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Changes in the Population Density of Pathogenic Microorganisms in Response to the Allelopathic Effect of *Thypha Latifolia*

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Aim. To determine the response of the populations of *Erysipelothrix rhusiopathiae* and *Leptospira interrogans* pathogenic microorganisms to the impact of broadleaf cattail (*Thypha latifolia*) root diffusates. **Methods.** Aqueous solutions of *T. latifolia* root diffusates were sterilized by vacuum filtration through the filters with 0.2-micron pore diameter. The experimental samples contained cattail secretions, sterile water, and cultures of *E. rhusiopathiae* or *L. interrogans*. The same amount of sterile water, as in the experimental samples, was used for the purpose of control, and the same quantity of microbial cultures was added in it. After exposure, the density of cells in the experimental and control samples was determined. **Results.** Root diffusates of *T. latifolia* caused an increase in cell density in the populations of *E. rhusiopathiae* throughout the whole range of the studied dilutions (1:10–1:10000). In the populations of the 6 studied serological variants of *L. interrogans* spirochetes (*pomona, grippotyphosa, copenhageni, kabura, tarassovi, canicola*), the action of broadleaf cattail root diffusates caused the decrease in cell density. A stimulatory effect was marked in the experimental samples of the *pollonica* serological variant of leptospira. **Conclusions.** The populations of *E. rhusiopathiae* and *L. interrogans* pathogenic microorganisms respond to the allelopathic effect of *Thypha latifolia* by changing the cell density. The obtained results provide the background to assume that broadleaf cattail thickets create favorable conditions for the existence of *E. rhusiopathiae* pathogen bacteria. The reduced cell density of *L. interrogans* in the experimental samples compared to the control samples observed under the influence of *T. latifolia* root diffusates suggests that reservoirs with broadleaf cattail thickets are marked by the unfavorable conditions for the existence of pathogenic leptospira (except *L. pollonica*).

Key words: *Erysipelothrix rhusiopathiae*, *Leptospira interrogans*, population density, allelopathic effect, *Thypha latifolia*.

INTRODUCTION

In course of many infectious diseases various elements of environments, in particular, soil and water, become the source of infection for the human being and animals. Some types of pathogenic microorganisms can not only stay passively in natural substrates, but also interact with some components of biota, and so, create so called "focal points of infection" within some territories. Bacteria *Erysipelothrix rhusiopathiae* and spirochete *Leptospira interrogans* belong to the microbes of the kind: both of them are able to exist within natural ecosystems [1].

Bacteria are Gram-positive, slim, either direct or slightly curved immovable rods never producing spores, capsules, or flagella. They are quite widespread in nature and resistant to numerous disastrous factors of environment during a long period of time. This kind of bacteria is pathogenic for many animals and also for a human being, while they cause diseases, erysipelas among them [2].

Spirochetes *L. interrogans* permanently move, look like slim threads with curved or butt ends. They target a great number of host organisms, mainly homoithermic animals and cause leptospirosis. The main source of infectious agent's transmission is fresh water reservoirs [2].

The above mentioned microorganisms and diseases caused by them have already been known for a long time before. Up to the moment, the preventive and cu-

rative measures for these diseases' treatment have been developed. The biological, cultural, biochemical and genetic features of their agents have also been investigated [2, 3]. Nevertheless, despite sufficient success in the infectious diseases control both as for the animals and the human being, the outbreaks of erysipelas and leptospirosis are reported in the territory of Ukraine from year to year. The same situation is fixed in many other countries all over the world [4−6].

The scientific literature overview states the data limitation as concerning the pathogenic microorganisms *E. rhusiopathiae* and *L. interrogans* existence in the environment [7–9]. While taking into account that the main source of infection of the human being with erysipelas and leptospirosis is water and soil exactly, the study of these agents' existence peculiarities in the mentioned environments become important to all practical purposes. The flood of accomplished explorations in ecology [10, 11] has proven that the sufficient influence upon the existence of the microbiocenosis (which include these pathogenic microorganisms as well) is made by the plant – by means of secretions of bioactive substances in the environment.

