil palm. It was aimed at developing a marker for the high yielding Tenera at the vegetative stage.

Materials and methods

Plant material for DNA extraction

DNA was isolated from fresh leaves of 4 Dura, 4 Pisifera and 13 Tenera oil palms obtained from the Nigerian Institute for Oil Palm Research (NIFOR), Benin City, Nigeria. DNA extraction was done using promega plant DNA extraction kit according to the manufacturers' instruction.

Primer design

In order to distinguish between shell alleles (Sh/sh), four allele specific primers (S20, S22, S32 and S33) were adopted from a previous study (Reyes *et al*., 2015) and combined as right and left primer. Three primer pairs specific to the shell gene

(S1, S2, S3) forward and reverse respectively were designed using primer blast on NCBI website. All 10 primer sequences were obtained and formulated by Inqaba (Table 1).

AS-PCR conditions

PCRs were performed with 10 µL volumes using Thermo Fisher Scientific PCR mix. The final concentrations were as follows: 1X Taq buffer, 2.5 mM, MgCl2, 0.32 mM, dNTP mixture, 0.25 µM for each primer, 0.5 U Taq and 10 ng genomic DNA. All reactions were performed using a Wagetech Projects Master Cycler (Eppendorf, Hamburg, Germany) with an initial denaturing cycle of 5 min at 95°C, 40 cycles of 30 s at 93°C, 1 min at 59°C, 1 min at 72°C, and a final extension cycle of 10 min at 72°C. The PCR products were visualized using GR green dye in 2% agarose gels.

Table 1.

Primer information for this study

Primer	Sequence	Amplified target	Reference
S1 (f)	AGTGCTGCCAAAGAAGGCT		This study
S1 (r)	TAAGTGACCAGGGTTGGCTG	*SHELL*	This study
S2 (f)	GGCGGTTTACAGGAGCAGAT		This study
S2 (r)	TAGCCTTTCTTTGGCAGCACT	*SHELL*	This study
S3 (f)	TTTGTTGCTTTTAATTTGCTTGAATACCTTT		Reyes *et al*., 2015
S3 (r)	TGGCTTGGCCATAGAACAAA	*SHELL*	Reyes *et al*., 2015
S20	TCAGCATCACAAAGGACAGACAACTCATAATCT	Sh/Sh, Sh/sh	Reyes *et al*., 2015
S22	CAGCATCACAAAGGACAGACAACTCATAAGCA	sh/sh	Reyes *et al*., 2015
S32	GCCGAAATGGACTGCTGAAGCAT	sh/sh, Sh/sh	Reyes *et al*., 2015
S33	GCCGAAATGGACTGCTGAAGAAA	Sh/Sh	Reyes *et al*., 2015

Results and discussion

A regular PCR was carried out to test the primers (S1and S2) for their responsiveness to the genome of Dura, Pisifera and Tenera palms (Figure 1). The allele specific primers adopted were also paired per allele (Sh/Sh, sh/sh, Sh/sh) and used to amplify the gene coding the three fruit forms. Products obtained were the same in the first and second combination (S20 and 33, S22 and 32). Amplification products in the third combination (S20 and 32) were the same in Pisifera and Tenera but different in Dura (Table 2).

Table 2.

Paired *SHELL* Allele Specific (A.S) primers as forward and reverse on Dura, Pisifera and Tenera genomes.

A.S primer	Shell allele	Pairs per Allele	Amplification in the fruit forms		
S20	Sh/Sh,Sh/sh	S20 &S33 (Sh/Sh)	Dura (100bp)	Pisfera (100bp)	Tenera (100bp)
S22	sh/sh	S22 &S32 (sh/sh)	Dura (100bp)	Pisfera (100bp)	Tenera (100bp)
S32	sh/sh, Sh/sh	S20 &S32 (Sh/sh)	Dura (100bp)	Pisifera (100bp & 300bp)	Tenera (100bp & 300bp)
S33	Sh/Sh				

S1 primer combined with AS primer pair S20&33 showed no difference between the fragments of Dura, Pisifera and Tenera S1 primer combined with AS primer pair S20&33 showed no difference between the fragments of Dura, Pisifera and Tenera. For the second set, (S1{S22&32}) the Tenera generated two bands of 600bp and 80bp which was different from the Pisifera (one band of 80bp). In the third set, (S1{S20&32}) similar banding pattern were observed in the three fruit forms. S2 in second and third combination with AS primers shows similar band patterns in all fruit forms (Figure 3). In the first combination, the fragment size of 400bp was not observed in the Pisifera form. S3 pimer in combination with AS primr pairs generated a unique band of 300 bpin Tenera fruit (Figure 4). A unique band of 300bp was observed in only the tenera fruit form when primer S3 was combined with primer S20 & S32. S3 primer combination with S20 and S32 generated a different PCR pattern as showed in Figure 5. The allele observed in the Tenera palms sampled was absent in Dura and Pisifera.

In order to find markers linked to the SNP mutation responsible for the variation in shell thickness of the oil palm, the present study employed a simple Allele Specific (A.S) PCR tool. This involved designing primer pairs from the shell gene and adopting shell Allele Specific (A.S) primers. The A.S primers (S20 and S32) used in identifying a marker for Tenera in this study were adopted from a previous research (Reyes *et al*., 2015) But there was no evidence for the discrimination of Tenera genotype with this prime pair. Our results showing the variantion in Dura could be due to homozygote dominant allele (Table 2), S20 & S32 is intended for the heterozygous allele (Sh/sh). This was previously shown by Corkey and Tinker, 2003.

Given that primer S20 & S32 is intended for the heterozygous allele (Sh/sh). According to Patent WO2016205240A3, Pisifera trees may be heterozygous at the SNP position of SEQ ID NO: 922 and SEQ ID NO: 923 as a result of having contribution from both Zaire and Nigerian derived lines. Several differences were

observed in the Pisifera genotype in the palms sampled for the second set of primers (2nd D4-T38) from Tenera being that there were two bands (600bp and 80bp) in Tenera and only one band (80bp) in Pisifera. This possibly maybe as a result of the primers tendency to target homozygote recessive allele in the Pisifera palms sampled.

Amplicons generated with S2 (S20&S33) in Dura and Tenera have the same size (Fig. 3). This may be due to the fact that primer S20 and S33 are for the heterozygote and homozygote dominant allele respectively (Reyes *et al*., 2015). It is also important to state that the Dura and Tenera genotypes are the shelled fruit forms, as such it is expected that they share similarities in alleles (Sathish and Mohankumar, 2007).

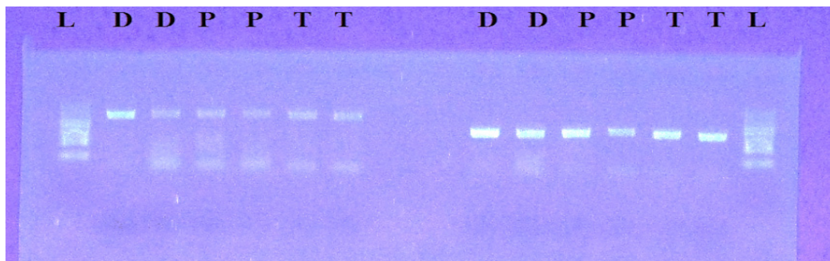

Figure 1. Electrophoregram of amplicons generated with S1 and S2 primers. (S1 and S2) designed. Primer S1: Right side, Primer S2: Left side.
Keys: **L**: 100bp ladder; **D**: Dura; **P**: Pisifera; **T**: Tenera.

Figure 2. Electrophoregram of amplicons generated with S1 and AS primers.
Keys: **L**: 100bp ladder; Upper part of gel (1st D4-T38): S20&S33; (2nd D4-T38): S22&S32; Lower part of gel (3rd D4-T38): S20&S32

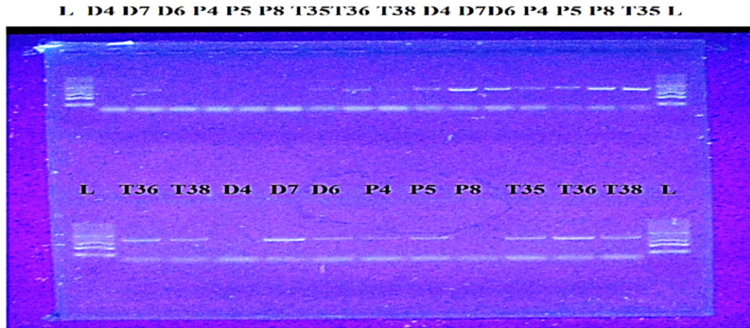

Figure 3. Electrophoregram of amplicons generated with S2 and AS primers.
Keys: L: 100bp ladder; Upper part of gel (1st D4-T38): S20&S33; (2nd D4-T38): S22&S32; Lower part of gel (3rd D4-T38): S20&S32.

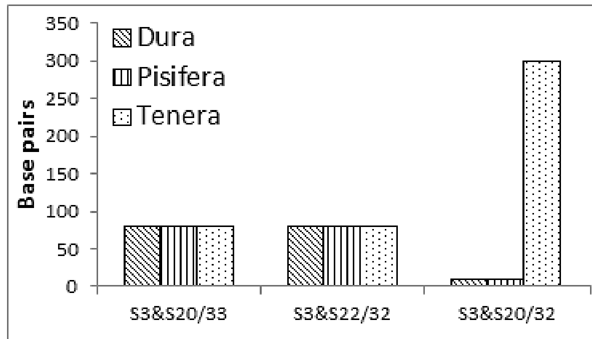

Figure 4. PCR product sizes obtained from using primer S3 in combination with the three allele specific primer pairs.

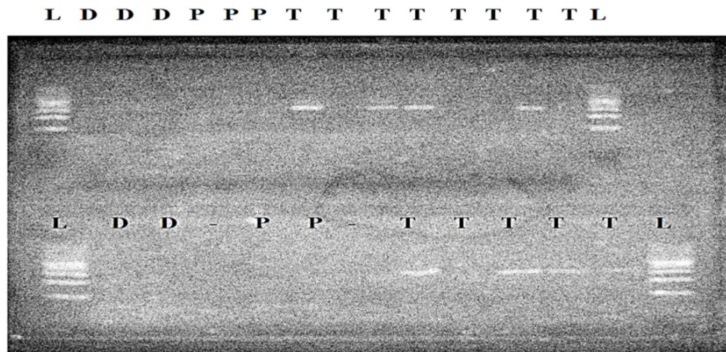

Figure 5. Electrophoregramm of amplicons generated with S3 and AS primers.
Keys: L: 100bp ladder; **-** : Nothing loaded; **D**: **Dura**; **P: Pisifera**; **T: Tenera**.

A unique marker for the Tenera fruit form was observed when primer S3 was used in combination with A.S primers S20 and S32. This amplification was done severally and in all the cases, a fragment size of 300bp was observed in about 75% of the Tenera palms sampled (Fig. 5). Researchers from the Indian Institute of Oil palm Research have developed a CAPS (Cleaved Amplified Polymorphic Sequence) marker which produced three alleles of sizes 550, 280 and 250bp in Tenera genotypes but their method required the use of a restriction enzyme for a digestion step (Babu *et al*., 2017). In the present study, PCR is all that was required to generate a reproducible marker for the Tenera fruit form.

Conclusions

The goal of any breeding programs, particularly in oil palm is to produce planting materials with higher oil yield; the Tenera fruit form is the preferred choice for commercial planting. Despite of the many advances which have been made in the production of hybrid oil palm seeds, the challenge of seed contamination is still significant, hence the need for the development of a molecular tool for accurate identification of Teneral forms.This study therefore provides a marker that can serve as an early screening aid to complement and certify the efforts of the hybrid seed producers who most times rely on morphological charcteristics, which can be very deceptive, for identification. This will dramatically reduce and possibly eradicate contaminants among the oil palm hybrid planting materials and eventually translate into increased income for growers and investors in the industry.

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Has the Fluocinolon-acetonid N ointment any effect on the kidneys and the thyroid gland structure and function?

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SUMMARY. Besides the naturally occurring glucocorticoids, there are many other synthetically produced glucocorticoids: dexamethasone, prednisolone, triamcinolone, triamcinolone acetonide, flumetazon, methyl prednisone and methylprednisolone. Corticosteroids are administered intravenously, orally, through inhalation directly onto the inflamed organ, eye drops and by applying skin ointments. Although long term use has its undesirable effects, e.g. high blood pressure, heart failure, diabetes and renal failure. Fluocinolon-acetonid N ointment is a synthetic derivate of the adrenocortical hormone, which is used for medical treatment purposes in dermatology. We also use it in our homes, mostly due to its anti-inflammatory effect, in the treatment of itching, and also in the acute keratosis. It is highly effective in serious, non contagious, dry skin inflammations, such as atopic eczema, seborrheic dermatitis, psoriasis, dermatitis or even in allergic reactions. In prolonged usage due to its liposoluble properties, it is easily absorbed into the bloodstream, which increases the chances of having side effects. The main objective of this study is to analyze the side effects of glucocorticoid excess when treatment is done with Fluocinolon- acetonid N ointment, to see if it has any effect on organs which have an important role in maintaining basal metabolism such as kidneys and thyroid gland. Our results demonstrate that fluocinolon treatment affects the structure and the function of kidneys and thyroid gland.

Keywords: glucocorticoid excess, kidney, thyroid gland

Introduction

Glucocorticoid, which is produced in the adrenal cortex, has the two most important representatives in the mammals: cortisol and corticosterone. Glucocorticoid hormones take part in inflammation, development and metabolic processes (Cain

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and Cidlowski, 2017). They regulate carbohydrate, protein, fat, calcium and bone metabolism and energy balance. Cortisol activates virtually all energy sources, so it can be quickly used by our organism (Stojanoski *et al.,* 2012). It heightens the blood sugar level, it starts the biolysis, and it even starts to break muscle proteins into free amino acids, if the energy level is not high enough in the body. The cortisol level increases in stress situations, but this hormone has a very important role at all levels which helps metabolism function correctly, as high blood pressure, it also regulates the body's use of protein, carbohydrate and fat. In adults, glucocorticoids are stress hormones with a wide range of physiological effects, which aid survival in environmental conditions that challenge homeostasis. They maintain blood flow and a supply of nutrients and oxygen to tissues when these resources are either scarce or in increased demand.

On the long term, high level of cortisol can cause serious problems in humans, reduces the pituitary ACTH secretion, this way areas such as adrenocortical will atrophy, the intellectual performance weakens, the immune system weakens also and the inflammatory processes in the body will become more common than in healthy organisms.

The mostly studied effect of glucocorticoid application was the impact on blood sugar level. The excess of glucocorticoids increases in the liver the storage of glycogen obstructs the movement of glycogen in the blood and it stimulates the gluconeogenesis (Nyirenda *et al.,* 2000; Hans *et al*., 2006; Waldron *et al.,* 2013; Ivy *et al.,* 2016). Glucocorticoids inhibit the uptake and usage of sugar in the muscle and adipose tissues (Grigoriadis *et al.,* 1988; Burén *et al.,* 2002; Lundgren *et al.,* 2004; Gounarides *et al.,* 2008). In a long term glucocorticoid application, initially insulin resistance appears and at the final stage diabetes mellitus can occur (Madar *et al.,* 1995).

Glucocorticoid excess has different effects on the cardiovascular system and kidneys, it raises the blood pressure (Grunfeld and Eloy, 1987; Singh *et al.,* 2012; Hunter *et al.,* 2014; Jeje and Raji, 2015b). In the vascular smooth muscle tissue glucocorticoids excess increases muscle fiber sensitivity to catecholamines. Glucocorticoids have an impact on many components of noradrenergic innervations: they increase the number of receptors, the receptors affect the connectivity to the G protein and the cAMP synthesis, induced catecholamine eliberation, furthermore it prolongs the effects of catecholamines on the smooth muscle, thus it rises the vascular and cardiac contractility (Fonyó, 2011). In addition to the direct effects of glucocorticoids, they can indirectly modify the vascular response, too. Glucocorticoids inhibit prostanoid synthesis, and this mechanism also prevents prostanoid induced vasodilatation (O'Sullivan *et al.,* 2013). The decreased level of glucocorticoids, in Addison-disease or after the removal of the adrenals, caused chronic low blood pressure in many patients (Fonyó, 2011). NaCl co transporter (NCC) is an important determinant of daily blood pressure variation. NCC activity in cells is regulated by

the circadian transcription factor per1. In vivo, circadian genes are entrained via the hypothalamic–pituitary–adrenal axis. Chronic corticosterone infusion increased bmal1, per1, sgk1, and tsc22d3 genes expression during the inactive phase. In the inactive phase pNCC was also elevated by corticosterone (Ivy *et al.,* 2016).

In the kidneys, it increases sodium retention in the renal tubules and potassium excretion. Long term treatment with synthetic glucocortikoids causes hypertension and low potassium levels. Dexamethasone treatment of female rats during pregnancy reduced renal mass and the number of nephrons in offspring rats (Martins *et al.,* 2003).

Long-term glucocorticoid treatment is associated with central obesity in humans, which is also typically observed in most patients with Cushing's syndrome. The mechanisms by which exogenous glucocorticoids alter metabolism and induce weight gain are poorly understood (Paggioli *et al.,* 2013).