The broadleaf cattail (*Thypha latifolia*) is widespread in the wetlands, bogs, plavs, flooded areas, also marshy and reedy banks [12, 13] where it grows in vast thickets. It has precious phytomeliorative peculiarities and great potential as a forage plant [14]. Following its spreading and prominent biomass created by this botanic species at the coastal parts of watersheds, the broadleaf cattail was selected for research of the response of the *E. rhusiopathiae* and *L. interrogans* populations to the allelopathic effect of this plant.

MATERIALS AND METHODS

Obtaining Plants and Root Diffusates Extraction Techniques. The *T. latifolia* specimens necessary for research were taken out of their natural sites of vegetation. The plants were dug out and extracted together with the portion of soil, whereto their root systems were. Aiming to avoid influence of the soil substance upon the results of the exploration, the soil was washed out by town water streams till complete exposal of root system. In course of such a procedure the plants roots are usually damaged, so the substances normally never secreted by the *T. latifolia* plants could appear in the experimental patterns. That is why, the exploration could be continued after complete healing of such wounds. For that purpose, the root systems of the plants were put into clean glass reservoirs filled up with tap water settled during two days before. After 10 days the water was poured out. This very procedure has been repeated thrice for getting rid of the soil rests, detritus and the greater part of tiny acquatic organisms that occurred to be in the reservoirs. At that, the plants themselves have never been taken out of these reservoirs, moreover, their positions have never been changed to prevent repetitious damages of their roots, because during this period of time not only wounds became healed, but also new bundles of tender knee roots developed.

For purpose of getting root diffusates, the fresh portions of water were added to the reservoirs with *T. latifolia* at the rate of 1:10 to the plants' biomass and seasoned during. seven days. During this period of time the plants were provided with natural temperature and lightening daily range (June, 2013). The water level was kept on previously marked tracks by means of adding fresh portions periodically.

The root diffusates extraction was based upon the technique *Obtaining Of Root Diffusates From the Plants Growing In Natural Biocenosis or In Filed* described in the paper [15]. At the same time, taking into account the specific character of our explorations, some changes were made. In particular, the roots of the plants were never cleaned with distillate water; the culture medium was made from tap water, but not from nutrient solution. The sufficient difference means the extent of exposure for obtaining the secretions. A. M. Hrodzynskyi recommends to season the plants in the cultural medium during 1−2 days. Following his evidences, the concentration of bioactive substances in the solution is raising up to the 2nd day and keep that rate up to the 4th one. After that the concentration is falling down – by reason of peculiar microorganisms enlargement that ruins the root secretions, obviously [15]. With due regard to these very peculiarities, the extent of exposure was prolonged up to seven days in the present explorations. Because naturally the secreting of bioactive substances by the plants is accompanied with their processing by microorganisms especially in the rhizosphere area [11, 15]. So, the obtaining conditions of *T. latifolia* root diffusates for further experiments were realistic as maximum.

Pathogenic Microorganisms Stock Cultures List and Ways of Their Cultivation. Microorganisms pure cultures were obtained for experiments performance from the Collection of the Institute of Veterinary of the NAAS of Ukraine.

L. interrogans spirochetes were cultivated in the Terskikh's medium laced with 5-per cent inactivated blood serum of a rabbit at temperature of 28.0 ± ± 0.3 ºC. The Terskikh's medium consists of phosphates buffer mixture and distillate water (1:9), pH 7.2−7.3. The rabbit's blood serum was inactivated with waterbath at temperature 56 ºC during 30 min twice with interval in 24 h.

The *L. interrogans* spirochetes are well-known due to their complicate intraspecific structure with great number of serovariants [2]. The stock cultures of the serovariants most often fixed within the territory of Ukraine as an agent of leptospiroses of both the animals and human being were chosen for the present exploration (Table 1).

E. rhusiopathiae bacterias (BP-2 IBM stock culture) were grown in brain-heart-infusion broth (AES Chemunex, France) at temperature of 36.7 ± 0.3 ºC during 48 h.

Experiments Preparation and Performance. The research of changes in pathogenic microorganisms' populations under the influence of broadleaf cattail's root diffusates were accomplished *in vitro*, though realistic as maximum.

The obtained solutions of *T. latifolia* root diffusates should be sterilized using the techniques preventing from changes of bioactive substances under the influence of high temperature, chemical agents or other aggressive factors. Owing to this, the obtained solution of the broadleaf cattail secretions was infiltrated in vacuum through 0.2 μm pore cellulose filters.