Side effects of gGlucocorticoid-based drugs on adult rat kidneys and thyroid gland were less studied. This article describes the side effects of treatment with Fluocinolon- acetonide N ointment, on the level of kidney and thyroid gland structure and function in adult Wistar rats. In addition we analyzed and measured t body mass and thyroid gland mass variation during the short term of treatment.

Materials and methods

Experiments were carried out in adult (60-day-old) male Wistar rats. The animals were kept under standardized bioclimatic conditions and fed on common rat chow, with water *ad libitum*.

Commercial Fluocinolon-N ointment containing 25 mg Fluocinolon-acetonid-N/100 g excipient, was applied topically to the skin at 2 cm^2, for four consecutive days, by smearing 50 mg ointment/100 g b.w on the inguinal region, the daily dose of Fluocinolon-acetonid-N being equals to 12.5 μg/100 g b.w. The animals were divided into the following groups: C-control group and FC –fluocinolon treated group.

After 16 hours of fasting and 24 hours following the cessation of treatments, the treated animals together with controls were sacrificed by exsanguinations. The body weight and the thyroid glands weights were measured with an accuracy of 0.00001 g immediately after excision. The significance levels were determined by parametric t-test. A $p<0.05$ was considered statistically significant.

For structural analysis the organs of slaughtered animals were fixed in Bouin liquid and afterwards processed in view of being embedded in paraffin. The fragments were sectioned at the Reichert-Austria type microtome with a thickness of 7 μ. The staining of kidney was made by means of hematoxilin-eosin method and for rendering

evident of the thyroid secretion, the staining of the sections was made by means of Hurduc and co-workers (Muresan *et al.,* 1974). The histological preparations obtained were examined on the Olympus microscope with digital camera.

Results and discussion

Body and thyroid gland weights change during treatment

The treated and control animals' body weight measured in our experiment are summarized in Table 1. Based on the table values of the control group during the treatment their body weight slightly increased, while in the body weight of treated animals with Fluocinolon-acetonid N a significant decrease can be observed.

Table 1.

Changes in body weight during treatment with Fluocinolon-acetonid N. ± the mean value illustrated. The % quantitative variation compared to control animals in body weight. P – significant

Animal group	1th day treat.	2th day treat.	3th day treat.	4th day treat.
C	181.7 ± 3.8	187.5 ± 3.9 +3.17%	188.6 ± 3.5 +3.82%	183.3 ± 3.9 +0.90% P> 0.5
FC	148.3 ± 4.5	145.1 ± 4.6 -2.15%	137. 8 ± 4.1 -7.09%	126.7 ±4.9 -14.56% P<0.01

There are a lot of data about the effect of glucocorticoid excess upon the fetal development. The hypothalamic–pituitary–adrenal (HPA) axis fulfills important functions during fetal development and the transition to extrauterine life. However, alterations to the HPA axis during fetal life, for example following pharmacological treatment with corticosteroids, may affect development and future health of the individual (Li *et al.,* 2013). Prenatal dexamethasone leads to low birth weight and compromises organogenesis (Martins *et al.,* 2003).

During the experiment in the treated animals parallel with the decrease in body weight we observed a slight decrease in the thyroid gland mass (Fig. 1). The magnitude of the reduction in thyroid gland mass was not significant.

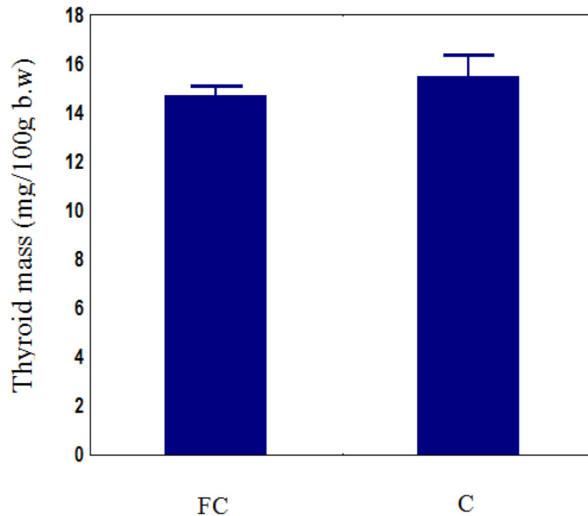

Figure. 1. Weight change in thyroid gland in treated (FC) and controlled (C) animals

Weight decrease of the thyroid gland can be associated with the decrease of the colloidal follicle diameter. Weight decrease of the thyroid gland is in harmony with our structural results.

Histological studies of the kidney in the control and treated group

Kidney sections from control animals displayed normal histological structure (Fig. 2), the outer part is the cortex, and the medulla is inside within the renal pelvis side portion.

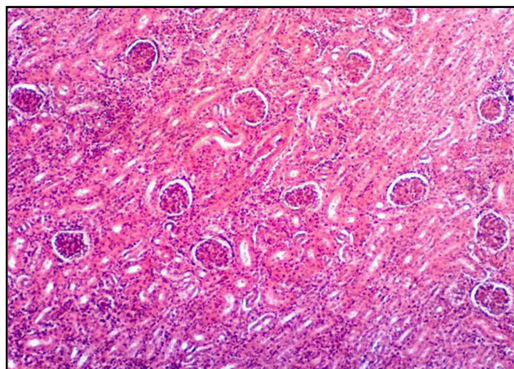

Figure 2. Renal histological section of the C-groups, Hematoxilin-eozin staining 10x

In animals treated with Fluocinolon-acetonid N, kidney sections are majorly different from the control animals, dystrophy of the renal tissues is clearly visible. The number of glomeruli is far less than in the control animal kidney sections. The few existing Bowman capsules shrunk, they blend into the environment of tubules, and they are hardly recognizable. The hialinoid is present in some places in the Bowman capsule, specifically in the vascular basement membrane which has become thicker (Fig.3 a, b).

In some parts of the renal tissues, on the wall of the renal tubules, necrotic lesions can be seen, where the epithelial cell nucleus has pyknotic, the cell membrane has also been destroyed. Among the renal tubules hyperemia is visible.

The bleeding and also the atrophic glomerulonephritis suggest that the Fluocinolon-acetonid N based treatment causes glucocorticoid excess due to its vasoconstrictor effect, glomerulus atrophy, and while in the rest of the kidney tissue hyperemia occurs, which leads to necrosis in the walls of the renal tubules. These findings are compatible with the data from other studies according to which glucocorticoid excess can produce very severe rat nephropathy (Kamphuis *et. al* 2007; de Vries *et al.,* 2010). From this we can speculated that glucocorticoids have vasoconstrictor effect on renal tissues. This is also proven by the fact that beta-adrenoreceptor stimulation significantly participates in glucocorticoid excess-induced metabolic and gravimetric alterations (Kis *et al.,* 2001; Kis and Crăciun, 2003 a, b).

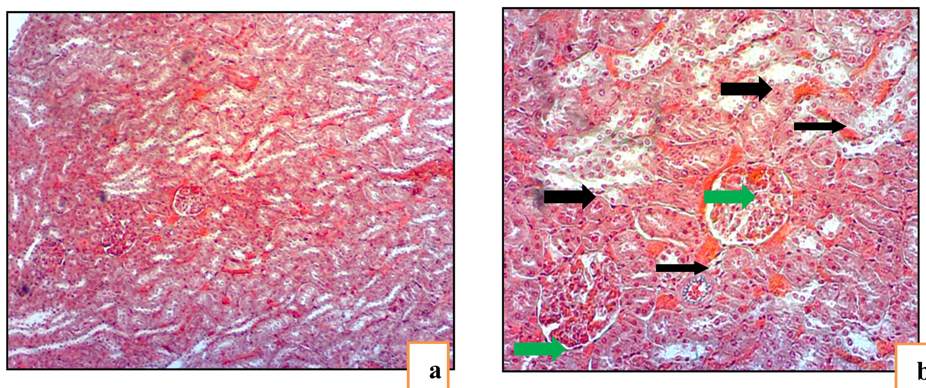

Figure 3. Renal histological section in treated group a) The renal section in FC-groups, the Bowman capsule shrunk, it is difficult to recognize, and also the numbers of normal glomeruli are less 10x. b) Nephrons dystrophy can be seen on the kidney section. The thin arrow points to the epithelial cell necrosis in the tubules, the thick points to the hemorrhage in the renal tissues and the green arrow to the hialinoid in the Bowman capsule, Hematoxilin-eozin staining, 20x

This experimental result is consistent with other similar research results, according to which the high amount of glucocorticoid is harmful, resulting in increased blood pressure (Hunter *et al.,* 2016; Ivy *et al.,* 2016; Jeje and Raji, 2017). In long-term treatment with glucocorticoids, increased blood pressure in the heart and vascular

system disease can occur which change kidney function. A number of experimental results demonstrate that dexamethasone treatment causes glomerulosclerosis, which significantly reduces the number of glomeruli (Singh *et al.,* 2012). Similar results are shown in the treatment with dexamethasone in mice, where the glomerulus number significantly reduced (Woods and Week, 2005). Wade and coworkers (1979) observed the thickening of renal tubule basic membrane after a treatment with cortisone.

Moritz and co-workers (2011), Woods and Weeks (2005), Ortiz and co-workers (2003) studied how exogenous glucocorticoids reduces the number of nephrons in fetal sheep kidneys. In dexamethasone treated pregnant sheep kidneys, the number of glomeruli decreases by 25%. Treated pregnant sheep's newborn lambs had a reduced number of nephrons in their adulthood, the arterial blood pressure was higher than in the non-treated pregnant sheep's offspring. The authors are assuming that the reduced nephron numbers have contributed in the hypertension formation.

After glucocorticoid treatment similar changes can be observed in other mammals such as rats, rabbits and mice (Ortiz *et al.,* 2003; Dickinson *et al.,* 2007; O'Sullivan *et al.,* 2013). Maternal administration of dexamethasone for 48 h early in rat kidney development results in offspring with a reduced nephron endowment. The authors hypothesized that dexamethasone may indirectly inhibit nephrogenesis by inhibiting ureteric branching morphogenesis (Shingh *et al.,* 2007).

Damage of the kidney tissues may explain the hematological effect of dexamethasone treatment. Prenatal cortisol excess inhibits erythropoietin production in fetal sheep and disturbed the maturation of erythrocytes (Jeje and Raji, 2015b).

Findings from this study suggest that Fluocinolon-acetonid N treatment could retard growth and subsequent development of nephropathy.

Histological studies of the thyroid gland in control and treated groups

The control animals showed normal structures of follicular thyroid, they were in different secretory phases. The follicle wall is made up of cubical epithelium (Fig 4 a, b).

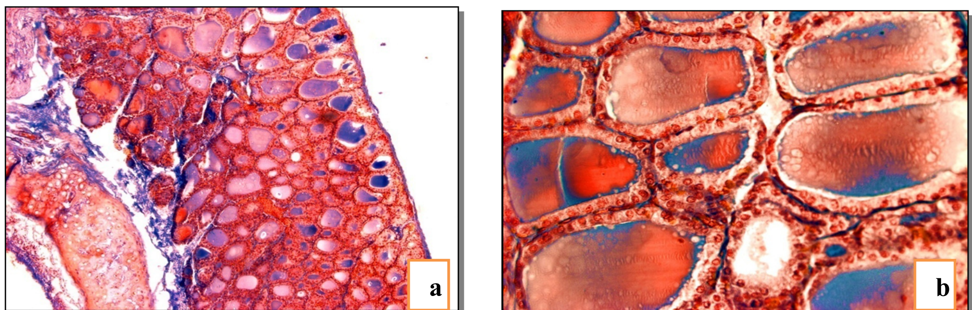

Figure 4 a) Thyroid histological section of the control animals, 1x. **b**) The thyroid follicle walls are covered with cubical epithelium, Hurduc staining, 40x

In the treated animals the thyroid show structural and functional changes in comparison to the thyroid tissue of the control animals (Fig. 5 a). Follicle diameters are much lower as compared to control animals, larger diameter edge of the gland follicles can be observed.

The number of smaller follicles is much higher than the ones in the controlled thyroid tissue. Towards in the central part of glandular tissues, the follicle diameter decreases, this occurs in empty follicles as well (Fig. 5 b).

The majority of follicles filled with colloid with a vesicular structure (Fig. 5 c), which suggests an increase in endocytosis of the thyroid hormones from the colloid.

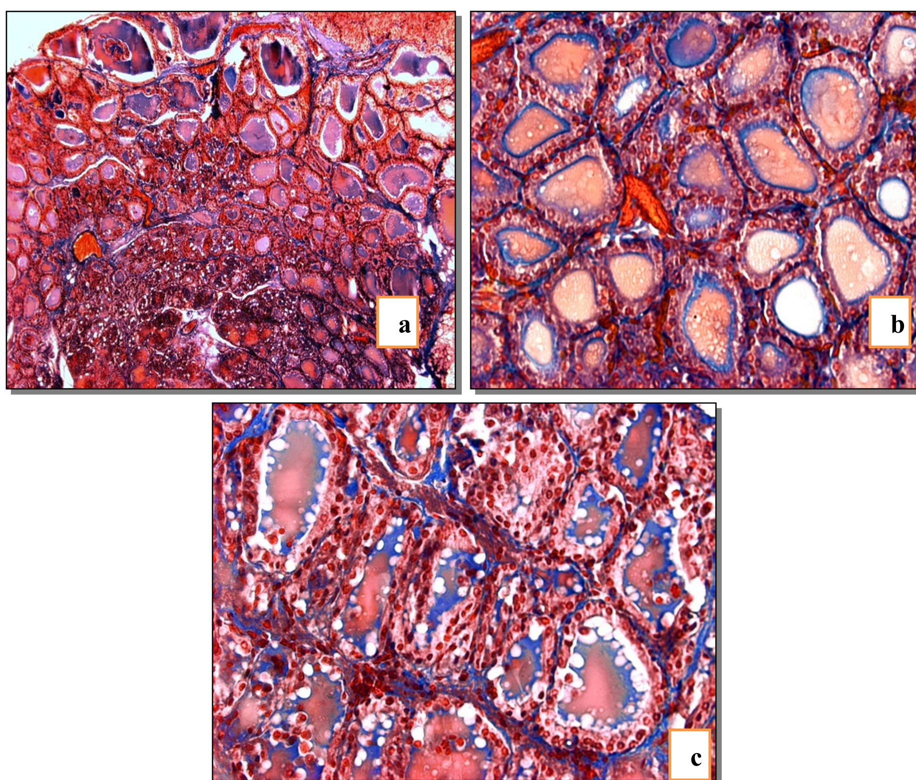

Figure 5 a) The thyroid of FC treated animals, 6x. **b**) Hormone generating follicles, 20x **c**) Vesicular structured follicles, Hurduc staining, 40x

As a result of the histological studies, we can conclude, that glucocorticoid excess induced increases of thyroid hormone releasing operation, which is reflected in the histological structure. This observation is consistent with bibliographic data,

according to which glucocorticoid excess increases the release of thyroid hormones. Literature data demonstrate that the thyroid gland is sensitive to glucocorticoid excess (Martino *et al* 2001; Menconi *et al* 2007; Nadolnik 2012).

Some results demonstrate that fluocinolon treatment effects hormone-producing pituitary cells, especially somatotrop, kortikotrop, gonadotrop and tireotrop cells. Fluocinolone treatment in Wistar rats caused ultrastructural changes in the tireotrop cells and reduction in secretory granules number, which leads to a reduction in the release of tireotrop hormone from pituitary gland (Kis and Crăciun, 2005). Fluocinolon treatment causes an excess of glucocorticoid-induced changes in hormone production in the thyroid regulation, this upsets the negative feed-back mechanisms and empties the thyroid which reduces the hormone production. The decreased hormone production is demonstrated by the finding that glucocorticoid excess causes significant weight loss in young and mature animals. The growth of various ages of Wistar rats were studied after treatment with Fluocinolon-acetonid N, and there has been a significant growth rhythm disruption in young animals which were born with reduced body mass. Their weight loss can be explained with the balance disruption of somatotropic and thyroxine / triiodothyronine hormone release (Kis and Crăciun, 2005).

Conclusions

1. The dose of Fluocinolon-acetonid N ointment used in the present study induced low body weight in adult male Wistar rats.
2. The glucocorticoid excess induced by Fluocinolon treatment increases the release of thyroid hormones.
3. In our experimental protocol the more sensitive organ to exogenous glucocorticoids is the kidney.
4. In our study the rat's kidney exhibited a discrete oligonephronia.

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The effects of antibiotics and antifungals added to plant culture media of *Triticosecale wittmarck*

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SUMMARY. The experiment aimed to study the antibiotics or antifungals effect to prevent the infection in *Triticosecale wittmarck* micropropagation, without affecting the inoculum. After identifying the pathogens, which are frequently affecting the *in vitro* culture media, respectively *Clostridium* sp. and *Cladosporium* sp., we made the antibiogram and antifungal susceptibility testing. Among antibiotics and antifungals, to which one of them were found to be sensitive, we chose to add the following to the culture media: V_1-gentamicin (GEN) - 40 mg/L, V_2-tetracycline (TET) - 10 mg/L, V_3- ciprofloxacin (CIP) - 40 mg/L, V_4-clotrimazole (CLO) - 50 mg/L, V_5-fluconazole (FLU) - 50 mg/L, V_6-terbinafine (TER) - 25 mg/L, having the culture medium, without anti-contaminants additions, as control lot. At the end of the experiment we concluded that anti-contaminants, used in concentrations recommended by literature, have prevented infections, but reduced the capacity for germination rate of triticale. Just terbinafine can be added in triticale culture media, but it is necessary to study other lower concentration that does not affect the growing of inoculum.

Keywords: antibiotics, antifungals, culture media, *Triticosecale*

Introduction

In vitro cultures, of all kinds, are exposed to contamination risks with different kinds of microorganisms. In agar media, the chemical composition and quality are less affected, respectively fungi mycelia or bacteria invasion are slower than compared to liquid media. However, usually in about one week after inoculation, the hyphae fungi invade the phyto-inoculum, the molds dampening the vitrocultures they infected (Turcuș and Cachiță-Cosma, 2009). Usually, the cultures initiated from explants taken from plant material grown in septic conditions, show a high occurrence of accidental infestations (Cachiță-Cosma and Ardelean, 2009).

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The reason of occurrence in such infections may be the exogenous (deficiencies in working technique) or endogenous nature (germs spread deep down in the intern structure of the explant, bacteria or mycosis located intercellular or endocellular) (Cachiță-Cosma and Ardelean, 2004). The micopropagation success will depend not only on preventing primary infections, but also preventing the occurrence of secondary infections, possible a few weeks after the inoculation.

Protecting the plants against these agents is mainly made with antibiotics and antifungals (Makovitzki *et al.,* 2007). For eliminating the infections, it is used antibiotics and antifungals treatment, or cultivating them on culture media with additional anti-contaminants (Thomas, 2004; Cachiță-Cosma and Ardelean, 2009; Kulkarni and Krishnamurthy, 2009; Smith, 2013), as mixture of methylchloro-isothiazolinone, methylisothiazolinone, magnesium chloride and magnesium nitrate (Guri and Kishor, 1998). The first research with antibiotics added in culture media involved vanilin and its derivatives (Knudson, 1974; McAlpine, 1974 cited by Thurston *et al.,* 1979).

Throughout the years, in many researches were made efforts for introducing certain antibiotics or antifungals, in order to prevent contamination of plants *in vitro* cultures (Arditti, 2008). Concentrations used by researchers varied: 0.1 mg/L metalaxyl, 1.0 mg/L actinomycin D, 2.5 mg/L amphotericyn B, 5 mg/L sodium omadine, 100 mg/L paromocyn sulfat, penicilin G, streptomicyn sulfate or pentochloronitrobezene. There are anticontaminats wich can be used in large concentrations: 300 mg/L ticarcilin, 400 mg/L ampicilin, 500 mg/L carbenicilin, 800 mg/L geneticin, 1000 mg/L cefotaxime, but most recommended concentration is between 10 –50 mg/L: tetracycline hydrochloride, thiabendazole, benomyl, cycloheximide, aureomycin, bavistin miconazole, mytomicin C, neomycin sulfate, rifampicin, vancomicyn (Arditti, 2008). Adding 50 mg/L gentamicin to the medium seemed to be useful, but not for the latent bacteria (Thomas, 2004). Amphotericyn B (92.7% pure,10 ppm), nystatin (4020 units mg/l, 25 ppm) and sodium omadine (90% pure, 5 ppm), penicilin G (1595 unit mg/L, 100 ppm), benomyl (50 % pure, 50 ppm), dowicide (97% pure, 5 ppm), gentamicyn (50 ppm), vancomicyn (50 ppm) used individually delayed *Cymbidium* shoots (Brown *et al.*, 1982).

The purpose of the present research is the study the antibiotic and antifungal effect on preventing infection of *in vitro* cultures, without affecting the development of *Triticosecale wittmarck* inoculum.

Materials and methods

Since the infection management techniques efficiency begins with a proper identification of the disease and/or the causal organism (Leonberger *et al.*, 2016), we have started to identify the pathogens in the *in vitro* cultures and the anti-contaminants to which they are sensitive (Cosma and Petruș-Vancea, 2017), to add them later in the culture media. Thus, two different species of microorganisms,

namely *Clostridium* genus and a fungus of *Cladosporium* genus, have been identified by diffusometric sensitivity testing methods (Costache and Colosi, 2008; Cercenado and Saavedra- Lozano, 2009). In the previous study (Cosma and Petruș-Vancea, 2017) we found out that the bacteria was sensitive to ciprofloxacin, levofloxacin, gentamicin, vancomycin, tetracycline, chloramphenicol, trimethoprim - sulfametoxazole, imipenem, and the fungus was sensitive to terbinafine, fluconazole, clotrimazole and posoconazole.

Plant material used in our experiments consists in triticale *(Triticosecale wittmack)* caryopses and research design is presented in table 1.

Culture media was standard Murashige-Skoog (1962) with Gamborg (1968) vitamins, solidified with agar-agar 8 mg/L and 5.7 pH, without growth regulators, which was placed in 5 mL glass containers with a size of 2/7 cm.

Antibiotic and antifungal concentrations were determined following references in the literature (Arditti, 2008). Understanding the effects of many important antibiotics in the plant physiological activity is still limited (Opriș *et al.,* 2013*).* In the orchid culture media, Thurston *et al.* (1979) recommended the combined introduction of anticontaminants, as well: benlate + nystatin + penicilin G + gentamycin + sodium omadine + graphite and the development of stock solutions for an easy work.

Table 1.

Experimental protocol

Experimental variants	Disinfection	Growth conditions	Measurement periods	Measurement types
V_0- without anti-contaminants (*control*) V_1-GEN-40 mg/L V_2-TET-10 mg/L V_3-CIP-40 mg/L V_4-CLO-50 mg/L V_5-FLU-50 mg/L V_6-TER-25 mg/L	15 min. 2% sodium hypochlorite +Tween 20 submersion, then followed by 5 sterile water washes (25 min.)	- 22-24 ˚C - white fluorescent lighting, light intensity 91800 µmol m^{-2} s^{-1} 16 h photoperiod	3 days 7 days	Embryonic root L. Coleoptile L. Leaf L. Germination % Survival % Infection level

Note: GEN-gentamicin, TET-tetracycline, CIP-ciprofloxacin, CLO-clotrimazole, FLU-fluconazole, TER-terbinafine, L.- length.

The inoculation was carried out in the sterile room, using horizontal flow cabinet, following the asepsis rules (Petruș-Vancea *et al.,* 2013).

Biometric data were mathematically processed in the Microsoft Excel 2013. The *t* test was performed using the SPSS for Windows to identify the significance of the difference from the control lot ($p<0.5$).

Results and discussion

At 3 days after germination, the germination percentage was increased in the control lot (V_0) and in the presence of gentamicin (V_1). The lowest germination rate was recorded at the *in vitro* group placed on culture media supplemented with tetracycline (V_3) (Fig. 1). The effect of tetracycline was reported to be inhibitory on germination by Turdeanu and Petruș-Vancea (2015), affecting *in vivo* triticale germination, but also by Mocanu and Petruș-Vancea (2016) in *in vitro* cultures.

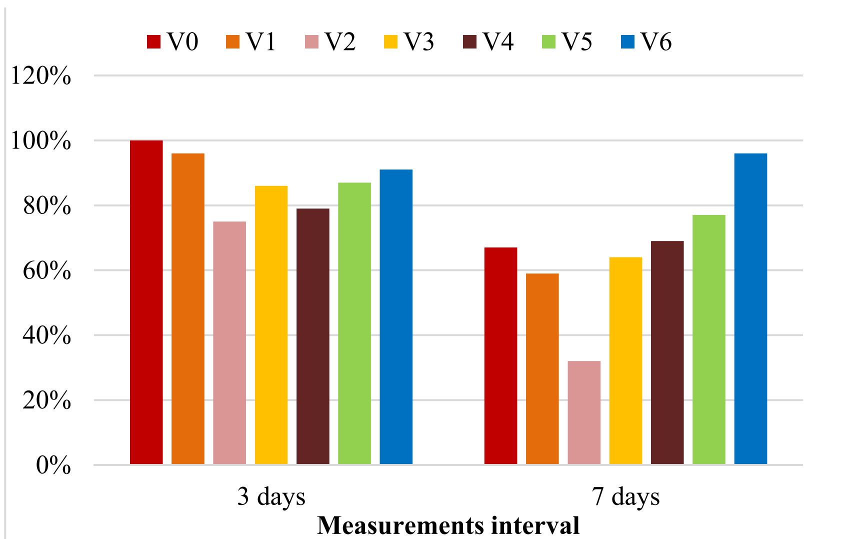

Figure 1. Percent expression of the *Triticosecale wittmarck* ***germination*** at **3 days** and the ***survival*** at **7 days** after inoculation on the following culture media: V_0 – control, MS-G, without anti-contaminants; V_1 – MS-G + gentamicin; V_2- MS-G + tetracycline; V_3- MS-G + ciprofloxacin; V_4- MS- G + clotrimazole; V_5- MS-G + fluconazole; V_6- MS-G + terbinafine, compared to the total of inoculated containers, which represents 100%.

Although germination process satisfactorily started, at 7 days after germination, there were large losses due to germinated plants necrosis, so the survival rates were diminished in all experimental variants, including the control, with the exception of the plants lot placed on culture media with the addition of terbinafine, a fungicide that caused necrosis to *Sequoia* minicuttings (personal data).

A different reaction was identified according to the ontogenetic plant period, respectively in the first days of life, immediately after germination (3 days) and during the growth period (7 days), when greater inhibitions could be measured (Fig. 1).

Even at 3 days after inoculation, *Cladosporium* infections occurred, especially on the control and antibiotic added to media, but also on the Clotrimazole antifungals addition. Infections in this case of culture initiation are caused by the cariopse explants insufficient sterilization, which conduct to over infection, to the impossibility of the fungicide to cope with the infection.

However, there was only one experimental variant, V_6, namely the terbinafine addition, with any infection (Fig. 2).

Moreover, the highest survival rate was recorded on the terbinafine medium, at 7 days after inoculation (Fig. 1), even higher than to the control. Instead, the growth indexes were small compared to the control (Table 2 and Fig. 3).

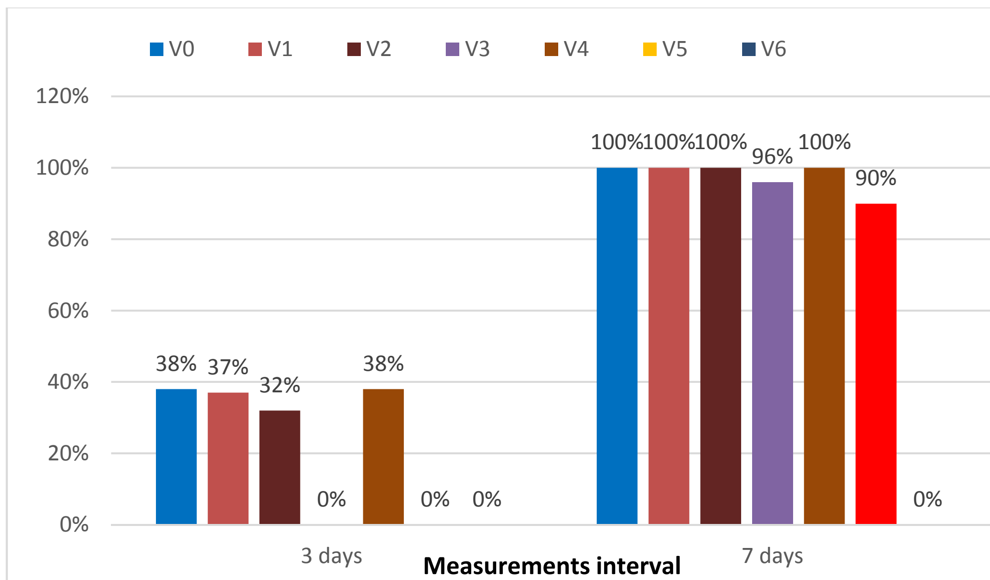

Figure 2. Percent expression of the *Triticosecale wittmarck* ***infection*** at **3 days** and **7 days** after inoculation on the following culture media: V_0 – control, MS-G, without anti-contaminants; V_1 – MS-G + gentamicin; V_2- MS-G + tetracycline; V_3- MS-G + ciprofloxacin; V_4- MS- G + clotrimazole; V_5- MS-G + fluconazole; V_6- MS-G + terbinafine, compared to the total of inoculated containers, which represents 100%.

Although the highest survival values were recorded in the terbinafine lot (V_6), respectively the null infection rate, from the plant growth indexes point of view, the highest inhibitions were registered (Table 2 and Fig. 3). Infected plants continued the *in vitro* growth process.

Cefotaxime was shown to eliminate contamination with *Xanthomonas campestris* pv.*pelargonii* and stimulate the growth of the *Pelargonium x domesticum* cv. 'Grand Slam' plant tissue cultures (Barrett and Cassells, 1994).

Table 2.

Plant growth indexes values, at **7 days** after inoculation on the following culture media: V_0 – control, MS-G, without anti-contaminants; V_1 – MS-G + gentamicin; V_2- MS-G + tetracycline; V_3- MS-G + ciprofloxacin; V_4- MS- G + clotrimazole; V_5- MS-G + fluconazole; V_6- MS-G + terbinafine (L.- length)

Types	Average (cm) ± standard deviation			Difference from control (cm) / significance of difference (p)					
	Root L.	Coleoptile L.	Leaf L.	Root L.		Coleoptile L.		Leaf L.	
V0	1.96±0.60	2.46±0.42	1.48±0.92		**p**		**p**		**p**
V1	1.93±0.72	2.78±0.43	1.23±0.91	-0.03	Ns	0.32	Ns	-0.25	Ns
V2	1.52±0.63	1.98±0.79	0.78±0.75	-0.44	Ns	-0.48	Ns	-0.70	Ns
V3	0.99±0.59	2.03±0.83	0.17±0.35	-0.97	*	-0.43	Ns	-1.31	***
V4	1.10±0.37	2.50±0.64	0.27±0.56	-0.86	Ns	0.04	Ns	-1.21	**
V5	1.50±0.58	1.35±0.55	0.58±0.61	-0.46	Ns	-1.11	*	-0.90	*
V6	0.4±0.18	0.7±0.26	0.00	-1.56	***	-1.76	***	-1.48	***

Note: $\bar{x} \pm S_{\bar{x}}$ - average (cm) ± standard deviation); p (significance): p>0.5 - non significance (Ns), p<0.5 - significance (*), p<0.1 - distinct significance (**), p<0.01 – very significance (***).

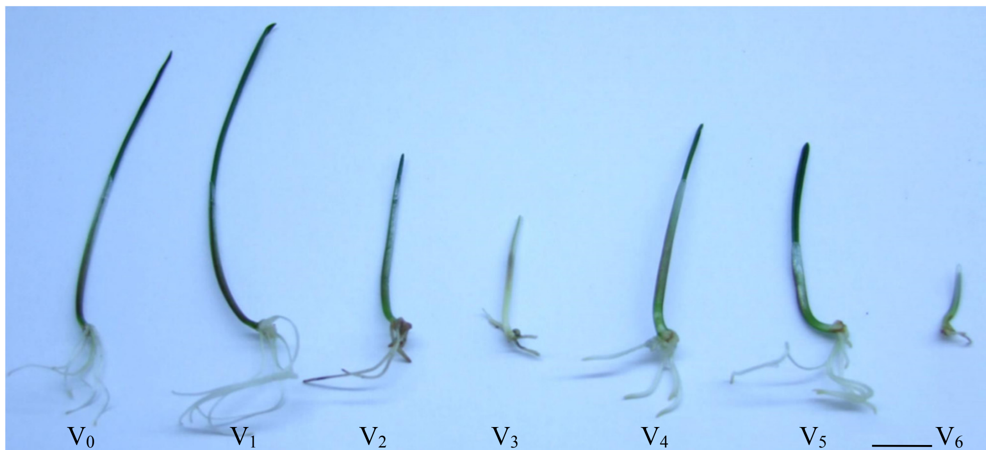

Figure 3. *Triticosecale wittmarck in vitro* cultivated plants at **7 days** after inoculation on the following culture media: V_0 – control, MS-G, without anti-contaminants; V_1 – MS-G + gentamicin; V_2- MS-G + tetracycline; V_3- MS-G + ciprofloxacin; V_4- MS- G + clotrimazole; V_5- MS-G + fluconazole; V_6- MS-G + terbinafine (bar means 1 cm).

Conclusions

1. Primary *in vitro* cultures initiated from triticale caryopses (*Triticosecale wittmarck*) in culture media with added antibiotics namely: gentamicin 40 mg/l, tetracycline 10 mg/l, ciprofloxacin 40 mg/l or antifungals like clotrimazole 50 mg/l and fluconazole 50 mg/l showed fungal infections.
2. Terbinafine 25 mg/l completely prevented the infections, but generated significant inhibitions of the growth indexes, therefore we consider necessary for the future to study the effect of a smaller concentration, which would not affect the plantlets growth.
3. It is necessary to continue researches in order to determine the antibiotics and antifungal combination, in a suitable concentration for preventing infections and proper growth of the *in vitro* plantlets, according to their species.

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Drinking water quality assessment in the Danube Delta Biosphere Reserve

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SUMMARY. This research is aimed mainly at the assessment of water quality of several drinking water treatment plants and groundwater wells, from the Danube Delta Biosphere Reserve and the habits of the local population in terms of water consumption and use. Thus, the study highlights the presence and distribution of prokaryotic and eukaryotic assemblages, specifically the algal communities which emerged as a dominant group in the investigated drinking water sources. The spatial variation of the pH, EC, turbidity, dissolved oxygen (DO), temperature, algae species and biomass was measured in five drinking water treatment plants (DWTP) and three groundwater wells from villages located in the Danube Delta Biosphere Reserve. No PCR product was present for either genes coding anatoxin, microcystin and saxitoxin. The bbe-Moldaenke technique identified the presence of sligthly more algal groups, than the light microscopic technique; both methods show clear results: the water treated in the drinking water treatment plants has significant algal biomass. The water quality indicated by the algal communities shows oligo-β-mesosaprobic conditions, despite the high number of taxa and individuals from Sf. Gheorghe and C. A. Rosseti samples, they reflect oligotrophic conditions.

Keywords: algae, cyanobacteria, cyanotoxins, drinking water.

Introduction

Due to the practical experience mentioned into international decree for many (particularly physico-chemical) parameters, the concentrations present in drinking water sources would rarely result in any breach of limit values (Commission Directive, 2015).

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On the other hand, microorganisms present in drinking water sources and the substances produced by them have been reported more frequently in similar studies performed in other countries. These findings could affect the health of people and their offspring (Cabral, 2010; Semenza *et al.*, 2012; Sweileh *et al.*, 2016).

Prokaryotic and eukaryotic microorganisms are naturally present in many water resources and can enter, grow and colonize water treatment and transport systems (including reservoirs, pipes or plumbing). Studies worldwide have reported the presence of protozoa, algae, invertebrate and fungi which can constitute a public health concern. The potential pathogenicity of some genera like *Acanthamoeba*, *Candida* and *Aspergillus*, can become a health concern by harboring a variety of pathogenic bacteria or by the presence of toxic compounds produced by cyanobacteria (Belila *et al.*, 2016; Fosso-Kankeu *et al.* 2008; Ibelings *et al.* 2014; Říhová Ambrožová *et al.*, 2009). Microorganisms in drinking water systems have the tendency to attach to any surface they come in contact with, resulting in so called “biofilms” (Douterelo *et al.*, 2014; Fish *et al.*, 2015). Despite of rigorous standards for regulatory purposes and systematic promulgation (modifications or completions of these), in the Romanian legislation no information can be found, about preventive measures that include control of the presence of algae and algal toxins or the presence of biofilms in the drinking water distribution systems (Decision 974, 2004; Law no. 458, 2012).

An overview of the drinking water quality in Europe by the reporting period 2011-2013, under the Drinking Water Directive 98/83/EC (Council Directive, 1998), showed that the development of the drinking water quality is based on the spatial scale of the Water Supply Zone. It could include a distinction between large and small water supply zones.

Small water supply zones serve less than 1,000 m^3/day and less than 5,000 people. In case of these water supplies, no obligation to report the drinking water quality every three years is needed (Overview of the drinking water quality in Romania, 2016). The water supply zone in the Danube Delta Biosphere Reserve could be included into the category of small water supply zone, due to the low number of inhabitants. Information provided by the national database on drinking water quality in the Danube Delta Biosphere Reserve is insufficient. The references on contamination with eukaryotic microorganisms are mentioned only in case of wells. This study is bound to bring more information and clarification about the quality of the drinking water in the Danube Delta and it shows the importance of conducting more similar studies.

Materials and methods

Drinking water sources

Inhabitants of the Danube Delta Biosphere Reserve have access to fresh water directly from the Danube River, through the drinking water treatment plants (DWTP) and drinking water distribution systems (DWDS) or groundwater wells.

The distribution network of the drinking water does not include a polyvinyl chloride (PVC) piping network in all locations from the the Danube Delta Biosphere Reserve. The routine maintenance of drinking water standards includes the periodical pumping of drinking water through the piping network in case of Sf. Gheorghe, Sulina, Chilia, Partizani, Pardina and Maliuc. There are no DWTPs and DWDSs in case of C.A. Rossetti, Letea and Vulturul.

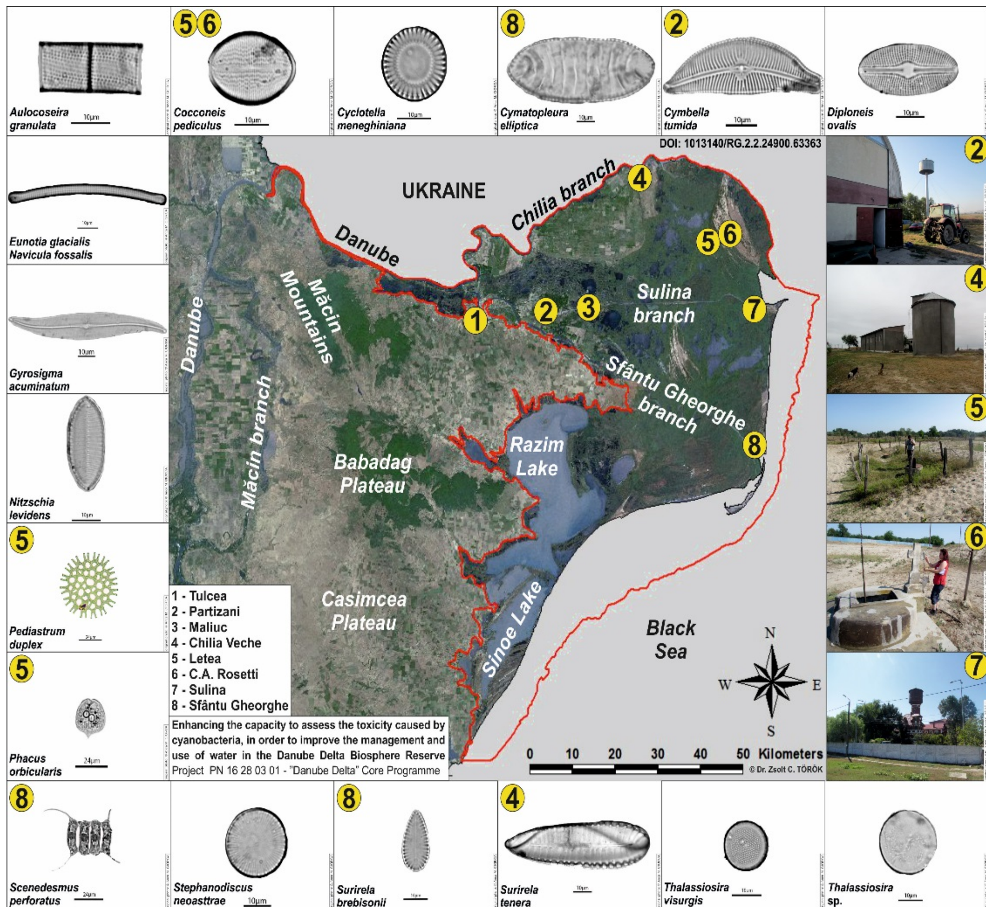

Figure 1. The sampling sites map (1. Tulcea, 2. Partizani, 3. Maliuc, 4. Chilia Veche, 5. Letea, 6. C. A. Rosetti, 7. Sulina, 8. Sfântu Gheorghe) sorrounded by photos of the algal taxa founded in the eight sites and photos from five sites

Sampling design

In situ measurements of water temperature, turbidity, dissolved oxygen concentration (DO) and electrical conductivity (EC) have been performed using HANNA HI 9829 Multiparameter, odors in water were also analyzed. Basic parameters examined in the present study were recommended by the Romanian legislation (Decision 974, 2004).

Ten liters of drinking water, were filtered *in situ* from every sampling site, using a 40 μm phytoplankton mesh size net. The resulting samples were used to determine the taxonomic composition and to measure the algae biomass, using bbe- Moldaenke technique with fluorescence spectrum excitation at 470nm LED (Light Emitting Diode) for green algae, 525nm LED for diatoms, 570nm LED for cryptophyceae and 610nm LED for cyanobacteria. Samples from DWTP (Sulina, Sf. Gheorghe and Chilia) and from groundwater wells (Letea and C.A. Rosetti) were stored in laboratory, in dark conditions for a period of seven days in opaque plastic containers (5L) and in transparent plastic containers (5L).

The qualitative assessment was made using light microscopic technique and the identifications were made to the species level (Krammer and Lange-Bertalot, 1986, 1988, 1991; Ettl, 1983). All samples were examined, before and after incubation for seven days. The quantitative assessment was determined by calculating the relative abundance, expressed as percentages. For phytoplankton roughly 400 individuals were counted in one drop of water, from every sample, at 40x magnification. In case of low number of individuals / sample, the entire surface of the slide was counted.

The taxonomic composition of the algal communities were analyzed from the following drinking water samples:

• "*in situ*" filtered samples from DWTPs in Sf. Gheorghe, Maliuc, Chilia, Partizani and from groundwater wells in Letea (1) and (2), C.A. Rosseti.

• before and after seven days of incubation from Sf. Gheorghe, Chilia, Sulina, Letea (1) and C.A. Rosseti.

DNA extraction and PCR amplification of cyanotoxin genes

The water samples were filtered through 0.2 μm-pore-size mixed cellulose ester membrane filters (Fioroni, France), followed by DNA extraction using ZR Soil Microbe DNA kit (ZymoResearch, USA), according to manufacturer's instruction. The optimal reaction component concentrations for the cyanotoxin gene amplification assays were as follows: 1 μL DNA, 5 μL MyTaq Red Mix 5X Buffer Solution, 0.3 μL of each 20 μM forward and reverse primers, 0.3 μL MyTaq Red 5u/μL and RNase/ DNase-free water to a final volume of 25 μL. Cycling protocols for each primer pair was used as recommended in the cited literature (Table 1).

Table 1.

Primer sets used in this study

Primer	Toxin	Sequence (F/R)	T_a°C	Product (bp)	Reference
***ana*C-gen**	Anatoxin	TCTGGTATTCAGTCCCCTCTAT/ CCCAATAGCCTGTCATCAA	58	366	Rantala-Ylinen *et al.*, 2011
***mcy*A-Cd1**	Microcistin	AAAATTAAAAGCCGTATCAAA/ AAAAGTGTTTTATTAGCGGCTCAT	59	297	Hisbergues *et al.*, 2003
***sxt*A**	Saxitoxin	GATGACGGAGTATTTGAAGC/ CTGCATCTTCTGGACGGTAA	60	125	Al-Tebrineh *et al.*, 2010

Results and discussion

Water supply and water use customs

The first water supply service in the Danube Delta was founded in 1920 in the town of Sulina, subsequently this service was extended to other locations from the Danube Delta Biosphere Reserve. Nonetheless, residents have continued to collect water directly from the Danube River for everyday household demands (drinking, cooking, and washing). The water supply service has achieved substantial and significant improvements after 1990, but these improvements are unable to provide the daily water requirements at a proper quantity and quality.

The available quantity of fresh water provided by the water supply services is not continuous during the summer time. The reported cubic meters per capita per days during summer period being as follows: 700-900 m^3/day at Sf. Gheorghe; 600 m^3/day at Sulina and Chilia. However, it is reported a less hours/day continuous water supply for the winter period, around 200 m^3/day.

The physico-chemical characteristics of water quality

The quality of the water is controlled and monitored at the DWTPs by the drinking water suppliers, private companies and by the Public Health Authority.

The research performed by the Danube Delta National Institute for Research and Development in 2016, deals with the assessment of the quality of water supplied to consumers, specifically from the point of view of chemical and biological contamination with algae and cyanobacteria toxins (Report of the project No PN 16 28 03 01, 2016). Cyanobacteria and its toxins are not included in national standards, therefore environmental protection agencies do not monitor their impact on the water quality. Concerns about monitoring and mitigating the negative effects of cyanobacteria and cyanotoxins on the environment, have been raised not only in the academic and scientific research field, but also at the European Enviroment Protection level (Falconer *et al.,* 2005, Nicholson *et al.*, 2001, Říhová Ambrožová *et al.,* 2009).

In the present evaluation of the water quality from the DWTPs and groundwater wells, took place in august 2016. All the analyzed water samples were odorless. *In situ* recorded parameters are presented in Figure 2.

The recorded pH values were in the range of standard values. In case of DWTPs the EC values were lower than in the case of drinking water from well sources and lower than standard value (Decision 974, 2004).

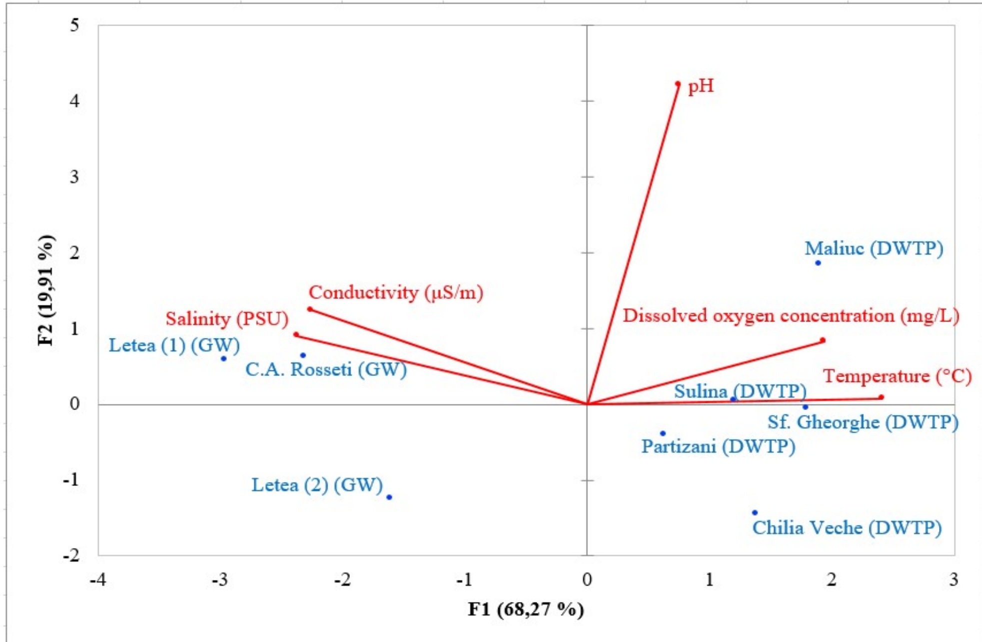

Figure 2. Principal Component Analysis (PCA) biplot (axes F1 and F2: 89.97 %) for the drinking water samples lakes and their aggregation based on physical and chemical parameters (abbreviations: DWTP-drinking water treatment plants, GW-groundwater well, PSU- Practical Salinity Unit)

Molecular detection of cyanotoxins

The presence of targeted cyanotoxin genes in the investigated environmental samples was verified by electrophoresis on a 1.0% agarose gel. No PCR product was present for either genes coding anatoxin, microcystin and saxitoxin. The concentration of cyanobacterial cells in drinking water samples is not always related to concentrations of cyanotoxins or genetic determinants (Szlag *et al.*, 2015). Possible factors causing the lack of cyanotoxin genes could be that some strains of potential toxin-producing species did not have the toxin gene, or that concentrations of cyanotoxin genes are lower in running- than stagnant water.

Algae and cyanobacteria in the drinking water supply

The evaluation of the response to the different treatment steps (decantation, filtration, chlorination), in case of algae and cyanobacteria was assessed at the entrance in the network distribution system. The influence of water treatment on the planktonic community structure was unnoticeable in case of Sf. Gheorghe, Chilia and Partizani (Fig. 3). The results showed higher values of the recorded algal biomass than at the sources from where water is extracted (The Danube River) before it enters the water plant.

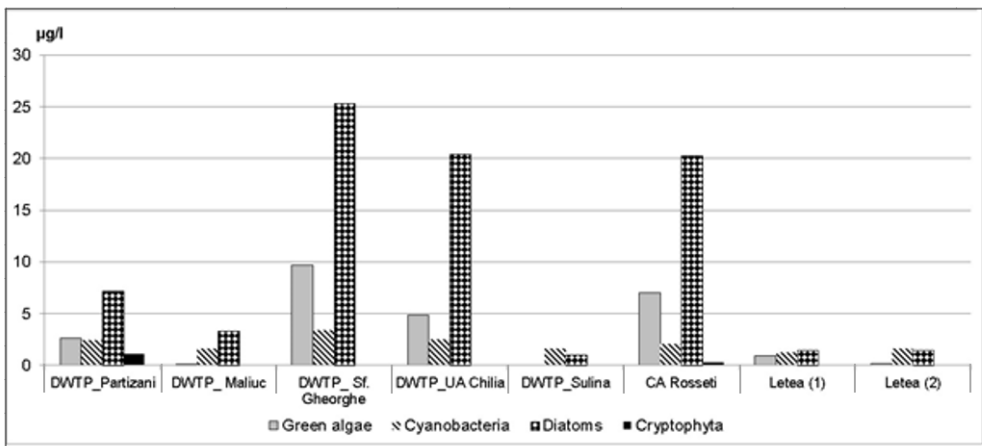

Figure 3. Algal biomass distribution in drinking water sources in the Danube Delta

The highest number of taxa were found in the "*in situ*" filtered samples from DWTPs from Sf. Gheorghe (35 taxa), Partizani and Chilia (21 and 20 taxa, respectively), Sulina (12 taxa) and only 8 taxa were found in samples from Maliuc. In comparison the samples from the groundwater wells were similar regarding the qualitative assessment: 27 taxa in C.A. Rosseti, 24 taxa in Letea (1) and only 10 taxa in Letea (2). Consequently three taxa were common in the analyzed samples: *Cyclotella meneghiniana*, *Stephanodiscus neoastraea* and *Thalassiosira weissflogii*.

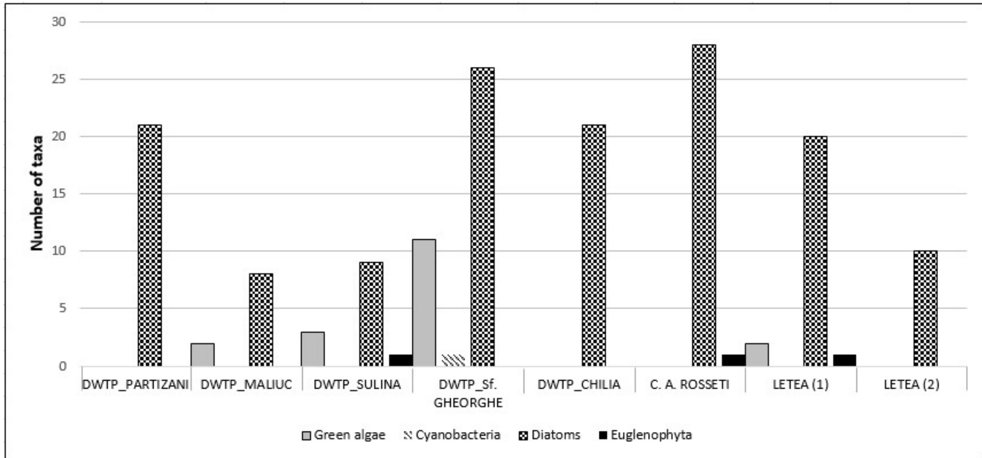

Figure 4. The taxonomic composition of the algal groups identified "*in situ*" the drinking water samples

Figure 3 and 4 show the similarity between the two methods of identifying the algal composition in water samples. Although bbe- Moldaenke technique identified the presence of sligthly more algal groups, both methods show clear results: the water treated in the DWTPs has significant algal biomass. In samples from the ground water Letea (1) and Letea (2) the algal biomass and taxonomic composition was the lowest. In Sulina green algae and other algal communities were found performing the light microscopy method. After analyzing the results, the water samples from DWTP Sf. Gheorghe registered the highest algal biomass (with the highest number of taxa), the dominating taxa being *Cyclotella meneghiniana*. The saprobic level indicated by this taxon is α-mesosaprobic, classifying the water quality in class III-IV (Van Dam *et al.*, 1994), which means the quality of the water is altered (in one drop of water 184 cells were found).

After seven days of incubating the water in transparent containers (TC) or in opaque containers (OC), the number of taxa dropped (Fig. 5). In each case the only identified taxa were diatoms, with only the siliceous frustule with no organic matter. These results could indicate that stored water bottles or cans, in dark conditions, are preferable for long-term use of the drinking water.

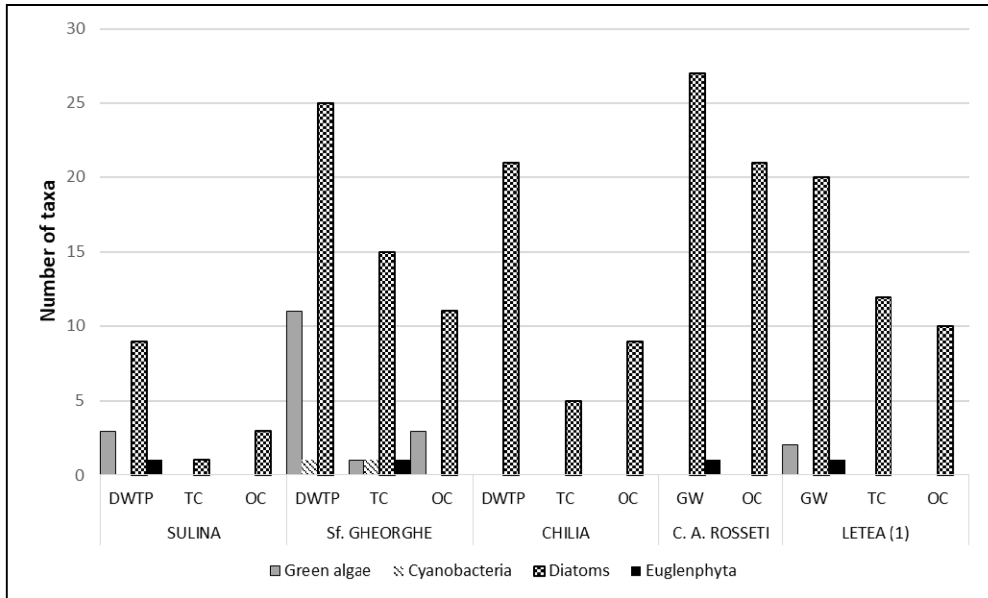

Figure 5. The taxonomic composition of the algal groups identified in the "*in situ*" drinking water samples compared to the incubated samples

The relative abundance was calculated for the following samples: DWTPs Partizani, Sf. Gheorghe and Chilia; groundwater wells C.A. Rosseti *in situ* and after incubation in an opaque can. In the samples from DWTP Sf. Gheorghe the class Mediophyceae (Centrales) was the only one present, with three taxa: *Cyclotella meneghiniana, Stephanodiscus neoastraea and Thalassiosira weissflogii*. These three taxa also ocupied more than 80% of the taxa identified in Partizani and Chilia. In the water samples from the ground water in C.A. Rosseti the diversity was higher. *Eunotia glacialis* being the dominant taxa (more than 50%), followed by *Diploneis elliptica* and *Achnanthes minutissima*. In Fig. 6 the correspondence analysis shows two distinct groups: DWTPs and groundwater. Because of the similarity between *Stephanodiscus neoastraea and Thalassiosira weissflogii*, in the correspondence analysis these species have been counted togheter (in order to avoid mislead identification).

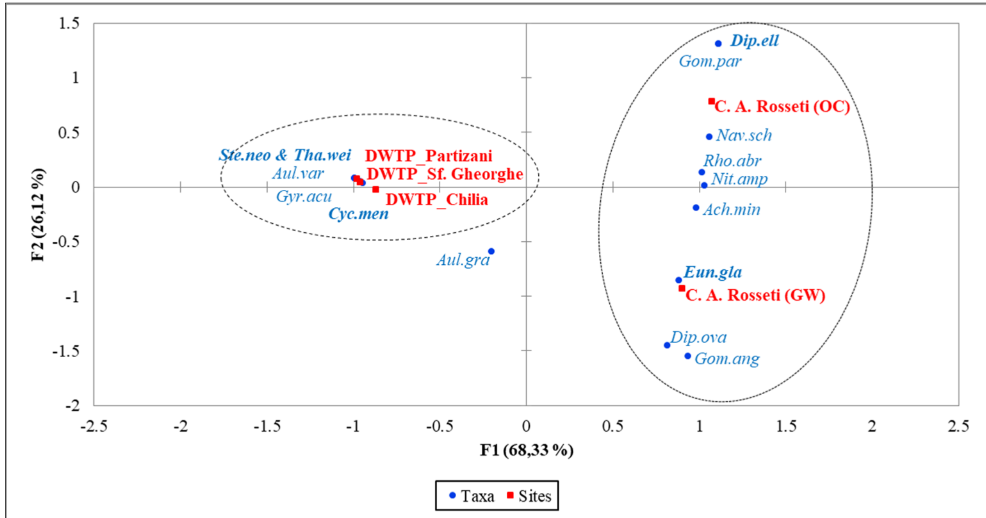

Figure 6. The correspondence analysis (axes F1 and F2: 94,45 %) showing the aggregation of sampling sites with the number of individuals from each algal taxa (abbreviations: *DWTP-drinking water treatment plants, GW-groundwater well, OC – opaque container; Ste.neo.- Stephanodiscus neoastraea*, *Tha.wei. - Thalasiosira wiesurgis, Aul.var. - Aulacoseira varians, Gyr.acu.- Gyrosigma acuminatum, Cyc.men. - Cyclotella meneghiniana, Aul.gra. - Aulacoseira granulata, Dip.ell. - Diploneis elliptica, Gom.par.- Gomphonema parvulum, Nav.sch.- Navicula schoenfeldii, Rho.abr.- Rhoicospahenia abreviatta, Nit.amp.- Nitzschia amphibia, Ach.min.- Achnanthes minutissima, Eun.gla.- Eunotia glacialis, Dip.ova.- Diploneis ovalis, Gom.ang.- Gomphonema angustum*.)

The water stored for household purposes can contain cyanobacteria, especially in rural areas without sewage systems or DWTP and DWDS, if water is collected from sources that presented a cyanobacterial blooming period. This phenomenon can be worsened if water is carried home and kept in transparent plastic containers. The presence of these microorganisms may affect the water quality, especially due to their ability to use carbon dioxide, light and minerals which allow them to grow and develop under these circumstances (Fosso-Kankeu *et al.*, 2008).

Overall the water quality indicated by the algal communities shows oligo-β-mesosaprobic conditions, but at every site the conditions change, because of the dominating bioindicator taxon. Even though the samples from Sf. Gheorghe and C. A. Rosseti had a higher number of taxa and individuals, they reflect oligotrophic conditions. The water quality could be at a lower level than expected, but as observed at the microscope samples, no high abundance of cyanobacteria was identified.

In the present study, after the incubation period (Table 2) a significant decrease of the total amount of chl-a and a slight increase in cyanobacteria was recorded in case of Sulina and C.A. Rosseti.

Table 2.

Concentration of chl-a and cyanobacteria biomass in DWTPs (abbreviations: CYAN - cyanobacteria, OC - opaque container; TC - transparent container; GW - groundwater wells, DWTP - drinking water treatment plants)

	DWTP/GW		**Incubation OC**		**Incubation TC**	
	chl-a (µg/l)	**CYAN (µg/l)**	**chl-a (µg/l)**	**CYAN (µg/l)**	**chl-a (µg/l)**	**CYAN (µg/l)**
Sulina	2.64	1.65	3.06	1.90	3.46	2.10
Sf. Gheorghe	38.52	3.44	21	2.92	14.82	2.68
Chilia	27.90	2.57	3.32	1.78	3.80	1.87
Letea (1)	3.72	1.30	3.46	1.51	3.45	1.42
C.A. Rosseti	29.62	2.09	10.19	2.17	7.43	1.84

Conclusions

Results revealed an undesirable amount of algal taxa in the drinking water samples, even after the treatment carried out in the DWTPs. In some cases the algal biomass was significantly reduced in the drinking water sampled from the wells, rather than from the Drinking Water Treatment Plants. The results obtained in this study should raise concern with the authorities and the legislation should contain information and toleration limits about the amount of cyanobacteria toxins and algae found in the drinking water.

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Neighbours vs. strangers discrimination in Water Rail (*Rallus aquaticus*)

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SUMMARY. Water Rail (*Rallus aquaticus*) manifest strongly intra- and interspecific aggressivity. In our study we test the Water Rail's reaction to the calls of conspecifics neighbours and strangers, to conclude if they express the "neighbour-stranger discrimination" (NSD) and / or "the dear enemy effect" (DEE) behaviour as evolutionary attributes that would reduce the energy consumption. Ten points located 100 m apart inside the Sic Reedbeds wetland were selected. In each point the acoustic reactions to spontaneous and playback of territorial Water Rail's call were counted for three days (18, 19 and 20 April 2011), between 6:00 – 8:00 p.m.. A statistically significant difference was found between the number of songs recorded after spontaneous activity and the number of calls recorded after playback. The results argues the hypothesis that individuals of this species recognize their neighbours and react less aggressively towards them. Moreover, our findings indicated that response to neighbours' calls follows a constant trajectory during the first or second half of the minute while the reaction towards foreigners is significantly losing intensity during the second half of the minute. The results suggest that the NSD and DEE are expressed in the Water Rail behaviour.

Keywords: dear enemy effect, intraspecific, neighbour-stranger discrimination.

Introduction

Most individuals of territorial species interact repeatedly with their conspecific neighbours, particularly during breeding season but also during their movements and migrations. They are competing for space, food and fertilization resources (Yezerinac *et al*., 1995; Webster *et al*., 2001; Segelbacher *et al*., 2005; Akçay *et al*., 2009). In

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many species, individuals manifest less aggressively towards their neighbours, a phenomenon called "Dear Enemy Effect" (DEE) or Fisher Phenomenon - Dear Enemy (Temeles, 1994).

Birds occupying closed habitats communicate primarily through calls. In these individuals, the capacity to differentiate the acoustic signals emitted by conspecifics has major implications for the recognition and discrimination of foreigners and neighbours, a phenomenon called "neighbour-stranger discrimination" (NSD). The phenomenon was described in birds species (Stoddard, 1996) as well as other taxonomic groups such as insects (Gordon 1989, Thomas *et al*., 1999), amphibians (Bee and Gerhardt, 2001), fish (McGregor and Westby, 1992), reptiles (Whiting, 1999), and mammals (Rosell and Bjørkøyli, 2002).

NSD and DEE manifestation are greatly beneficial for the individuals in the sense that by reducing aggressiveness, individuals can afford more time to other activities (feeding, courtship) while minimizing the risk of injuries resulting from direct confrontations (Akçay *et al*., 2009).

Meanwhile, there are some costs of reduced aggressiveness, which may lead to giving up the opportunity to enlarge the territory or even losing territory to a neighbour who manifests aggressiveness (Akçay *et al*., 2009). In this regard it would be more effective for the DEE to be conditioned by the level of aggression manifested by neighbours. Thus, an individual may manifest aggressiveness towards the aggressive neighbour or not (Akçay *et al*., 2009).

Therefore, recognising neighbours and reducing aggressiveness towards them decreases energy consumption in the individual and improves its chances of survival. Generally, neighbours defend their own territory, which they already occupied, and are not a danger to others, whereas an intruder will try to gain new territory, at the expense of those who already occupy it (Stoddard, 1996).

Referring to NSD, studies have been conducted on 27 species of Passeriformes, with a single case showing that individuals react more aggressively to the song of intruders than that of neighbours (Falls, 1982; Lambrechts and Dhondt, 1995; Stoddard, 1996; Lovell and Lein, 2004).

Meanwhile, studies on a species of Ralid (*Gallirallus philippensis*) (Lachish and Goldizen, 2004) argue that the lack NSD and DEE is due to territorial instability that characterizes this species, a phenomenon which is also common in Strawberry poison Frog (*Dendrobates pumilio)* (Bee, 2003) and is explained from the same perspective.

On the other hand, recent studies conducted on a species of the family Tyrannidae (*Empidonax alnorum*), demonstrates that they recognize and react less aggressively to the song of neighbours (Lovell and Lein, 2004)

The Water Rail (*Rallus aquaticus*) is a species that expresses strong aggressiveness, both interspecific and conspecific, not only during establishing territory, courtship and egg incubation but also during rearing, thus including the entire

breeding season, of over five calendar months (Ripley, 1977; Taylor, 1998; Ciach, 2007). Because it is a species that strongly expresses that behaviour, its energy consumption is high. In this case, neighbour recognition (NSD) and lowering aggression towards them (DEE) would be an evolutionary attribute that would reduce consumption of energy, increasing the individual's chances of survival.

In this context our study aims are (i) to highlight if there is a difference in the Water Rail's reaction to the calls of neighbours or strangers, to conclude if they express the NSD and (ii) to analyse the intensity of these reactions to determine the degree of aggressiveness manifested towards neighbours and intruders in term to emphasize if they exhibit or not the DEE behaviour.

Materials and methods

The experiments were carried out at the Sic Reedbed, a 252.68 ha wetland which is 98% covered by the reed *Phragmites australis* (David, 2008). Located in the Fizeş Basin, in the central part of the Transylvanian Plain in Romania (24°10' E; 46°50' N) the Sic Reedbed is the largest reedbed in Transylvania (Stermin *et al.*, 2012). The density of the vegetation is around 400 plant stems per square meter and the water level does not exceed 1.5 m, generally ranging between 20 and 50 cm (Stermin *et al.*, 2012), with a high Water Rail population density (1 pair/ha) (Stermin *et al.*, 2013).

Ten points, located 100 m apart, were chosen inside the Sic Reedbeds, in a transect that crosses the marsh area. This distance was considered large enough since sounds emitted in one point would not be audible in the neighbouring points. From our observations, the sounds used (playback) in this experiment were not audible over a distance of 30-40 m due to the noise barrier created by dry vegetation. A sound intensity similar to that of a natural song was used throughout the experiment. Because the width of the reeds in the area is approximately 450 m, it was more efficient to choose observation points inside the reeds, covering a radius of 360 degrees.

The experiments lasted for three days: 18, 19 and 20 April, in the first part of the breading season, when the territories were established and neighbours had already time for social interaction (Ripley, 1977; Taylor, 1998; Stermin *et al.*, 2012), between 6:00 – 8:00 p.m. the day time when the birds express a high call activity (Stermin *et al.*, 2013). The following study methodology was applied: in each point, we waited for a Water Rail's spontaneous reaction from inside a radius of 20 m from the point. From one minute after the moment the spontaneous reaction began, all acoustic reactions of its conspecifics were recorded, taking note of the number and time lapse between calls. After another minute, a playback of territorial calls of the species was played. Mean playback intensity and duration (20 seconds) coincided

with the intensity and duration of a natural song by individuals. From one minute after playback began, reactions by surrounding Water Rails as well as their duration were noted.

The principle behind the methodology was based on the fact that the number of reactions by surrounding birds after spontaneous activity of an individual is characteristic aggressiveness towards neighbours who are already known, and the number of reactions after playback would characterize aggression shown to outsiders. The data were interpreted by applying ANOVA.

Results and discussion

A statistically significant difference was found between the number of songs recorded after spontaneous activity and the number of songs recorded after playback ($F\ (1,62) = 14.400$, $p = 0.00034$) (Fig. 1).

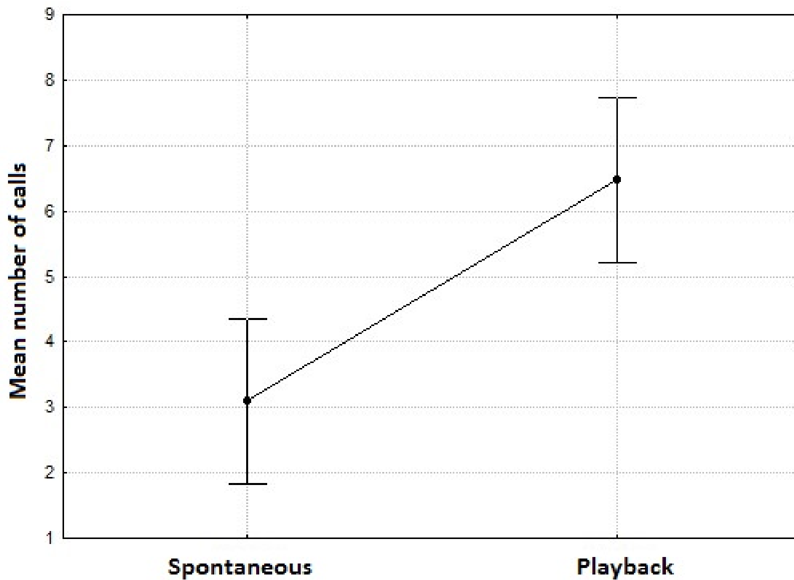

Figure 1. Mean calls numbers after spontaneous and playback activity.

Regarding time distribution, for the spontaneous activity during the one minute experiment, songs were grouped for the first half (the first 30 seconds) and second half (last 30 seconds). In this respect no statistically significant difference was found ($F\ (1,62) = 0.176$, $p = 0.676$) (Fig. 2).

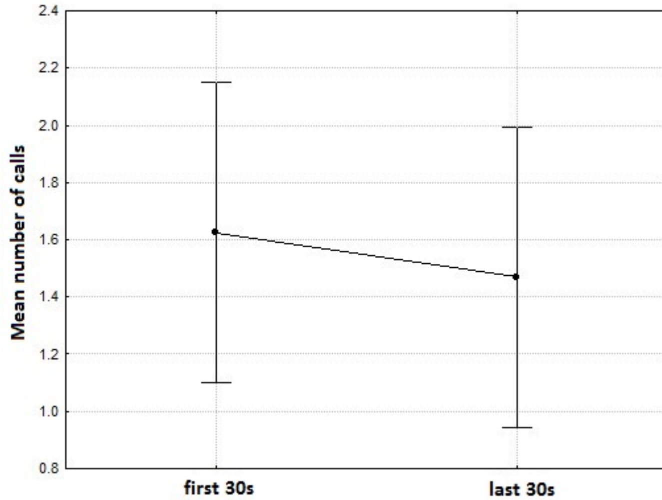

Figure 2. Mean calls distribution after spontaneous and playback reaction, by time of emission (first and second 30 s intervals)

Regarding the reactions recorded after playback, a statistically significant difference was noted between the mean number of songs issued during the first and the last half minute which followed the playback ($F(1,62)$ = 4.089, p = 0.0474) (Fig. 3).

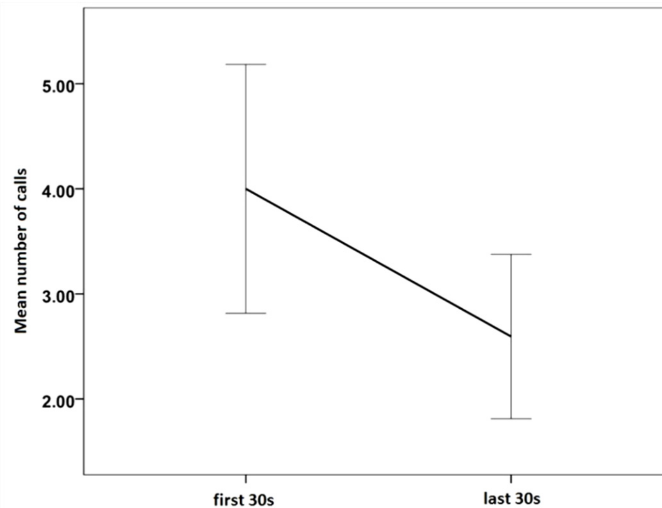

Figure 3. Distribution of mean calls number emitted after playback, by time of emission (first or last 30 s intervals)

As far as the difference between the average number of songs issued during the two time periods (first and last 30 seconds) in either approach (spontaneous reaction and playback), a statistically significant difference has been found ($F(3,124) = 8.437$; $p = 0.00004$). Analyzing the differences between mean number of songs issued during each period within each method (spontaneous and playback), it became clear that the mean number of reactions to playback during each period is significantly higher than the mean number of reactions to spontaneous activity (Table 1, Fig. 4).

Table 1.

Fisher LSD test results regarding the differences between mean calls number emitted after spontaneous reaction and playback during each time period (I – first 30 s, II – last 30 s) (Significant results ($p < 0.05$) are in bold).

	Spontaneous I	Playback I	Spontaneous II	Playback II
Spontaneous I		**0.000056**	0.781290	0.068694
Playback I	**0.000056**		**0.000019**	**0.021030**
Spontaneous II	0.781290	**0.000019**		**0.036463**
Playback II	0.068694	**0.021030**	**0.036463**	

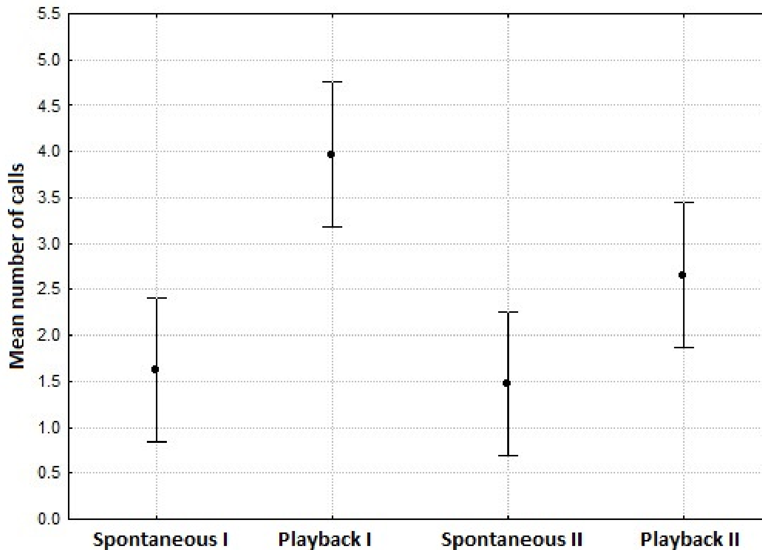

Figure 4. Distribution of mean calls number emitted after spontaneous reaction and playback, during each time period (I – first 30 s, II – last 30 s)

According to the results, the bird's reactions to playback were almost double in intensity at compared to the reaction to spontaneous calls of indigenous bird. This case argues the hypothesis that individuals of this species recognize their neighbours and react less aggressively towards them.

Also, the response to neighbours' songs follows a constant trajectory, with no differences between the numbers of responses issued during the first or second half of the minute. Instead, the reaction towards foreigners is very strong at the beginning, significantly losing intensity during the second half of the minute, towards the point of matching the intensity manifested towards neighbours.

This behaviour leads to minimizing the energy consumed by acts of aggression. By tolerating neighbours, individuals spend more time and energy with courting or caring for offspring (Ydenberg *et al.,* 1988; Temeles, 1994). One hypothesis argued by Temeles (1994) postulates that some species are more aggressive towards strangers because they may lose both their territory and partner, while with neighbours, only the partner can be lost since the neighbours already have well established territories.

However, the tolerance shown towards neighbours in other species (*Thryothorus ludovicianus*) (Hyman, 2002, 2005) is explained by pair monogamy and the lack of "extra-pair copulations" EPCs (phenomenon in which one partner mates with other individuals within a breeding season) (Haggerty *et al*., 2001), while in cases where EPC is present, DEE is not manifested, individuals reacting very aggressively towards neighbours in order to prevent them mating with their partners (Akçay *et al*., 2009).

No genetic studies have been conducted on the Water Rail to support or refute this species' monogamy, but the lack of aggression towards neighbours may support the hypothesis of genetic monogamy.

Conclusions

Water Rail can differentiate between neighbours and strangers expressing NSD behaviour and also DEE reacting less aggressively towards calls that are familiar than to new calls. The main reason may be energy saving in the context that this species frequently manifests aggressive territorial behaviour and often with high intensity.

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Screening for phenotypic and genotypic resistance to antibiotics in Gram positive pathogens

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SUMMARY. Gram positive bacteria such as methicillin resistant *Staphylococcus aureus*, vancomycin resistant enterococci or multidrug resistant *Streptococcus* spp. are increasingly involved in severe infections with serious clinical consequences. The aim of this study is to investigate phenotypic and genotypic resistance traits in Gram positive pathogens isolated from clinical specimens in Cluj-Napoca, Romania. A total number of 31 *Enterococcus* spp., *Staphylococcus* spp. and *Streptococcus* spp. strains were subjected to antimicrobial susceptibility testing by disc diffusion, while the carriage of 26 antibiotic resistance genes and of class 1 integron was assessed by PCR. Bacterial pathogens included in this study were mostly susceptible to folate pathway inhibitors (100%), oxazolidinones (97%), fosfomycins (93%) and glycopeptides (92%). Enterococci, staphylococci and streptococci displayed high levels of phenotypic resistance to penicillins, tetracyclines and macrolides, a percentage of 42% being multidrug resistant. The strains under this study proved to be able to produce β-lactamase enzymes encoded by the *TEM-1* gene and aminoglycoside modifying enzymes due to the carriage of *aac(6')-Ie-aph(2")* gene, to possess ribosomal protection mechanisms for macrolide and tetracycline resistance associated with *ermB*, *ermC* and *tet(M)* genes and to bear efflux genes *tet(A)*, *tet(B)*, *tet(C)* ant *tet(L)*. Class 1 integron integrase was detected in 16% of the isolates, but no significant correlations were found between the carriage of *intI1* gene and the phenotypic or genotypic resistance among the Gram positive pathogens investigated.

Keywords: AMR, MDR, ARG, *Enterococcus* spp., *Staphylococcus* spp., *Streptococcus* spp.

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Introduction

Gram positive bacteria are common causes of severe infections, methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE) or multidrug resistant (MDR) *Streptococcus* spp. being of particular concern. The percentage of severe nosocomial infections such as bloodstream infections caused by antimicrobial resistant (AMR) Gram positive bacteria is increasing (Rice, 2006; Attman *et al*., 2015). For instance, penicillin and macrolide resistance is associated with a higher mortality rate in cases of pneumococcal community acquired pneumonia and bacteraemia; infections due to MRSA and VRE are also associated with higher mortality rates, prolonged length of hospital stay, and increased costs (Lode, 2009; Flores-Mireles *et al*., 2015). As a whole, MDR Gram positive pathogens are rapidly becoming an urgent and sometimes unmanageable clinical problem, not only in pneumonology, oncology and urology wards, but also in intensive-care units (Cornaglia, 2009). The serious clinical consequences of AMR among Gram positive pathogens emphasize the importance of efforts to limit their emergence and spread. Susceptibility data can be used by healthcare providers to make rational choices about the use of antimicrobial agents. Furthermore, susceptibility data can guide policy-makers to track and prevent the spread of antimicrobial resistant organisms. Finally, awareness on bacterial resistance traits may stimulate interest in developing new antimicrobial agents and therapies (Lode, 2009).

Previous epidemiological surveys in Romania have focused on the prevalence of Gram positive bacterial infections without molecular screening (Simon *et al*., 2010; Stoian *et al*., 2013; Pîrvănescu *et al*., 2014), which was performed often in Gram negative pathogens (Crăciunaş *et al*., 2010; Flonta *et al*., 2011, Székely *et al*., 2013).

This study investigates antimicrobial susceptibility in Gram positive pathogens isolated from clinical specimens in Cluj-Napoca, Romania. Furthermore, *Enterococcus* spp., *Staphylococcus* spp. and *Streptococcus* spp. isolates displaying phenotypic AMR were screened for multiple mechanisms of genetic resistance. Enzymes involved in antibiotic modification (β-lactamases, aminoglycoside modifying enzymes, alteration of antibiotic target site (peptidoglycan precursors conferring resistance to glycopeptides), protecting proteins (for the DNA- enzyme complexes from the action of quinolones), ribosomal protection mechanisms (for macrolide and tetracycline resistance), efflux proteins (for tetracycline resistance) have been investigated by the PCR screening for 26 antibiotic resistance genes (ARGs). Also, the association of phenotypic and genotypic resistance with the carriage of class 1 integron was assessed in all clinical isolates.

Materials and methods

Bacterial isolates

Clinical specimens of urine and various secretions were collected in a private laboratory from Cluj-Napoca during January-March 2016. After the inoculation of samples into specific culture media including Blood agar, Chapman agar and Bile

Aesculin agar, incubated at 37 °C overnight, presumptive colonies were purified and recultured. The isolates were identified according to standard methods by colony appearance, pigment production, catalase test, oxidase test, Gram staining. Latex agglutination kits (Oxoid) were used for additional serological confirmation.

Antimicrobial susceptibility testing

Enterococcus spp., *Staphylococcus* spp. and *Streptococcus* spp. strains isolated from clinical samples were subjected to antimicrobial susceptibility testing as per the reference guidelines (EUCAST, 2014) against the following antibiotic classes: penicillins, cephems (except for enterococci), glycopeptides, lipopeptides, florquinolones, aminoglycosides, macrolides (except for enterococci), tetracyclines, folate pathway inhibitors (only for staphylococci), oxazolidinones and fosfomycins (only for enterococci). *Enterococcus* spp. were tested for AMR against 9 individual drugs: penicillin, ampicillin, vancomycin, ciprofloxacin, levofloxacin, gentamicin, tetracycline, linezolid and fosfomycin. *Staphylococcus* spp. were tested for AMR against 12 individual drugs: penicillin, cefoxitin, daptomycin, ciprofloxacin, levofloxacin, gentamicin, erythromycin, clarithromycin, tetracycline, trimethoprim-sulfamethoxazole, linezolid and fosfomycin. *Streptoococcus* spp. were tested for AMR against 10 individual drugs: penicillin, ampicillin, cefoxitin, ceftriaxone, cefepime, vancomycin, daptomycin, levofloxacin, erythromycin and linezolid. Antibiotic susceptibility testing was performed by disk diffusion in Mueller-Hinton agar plates (Farkas, 2016). Following overnight incubation, the inhibition zone diameters were measured and the results were interpreted according to CLSI guidelines (CLSI, 2015).

ARG screening

Gram positive pathogens displaying phenotypic resistance to at least one antibiotic were further included in the screening of ARGs conferring resistance to the corresponding antimicrobials. In addition, carriage of class I integron integrase gene was screened in all isolates. PCR amplifications were performed in 25 µl reaction mix containing: 12.5 µl DreamTaq Green PCR master mix (2x), 10.25 µl nuclease-free water, 0.125 µl each primer to a final concentration of 0.5 µM, and 2 µl bacterial suspension adjusted to a 0.5 McFarland standard. The following reaction conditions were set up using a thermocycler TProfessional Trio (Analytik Jena, Germany). After the initial denaturation at 94°C for 5 min, the cycle for denaturation, annealing and synthesis was 35-fold repeated and followed by a final elongation at 72°C for 5 min (Table 1). Amplified PCR products were separated in 1.5% agarose gel in 1 x TBE buffer and stained with ethidium bromide 0.5 µg/ml. Data acquisition and analysis were performed using the BDA Digital Compact System and BioDocAnalyze Software (Analytik Jena, Germany). PCR primers and molecular reagents were purchased from Cleaver Scientific, Eurogentec, Lonza and Thermo Fisher Scientific.

Table 1.

Primers and PCR conditions used in this study

Antibiotic class	Target gene	Primers sequence F/R (5' – 3')	Amplicon size (bp)	Annealing temperature and time
Penicillins	*TEM-1*	GGTCGCCGCATACACTATTC/ ATACGGGAGGGCTTACCATC	500	57°C 45 s
	TEM-2	AAGTAAAAGATGCTGAAGATAAGTTGG/ GATCTGTCTATTTCGTTCATCCATAG	737	61°C 45 s
	SHV-1	GCGTTATATTCGCCTGTGTATTAT/ GCCTGTTATCGCTCATGGTAATG	385	61°C 45 s
Cephems	*AmpC*	AGAAGGACCAGGCACAGATC/ CTCGGCATTGGGATAGTTGC	455	57°C 45 s
Glycopeptides	*vanA*	GCTATTCAGCTGTACT/ CAGCGGCCATCATACGG	781	51°C 45 s
	vanB	CGCCATATTCTCCCCGGATAG/ AAGCCCTCTGCATCCAAGCAC	600	61°C 45 s
Florquinolones	*qnrA*	AGTTTGATGGTTGCCGCTTT/ TCTTCATTGATCTGCACGCC	541	53°C 45 s
	qnrB	TCGTGCGATGCTGAAAGATG/ CCGAATTGGTCAGATCGCAA	368	55°C 45 s
	qnrS	TGATCTCACCTTCACCGCTT/ GAGTTCGGCGTGGCATAAAT	496	55°C 45 s
Aminoglycosides	*aac(3')-I*	ACCTACTCCCAACATCAGCC/ TCTTCCCGTATGCCCAACTT	329	55°C 45 s
	aac(3)-IIIa	GCATGCCTCACTTAAAGCGA/ ACCGTTTCTTCCAAGCATCG	514	55°C 45 s
	aac(6')-Im	GGCTGACAGATGACCGTGTTCTTG/ GTAGATATTGGCATACTACTCTGC	482	61°C 45 s
	aac(6')-Ie-aph(2")	CCAAGAGCAATAAGGGCATA/ CACTATCATAACCACTACCG	400	53°C 45 s
	aac(6')-II	AGCGACCGACTCTTGATGAA/ GGCTTGTCGTGTTTGAACC	414	53°C 45 s
	aph(2)-Ib	CTGAACACAGCAGCGACTAC/ TTGTAATCGCCATGCACCAG	646	55°C 45 s
	ant(4')-Ia	GTCAAAAACTGCTAACACAAG/ AATAATACTGCTAACGATAAT	135	53°C 30 s

Table 1. continued

Macrolides	*ermA*	GAACCAGAAAAACCCTAAAGACAC/ ACAGAGTCTACACTTGGCTTAGGATG	513	61°C 45 s
	ermB	GAAAAGGTACTCAACCAAATA/ AGTAACGGTACTTAAATTGTTTAC	639	51°C 45 s
	ermC	CGTAACTGCCATTGAAATAGACC/ GTGAGCTATTCACTTTAGGTTTAGG	356	61°C 45 s
	mefA	CATCGACGTATTGGGTGCTG/ CCGAAAGCCCCATTATTGCA	516	55°C 45 s
Tetracyclines	*tet(A)*	GCAAGCAGGACCATAATCGG/ GCCGATATCACAGATGGGGA	572	57°C 45 s
	tet(B)	GGTTAGGGGCAAGTTTTGGG/ ATCCCACCACCAGCCAATAA	541	57°C 45 s
	tet(C)	TGAGATCTCGGGAAAAGCGT/ AAAGCCGCGGTAAATAGCAA	460	53°C 45 s
	tet(K)	AGGATCTGCTGCATTCCCTT/ AGCAAACTCATTCCAGAAGCA	822	53°C 45 s
	tet(L)	TATTCAAGGGGCTGGTGCAG/ CGGCAGTACTTAGCTGGTGA	545	57°C 45 s
	tet(M)	CCGTCTGAACTTTGCGGAAA/ CAACGGAAGCGGTGATACAG	627	55°C 45 s
Class 1 integron integrase	*intI1*	CGTGCCGTGATCGAAATCCAG/ TTCGTGCCTTCATCCGTTTCC	371	60°C 45 s

Statistical analysis

Frequencies and proportions of AMR isolates, the abundances of MDR, ARGs and *intI1* bearing strains were calculated. MDR was defined as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al*., 2012).

Inferential statistics by co-dependency was analysed to verifiy whether occurrences of phenotypic and genotypic resistance were correlated with *int1I* carriage. Two-sided 5% significance levels were used to identify statistically significant results. Statistical analyses were performed using the Real Statistics Resource Pack software for Microsoft Excel (Zaiontz, 2015), with a significance level of p = 0.05.

Results and discussion

Bacterial isolates

A total number of 31 Gram positive pathogens were isolated in this study and selected for AMR and ARG screening. *Enterococcus* spp. (11 isolates) were isolated from urinary tract infections (nine strains) and from secretions (two strains). *Staphylococcus* spp. (17 isolates) obtained from urocultures (three strains) and from secretions (14 strains) were identified as *S. aureus* (16 isolates) and *S. saprophyticus* (one isolate). The three isolates of *Streptococcus* spp. were obtained from urinary tract infectionss (two strains) and from secretions (one strain), being confirmed as group B *Streptococcus*, group B β-hemolytic *Streptococcus* and group C *Streptococcus*.

Antimicrobial susceptibility

Gram positive pathogens included in this study were mostly susceptible to folate pathway inhibitors (100%), oxazolidinones (97%), fosfomycins (93%) and glycopeptides (92%) (Table 2). *Enterococcus* spp., *Staphylococcus* spp. and *Streptococcus* spp. isolates displayed phenotypic resistance from one to six individual drugs, belonging to up to five antimicrobial categories (Fig. 1). High levels of resistance were observed for penicillins (80%), tetracyclines (72%) and macrolides (58%). A percentage of 42% clinical isolates were multidrug resistant (Fig. 2), MDR etiology being the following: two out of three strains of *Streptococcus* spp., seven out of 17 *Staphylococcus* spp. and four out of ten *Enterococcus* spp. Seven out of 17 *S. aureus* were MRSA while only one out of ten *Enterococcus* spp. were VRE.

Table 2.

Antimicrobial susceptibility of Gram positive isolates

Antibiotic category	**Susceptible isolates**			
	Enterococci (%)	**Staphylococci (%)**	**Streptococci (%)**	**Total (%)**
Penicillins	5 (45)	15 (88)	0 (0)	6 (19)
Cephalosporins	-	12 (71)	3 (100)	15 (75)
Glycopeptides	13 (93)	-	3 (100)	12 (92)
Lipopeptides	-	8 (50)	1 (33)	11 (58)
Florquinolones	8 (80)	15 (94)	2 (67)	26 (87)
Aminoglycosides	9 (82)	15 (94)	-	25 (89)
Macrolides	-	6 (38)	1 (33)	5 (26)

Table 2. continued

Tetracyclines	1 (9)	4 (57)	-	5 (28)
Folate pathway inhibitors	-	17 (100)	-	17 (100)
Oxazolidinones	10 (91)	5 (100)	3 (100)	28 (97)
Fosfomycins	10 (91)	3 (100)	-	13 (93)

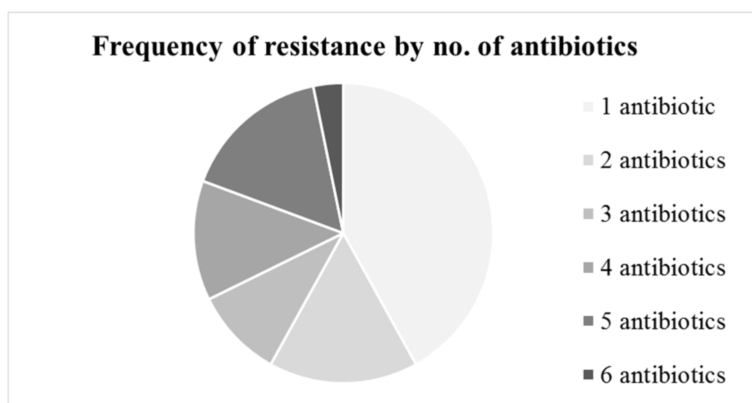

Figure 1. Percentage of strains exhibiting antimicrobial resistance

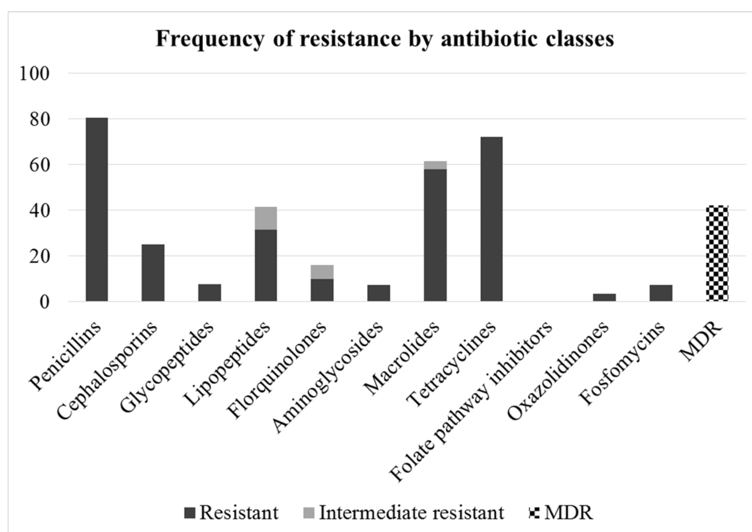

Figure 2. Percentage of resistant, intermediate resistant and MDR strains

ARG screening

Of 25 Gram positive pathogens displaying resistance to penicillins, six (24%) carried the *TEM-1* β-lactamase, while the *TEM-2*, *SHV-1* and *AmpC* genes were not identified in this study. Although *TEM-1* is the most common plasmid-mediated β-lactamase of resistant enteric Gram negative bacilli (Paterson and Bonomo, 2005), this gene was present in four *Enterococcus* spp. isolates and in two *Staphylococcus aureus* strains, one being methicillin resistant (Table 3). *TEM-1* is able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended-spectrum cephalosporins. It is inhibited by clavulanic acid (Paterson and Bonomo, 2005).

Aac(6')-Ie-aph(2"), a gene encoding aminoglycoside modifying enzymes was detected in two strains phenotypically resistant to gentamycin (100%), both *Enterococcus* spp. (Table 3). The genes *aac(3')-I*, *aac(3)-IIIa*, *aac(6')-Im*, *aac(6')-II*, *aph(2)-Ib*, *ant(4')-Ia* were not present. The bifunctional *aac(6')-aph(2")* gene, resulted from the fusion of two aminoglycoside modifying enzyme genes, confers high-level resistance to all clinically useful aminoglycosides except streptomycin. The genes responsible for high-level aminoglycoside resistance have in most cases been identified on plasmids and on transposons (Behnood *et al.*, 2013).

Resistance to macrolides was found to be mediated by ribosomal methylation associated with *ermB* gene in *Streptococcus* spp. and with *ermC* gene in MRSA (Table 3), while the *ermA* gene was not present. Methylation of the ribosomal drug binding site, which confers resistance to macrolides, lincosamides, and streptogramin group B (MLSB) may be inducibly or constitutively expressed (Leclercq, 2002). Previously, the most prevalent mechanism of macrolide resistance was also associated with *ermB* gene in *Streptococcus pneumoniae* (Kresken *et al.*, 2004; Reinert *et al.*, 2008). Drug efflux conferring low-level resistance to erythromycin but not clindamycin, encoded by the *mefA* gene, was not detected in this study.

Tetracycline resistance was found to be encoded by *tet(A)* (8%), *tet(B)* (15%), *tet(C)* (23%), *tet(L)* (23%) and *tet(M)* (69%) (Table 3). Of three *Staphylococcus aureus* isolates resistant to tetracycline, one MRSA strain was positive for both *tet(B)* and *tet(C)* genes. Of ten *Enterococcus* spp. isolates displaying phenotypic resistance, one carried *tet(A)* and *tet(B)* genes, two carried *tet(C)*, three carried *tet(L)* and nine carried *tet(M)* genes. *Tet(K)* was not found among the Gram positive pathogens under this study. Many tetracycline efflux pumps, such as *tet(A)*, *tet(B)* and *tet(C)* genes have been described as specific for Gram negative bacteria. These proteins share similarities with other efflux proteins involved in multiple drug resistance, quaternary ammonium resistance, together with chloramphenicol and quinolone resistance. The *tet(K)* and *tet(L)* genes are generally found on small transmissible plasmids in Gram-positive bacteria (Roberts and Schwartz, 2015). The *tet(L)* gene has often been linked to the trimethoprim resistance gene *dfrK* (Roberts, 2012). The tetracycline ribosomal

protection protein *Tet(M)* confers resistance by catalyzing the release of tetracycline from the ribosomes and also by directly interacting and altering the conformation of the tetracycline binding site. It is tough to be of Gram positive origin, where a mosaic of *tet* genes but also a combination of *tet(M)* and *erm(B)* was identified (Roberts and Schwartz, 2015), being often found in clinical strains of *Enterococcus* spp. and *Staphylococcus* spp. (Schmitz *et al*., 2001; Anderson *et al*., 2017).

The only one VRE isolate was not positive for *vanA* or *vanB* genes. Neither the quinolone resistance mechanisms encoded by *qnrA*, *qnrB* and *qnrS* genes were detected in the three Gram positive pathogens phenotypically resistant to quinolones.

All the Gram positive pathogens were screened for class 1 integron integrase *intI1*, which was found in five out of 31 isolates.

Table 3.

Antimicrobial resistance mechanisms of Gram positive isolates

Antibiotic category	**Genetic mechanism of resistance**	**Prevalence of ARGs**			
		Enterococci (%)	**Staphylococci (%)**	**Streptococci (%)**	**Total (%)**
Penicillins	*TEM-1*	4 (67)	2 (13)	0 (0)	6 (24)
Aminoglycosides	*aac(6')-Ie-aph(2")*	2 (100)	-	-	2 (100)
Macrolides	*ermB*	-	0 (0)	2 (100)	2 (20)
	ermC	-	1 (13)	0 (0)	1 (10)
Tetracyclines	*tet(A)*	1 (10)	0 (0)	-	1 (8)
	tet(B)	1 (10)	1 (33)	-	2 (15)
	tet(C)	2 (20)	1 (33)	-	3 (23)
	tet(L)	3 (30)	0 (0)		3 (23)
	tet(M)	9 (90)	0 (0)	-	9 (70)
Class 1 integron	*intI1*	1 (9)	3 (17)	1 (33)	5 (16)

No significant correlations were found between the carriage of class 1 integron integrase AMR, MDR or ARG detection in Gram positive pathogens isolated from clinical samples under this study.

Conclusions

This study emphasizes the high-level of antimicrobial resistance, multidrug resistance and different mechanisms of genetic resistance among the Gram positive pathogens. *Enterococcus* spp., *Staphylococcus* spp. and *Streptococcus* spp. isolates

displayed high levels of phenotypic resistance to penicillins, tetracyclines and macrolides. A percentage of 42% clinical isolates were MDR strains. Gram positive pathogens are able to produce β-lactamase enzymes encoded by the *TEM-1* gene, aminoglycoside modifying enzymes due to the carriage of *aac(6')-Ie-aph(2")* gene, possess ribosomal protection mechanisms for macrolide and tetracycline resistance associated with *ermB, ermC* and *tet(M)* genes and bear efflux genes *tet(A)*, *tet(B)*, *tet(C)* ant *tet(L)*. No significant correlations were found between the carriage of class 1 integron integrase AMR, MDR or ARG detection in Gram positive pathogens isolated from clinical samples under this study. Class 1 integron integrase gene was detected in 16% of the isolates, but no significant correlations were found between the carriage of *intI1* and the phenotypic or genotypic resistance among the Gram positive pathogens investigated.

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Plant growth response and nitrate reductase activities of roots of *Chromolaena odorata* in a model spent lubricating oil-polluted soil

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SUMMARY. The ability of *Chromolaena odorata* propagated by stem cuttings and grown for 50 days in the soil containing five (5) different concentrations of spent lubricating oil (SLO) in soil (0, 1, 3, 6, 9 and 12 % SLO), was investigated. The experiments were watered daily at 70% moisture field capacity. Parameters such as number leaves per plant, shoot length, plant height as well as nitrate reductase activities were measured. Shoot length as well as leaf number were significantly ($p < 0.05$) reduced, compared to the control treatment. Results also showed that nitrate reductase activities increased slightly with time. However, beyond 40 days, nitrate reductase activity was not detected in 3% - 12% w/w oil-in-soil treatments, respectively. Pollution indices such as Contamination factor and Hazard Quotient, used in the present study indicated significant reduction in contamination values upon sowing of *C. Odorata*. Values obtained from Bioaccumulation Quotients also indicated that the plant was able to significantly bioaccumulate elements such as Fe, Cu and Ni present in the SLO-polluted soil.

Keywords: bioaccumulation, *Chromolaena odorata*, enzymes, nitrate assimilation, nitrate reductase, phytoremediation.

Introduction

In a farming ecosystem, the soil is the furthermost prized component. However, environmental sustainability is principally subject to appropriate soil maintenance. Sustainable usage of this natural resource is unquestionably obligatory for better agricultural productivity. Soil pollution by crude oil, petroleum products,

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or other waste petroleum materials is a major ecological concern in many third world countries, including Nigeria. The disposal of spent engine oil in the big cities has been persistently problematic since many automobile mechanics dispose these oils indiscriminately either in gutters or open lands. Nearby farms are affected directly or indirectly from run-offs during rainfall, when these oil materials are deposited therein. This practice adversely affects plants, soil microbes and other soil fauna (Adenipekun *et al.*, 2008).

Oil pollution in whatever form is toxic to plants and soil microorganisms (Adedokun and Ataga, 2007). In an era when most people clamour for organic foods, restoration of oil-polluted soils for purposes of farming may not necessarily be possible through the use of conventional methods like application chemical treatments or even the use of physical methods which may not remove the oil completely. A more cost-effective but slow method, known as phytoremediation, is currently making waves in environmental biorestoration science.

It is however pertinent to note that the selection of phytoremediation methods is usually preceded by a knowledge of the plant's physiology or growth response as well as phytoaccumulation capacities when exposed to the contaminant. One of such physiological response is its nitrate assimilatory capacity; a very important factor considering the role nitrogen plays in plant's vegetative development. Nitrate reductase activity is one of several enzymatic systems that have significant correlation with nitrate assimilation.

The nitrate assimilatory pathway is facilitated by two enzymes, nitrate reductase (EC 1.6.6.2) and nitrite reductase (EC 1.7.7.1). These enzymes catalyze the stepwise conversion of nitrate to nitrite (nitrate reductase), and nitrite to ammonia (nitrite reductase). Before they can be incorporated into amino acids, nitrate absorbed by plants must first be reduced to ammonium (Fan *et al.,* 2002). The first step in the conversion of nitrate is catalyzed by nitrate reductase (NR). Lexa *et al.* (2002) described this substrate-inducible plant enzyme as essential in nitrogen assimilation. Activity of this enzyme is considered to be a limiting factor for growth and protein production in plants (Jackson *et al.*, 2008).

As many scientists use a plant's capacity to assimilate nitrate nitrogen as one of several indicators of changes in the environment, NR activity measurements becomes a much more reliable choice (Mahan *et al.,* 1998; Ghoulam *et al.,* 2002). These authors have previously reported NR activity inhibition due to plant's exposure to metals such as copper and salinity. Ghoulam *et al.* (2002), Mahan *et al.,* 1998) also reported significant decreases in NR activity due to drought. In the present study therefore, phytoremediative capacities of the test plant, *Chromolaena odorata* is measured against NR activity for selected periods. The test plant was chosen because of previous reports of its capacity to survive oil contamination (Anoliefo *et al.*, 2003, 2006).

Materials and methods

Garden soil was collected from an area measuring 10 m x 10 m marked on a land. Care was taken to ensure that soil was obtained from an area that had never been impacted with petroleum oil spill. Soil was analysed for selected chemical parameters before use (Table 1). Thereafter, 10 kg soil each was placed in large buckets. Spent lubricating oil (SLO) was obtained as pooled sample from an auto-mechanic workshop.

The emphasis on the study was the test plant's capacity for heavy metal remediation in a polymetallic soil. The polymetallic soil environment in this study was created by contaminating soil with spent lubrication oil. The choice is based on the report of Whisman *et al.* (1974); Ikhajiagbe and Anoliefo (2012) that most heavy metals such as V, Pb, Mn, Cd, Cr, Ni, and Fe were present in high quantities in spent lube. The soils in each bucket were then mixed thoroughly with SLO in 5 different levels: 0, 1, 3, 6, 9 and 12 % w/w. The 100 g SLO measured 135.2 ml. The control was not contaminated with SLO. Treatments and control existed in replicates of 5. The entire set up was left in a well-ventilated screen house for 1 month to attenuate, without mechanically disturbing the soil.

Afterwards, fresh stem cuttings of *Chromolaena odorata* (girth, 1.89±0.42 cm; length 30.00±0.00 cm) were obtained for the study from a fallow area. These stem cuttings were obtained from midway through the plant axil. Care was taken to ensure that the stem was devoid of injury prior to use. Single stem-cuttings were sown per bucket at a depth of 10cm. the set up was observed for phytoremediative capabilities for 50 days as well as nitrate reductase activity. Total plant accumulated figures for HM were determined by atomic absorption spectrophotometry (model, Buck Scientific 210 VGP), according to the methods of SSSA (1971) and AOAC (2005). Nitrate reductase activity (NRA) of root sample was determined according to Stewart *et al.,* 1972) with slight modifications from Ajakaiye (1987) and Cerqueira *et al.* (2009). Means were separated by using the Least Significant Difference $p \leq 0.05$ significant level. The Superior Performance Software System (SPSS) (version 16.0 for windows) package was used for statistical analyses.

In order to compare remediative success of the test plant, residual metal contents were compared with standard benchmark (Efroymson *et al*., 1997a,b; Cal-EPA, 2005).

Contamination Factor (CF)

CF expresses the ratio of the eventual concentrations of pollutant and its pre-industrial concentration (Ikhajiagbe, 2010).

$$CF = \frac{\text{Concentration of pollutant at the specific date of concern}}{\text{Background/Pre-industrial Concentration, before pollution}}$$

If CF > 1, the implication is that the inherent contamination due to that particular pollutant is as a result of the amendment by the researcher.

Bioaccumulation Quotient (BQ)

BQ expresses the possibility of the contaminant being significantly accumulated in plant parts, thereby posing health threats (Ikhajiagbe and Anoliefo, 2012). The Bioaccumulation Quotient is expressed

$$BQ = \frac{\text{Concentration of accumulated pollutant in the accumulant}}{\text{Concentration of accumulated pollutant in Soil (Source)}}$$

When BQ > 1= Significant accumulation in of the pollutant is implied.
When BQ < 1= Bioaccumulation is not of significant effect.

Results and discussion

The chemical parameters of the garden soil used for the study has been presented on Table 1. As provided, when CF > 1, the implication is that the inherent contamination due to that particular pollutant is as a result of the amendment by the researcher. Contamination factor after 50 days of plant exposure to polymetallic oil-polluted soil showed higher than unit values for Mn and Cu in soils due to SLO pollution. The meaning is that remediation may not have been entire after 50 days exposure (Table 2). Heavy metal accumulation in whole *C. odorata* plant at 50 days after sowing has been presented on Table 3. Accumulations in SLO-polluted soils significantly differed from those in the control soil thus indicating the capacity of *C. odorata* to accumulate metals in polymetallic soils. Accumulated Mn ranged from 1.2 – 5.8 mg kg^{-1} per dry wt. of whole plant. Accumulation of Cd in *Chromolaena* plants was below detection in both polluted and control soils. Results showed significant accumulation of Cu in plant parts of *Chromolaena odorata* (Table 4). Significant bioaccumulation quotient values were also recorded for Fe and Ni in polluted soils. Significant accumulation in of the pollutant is implied when BQ > 1.

The toxic impact of spent lubricating oil on soil fauna and flora relies basically on its hydrocarbon and heavy metal composition. Although most heavy metals may not be necessarily present in unused lubricating oils, however after they have been used in motor vehicles and other heavy machines, under very high temperatures, heavy metals such as V, Pb, Al, Ni, and Fe, which were below detection in unused engine oil, eventually give high mg kg^{-1} values in used oil (Whisman *et al.,* 1974).

The occurrence of these heavy metals in soil, especially in high concentrations is very distressing, particularly in their relationships with plant growth. Not only these metals, but hydrocarbons which have, in previous studies shown significant phytotoxic effects, in most cases, leading to plant death (Ikhajiagbe, 2010). The bioavailability of these contaminants to plants in the polluted soils is the starting point to a resultant phytoxic impact on the growing plant population inn affected soil. Metals, whether absorbed in very low quantities or not, are capable of replacement of essential metals in pigments or enzymes, thereby unsettling their

function (Henry, 2000). However, plant resistance to these environmental pollutants has been demonstrated in previous studies (Wong and Chu, 1985; Vwioko and Fashemi, 2005). Plant tolerance to these contaminants is majorly a factor of its capability for selective absorption from soil solution by its roots. Contaminants, like metals may be bound to exterior exchange sites on the root and not actually taken up (Efroymson *et al.,* 1997a,b). In some other cases, when absorbed by plants, they are stored in bio-unavailable forms in harvestable plant parts (phytoextraction).

Table 1.

Chemical parameters of garden soil used in the study

Parameters	**Soil (per Dry wt.)**
Ph	5.62
Electric conductivity (µs cm^{-1})	237.24
Total organic carbon (%)	0.49
Total Nitrogen (%)	0.11
Exchangeable acidity (meq.$100^{-1}g^{-1}$)	0.23
Fe (mg kg^{-1})	212.32
Cu (mg kg^{-1})	2.90
Cd (mg kg^{-1})	ND
Mn (mg kg^{-1})	12.76
V (mg kg^{-1})	0.08
Pb (mg kg^{-1})	0.03
Ni (mg kg^{-1})	1.02
Total hydrocarbon content (mg kg^{-1})	191.02

Table 2.

Contamination factor for heavy metal composition of oil polluted soil 50 days after sowing *C. odorata.*

Treatments	Mn	Fe	Cu	Pb	V	Ni	Cd
	Background concentration (mg kg^{-1})*						
	12.76	212.32	2.90	0.03	0.08	1.02	ND
	Contamination factors (units)						
0%	0.92^{b}	0.96^{a}	0.74^{c}	0.00^{c}	0.00^{d}	0.00^{d}	0.00^{c}
1%	1.42^{b}**	0.06^{b}	0.47^{c}	0.14^{bc}	0.13^{cd}	0.06^{cd}	0.00^{c}
3%	1.90^{ab}**	0.10^{b}	0.78^{c}	0.22^{bc}	0.28^{bc}	0.07^{bcd}	0.00^{c}
6%	2.01^{a}**	0.12^{b}	1.42^{bc}**	0.39^{ab}	0.34^{b}	0.09^{abc}	0.05^{b}
9%	2.28^{a}**	0.14^{b}	3.30^{ab}**	0.53^{ab}	0.53^{a}	0.14^{abc}	0.08^{ab}
12%	2.81^{a}**	0.17^{b}	4.28^{a}**	0.66^{a}	0.63^{a}	0.16^{a}	0.12^{a}
LSD (0.05)	1.06	0.26	1.13	0.38	0.18	0.08	0.04

**Background concentration here refers to the natural concentration of the elements in the soil prior to contamination with SLO. (See Table 1)*

***If CF > 1, Contamination is due SLO application to soil.*

Means of the same column with similar alphabetic superscripts do not differ from each other (p>0.05)

Nitrate reductase activity (NRA) in developing *C. odorata* plants was reported in 20, 30, 40 and 50 days (Table 5). After 20 days, NRA was 0.311 $\mu Mhr^{-1}g^{-1}$ in the control, compared to 0.157 – 0.287 $\mu Mhr^{-1}g^{-1}$ in pants exposed to oil-polluted soil. This decrease in NRA is in accordance with increasing oil concentration in the soil. This was similar for the 30th, 40th and 50th days respectively. NRA increased with the age of plants. However, NRA was undetected for plants in 12% oil-in-soil treatments on the 50th day. This was probably because plants did not survive at 50 days.

Table 3.

Heavy metal accumulation in whole *C. odorata* plant at 50 days after sowing

Heavy metals	1%	3%	6%	9%	12%	Control	LSD (0.05)
	Metal conc. in soil (mg kg^{-1})						
Mn^{2+}	3.8^{a}	4.2^{a}	4.7^{a}	5.3^{a}	5.8^{a}	1.2^{b}	2.3
Fe^{3+}	164.2^{de}	191.4^{cd}	253.1^{bc}	299.0^{ab}	341.3^{a}	100.2^{e}	86.3
Pb^{2+}	ND^{c}	ND^{c}	0.049^{bc}	0.103^{ab}	0.185^{a}	ND^{c}	0.094
Cu^{2+}	1.54^{ab}	1.93^{ab}	2.30^{a}	2.78^{a}	2.24^{a}	0.63^{b}	1.38
V^{2+}	ND^{c}	0.06^{c}	0.14^{bc}	0.29^{ab}	0.40^{a}	ND^{c}	0.22
Ni^{2+}	ND^{c}	0.27^{c}	1.43^{b}	1.95^{ab}	2.65^{a}	ND^{c}	0.98
Cd^{2+}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.001

Means of the same row with similar alphabetic superscripts do not differ from each other ($p>0.05$). NA not available. Means of the same column with similar alphabetic superscripts do not differ from each other ($p>0.05$). ND not detected (<0.0001 mg kg^{-1})

Table 4.

Bioaccumulation quotient for heavy metals accumulated in whole *C. odorata* plant at 50 days after sowing

Heavy metals	Cu	Pb	Cd	Fe	Ni
		Benchmark* (mg kg^{-1})			
	0.20	5.00	0.01	5.00	0.20
		Bioaccumulation quotient			
0%	3.15^{b}**	0.00^{b}	0.00^{a}	4.80^{d}**	0.00^{c}
1%	7.70^{ab}**	0.00^{b}	0.00^{a}	32.80^{c}**	0.00^{c}
3%	9.65^{ab}**	0.00^{b}	0.00^{a}	38.20^{bc}**	1.35^{c}**
6%	11.50^{a}**	0.01^{ab}	0.00^{a}	50.80^{ab}**	7.15^{b}**
9%	11.20^{a}**	0.02^{ab}	0.00^{a}	59.80^{ab}**	9.75^{ab}**
12%	13.90^{a}**	0.04^{a}	0.00^{a}	68.20^{a}**	13.25^{a}**
LSD (0.05)	6.92	0.03	0.001	22.64	5.35

**Benchmark given is FAO recommended Maximum concentration of trace elements for crops (FAO, 1985).*
*** Significant accumulation in of the pollutant is implied.*
Means of the same column with similar alphabetic superscripts do not differ from each other ($p>0.05$)

Plant survival under conditions of stress relies greatly on its nitrogen assimilation capacities. One of numerous biochemical explanations on improved nitrogen utilization by plants is dependent on an understanding of its nitrate reductase (NR) activities. NRs, which are molybdoenzymes, reduce nitrate to nitrite (Solomonson *et al.,* 1990). This reduction reaction is critical for the assembly of protein in most plants, as nitrate is the predominant source of nitrogen in fertilized soils (Marschner and Petra, 2012). The nitrate uptake system in plants must be versatile and robust because plants have to transport sufficient nitrate to satisfy total demand for nitrogen in the face of environmental stress. This is very important for metabolic processes including chlorophyll synthesis, photosynthesis, as well as antioxidative defences.

NR is found largely in the cytosols of root epidermal and cortical cells as well as in shoot mesophyll cells (Rufty *et al*., 1986; Vaughn and Campbell, 1988; Fedorova *et al.,* 1994). Incidentally, these are also sites of heavy metal bioconcentration. The possibility of direct impact on NR activity therefore suffices. This study showed that waste oil contamination (heavy metals and hydrocarbons) negatively affected NR activity of the test plant. Increased time of exposure as well as concentration were two important factors to recon in their phytotoxic effects.

Table 5.

Nitrate reductase activity in developing plants

Nitrate reductase activity ($\mu M\ hr^{-1}\ g^{-1}$)	Number of days after sowing			
	20	30	40	50
Control	0.311[a]	0.434[a]	0.587[a]	2.086[a]
1 %	0.287[a]	0.415[ab]	0.531[ab]	0.957[b]
3 %	0.283[a]	0.323[bc]	0.385[abcd]	0.142[c]
6 %	0.198[b]	0.304[c]	0.312[bcd]	0.103[c]
9 %	0.146[c]	0.196[d]	0.229[cd]	0.093[c]
12%	0.157[c]	0.178[d]	0.174[d]	ND[c]
LSD (0.05)	0.032	0.103	0.209	0.242

ND= Not Detected (below value of 0.0001). *Means of the same column with similar alphabetic superscripts do not differ from each other (p>0.05)*

As earlier reported, the inhibitory effect of the used oil could partly be attributed to the toxic nature of some of its constituents on this enzyme. It has been reported that polycyclic aromatic hydrocarbons (PAH) is a toxic and recalcitrant portion of used engine oil (Wang *et al.,* 2000). Heavy metals affect the activities of a wide range of enzymes. A number of metals, like Pb have been reported to interfere with the free –SH groups of plant enzymes, others block the –COOH group. Burzynski (1987) reported significant decreases in nitrogen assimilation and enzyme activity due to heavy metal contamination.

NR activity in the study was only investigated in the first 50 days of the plant life. During this period, it was observed that NR activity increased steadily. This however does not provide the adequate information required for ascertaining impact of plant age on NR activity.

Conclusions

The capability for bioconcentration of heavy metals *Chromolaena odorata* has been reported in this study. Although the test plant may have been reported in earlier studies to be resilient to used oil contamination; the study showed that this depends on the concentration of the contaminant. Having survived in concentrations of as much as 12%, it is suggested that more phytotoxicity studies be conducted on the plant upon exposure to oil concentrations above 12% so as to be able to place a peg on possible benchmark concentration for survival.

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==ERRATUM==

STUDIA UBB BIOLOGIA, Volume 62 (LXII), Special Issue, 2017, pp. 132-133

Title: Ecology and chorology of *Pulmonaria mollis* Wulfen ex Kern. s.s. – spying migration routes?

Author(s): Maria Janicka

In this summary, at the end of paragraph 3, a mistake was made, the omitted text was underlined in red.

Ecology of *P. mollis* s.s may explain its contemporary dispersed range, but it brings only undirect information about migration routes. It seems that mild and wet climate of Atlantic period has facilitated occupying the new areas of C Europe, but agriculture management has stopped or modified this process.

We ask the readers to make corrections, and those affected by errors please accept the apologies of the Editorial and Publishing House.