The test samples contained *T. latifolia* root diffusates diluted to 1:10, 1:100, 1:1,000, and 1:10,000 with corresponding amounts of sterilized tap water and 0.1 cm^3 of *E. rhusiopathiae* bacteria cultures. The like ratios of *E. rhusiopathiae* cultures and sterilized tap water were used as the control samples. The *E. rhusiopathiae* stock cultures were taken out for inoculation from one and the same tube, so, the initial density of bacteria was equal both in test and control samples.

E. rhusiopathiae bacterias' density population was determined after 48-h exposure of samples preserved at temperature of 18−20 ºC. The 0.1 cm^3 samples were plated at nutrient agar (AES Chemunex) at serial dilutions $1 \cdot 10^{-3}$ and $1 \cdot 10^{-4}$ and cultivated during 72 h at temperature of 36.7 ± 0.3 ºC. The grown microorganisms colonies were calculated using the MBC-10 microscope. After that the average number of alive bacteria in 1.0 cm^3.

The response of *L. interrogans* to the influence of broadleaf cattail's root diffusates was examined under the following conditions: the test samples contained 0.4 cm^3 of secretions' sterilized dilution and 0.1 cm^3 of leptospira culture. The test samples contained the analogous ratios of sterilized tap water and spirochetes' culture. The spirochetes' density in the samples were determined after 24 h by means of the leptospira counting cells technique [17].

The results obtained in consequence of performed explorations were processed with standard statistical techniques [18].

RESULTS AND DISCUSSION

E. rhusiopathiae cells density inventory test and control samples with subsequent comparison proved that

Table 1. *L. interrogans* Spirochetes Stock Cultures Chosen For the Experiments

Serogroup	Serovariant	Stock Culture	Abbreviation
Sejroe	*pollonica*	*493 Poland*	*L. pollonica*
Hebdomadis	*kabura*	*Kabura*	*L. kabura*
Tarassovi	*tarassovi*	*Perepelicyn*	*L. tarassovi*
Pomona	*pomona*	*Pomona*	*L. pomona*
Grippotyphosa	*grippotyphosa*	*Moskva V*	*L. grippotyphosa*
Canicola	*canicola*	*Hond Utrecht IV*	*L.canicola*
Icterohaemorrhagiae	*copenhageni*	*M 20*	*L. icterohaemorrhagiae*

Table 2. *E. rhusiopathiae* Cells Density In Test and Control Samples Upon Influence of *T. latifolia* Root Diffusates

E. rhusiopathiae Cell Density, mln/cm^3				
Secretions Dillutions				Control
1:10 (t = 21,50)	*1:100* (t = 27,67)	*1:1000* (t = 4,34)	*1:10000* (t = 4,60)	
44,50	8,20	3,00	2,40	2,40
50,30	7,80	2,60	3,20	1,50
39,20	8,40	3,10	3,40	1,40
42,20	7,90	3,30	2,70	2,10
40,00	8,10	2,70	3,00	1,80
47,60	7,50	2,40	2,90	2,00
Arithmetic Average				
43,97	7,98	2,85	2,93	1,87

Note: t – Student's coefficient; for all dilutions t_{cr} = 2,23 − critical value of characteristic t; P = 0,05.

broadleaf cattail's root diffusates provide stimulatory influence upon these microorganisms' populations (Table 2). Foremost, the stimulatory effect was reported in the samples with insufficient dilutions (1:10) of the trial plants' root diffusates. On the average, *E. rhusiopathiae* cells density was in the test samples 23.5 times larger than in the control ones at this dilution rate.

The set of tests with 1:100 dilution of broadleaf cattail's root diffusates the intensity of stimulatory impact on the *E. rhusiopathiae* bacteria populations remarkably decreased, though it was still notable enough, while the cells density was 4.3 times higher than in the control samples. The further reduction of broadleaf cattail's root diffusates concentration in the test samples (1:1,000 and 1:10,000) caused the decrease of stimulatory parameters as for the *E. rhusiopathiae* population. However, even so, difference in cells density for test and control samples was notable (approx. 1.5 times) and statistically-valid. The analysis of the obtained results as concerning the impact of the broadleaf cattail's root diffusates on the trial *L. interrogans* spirochetes reported the following peculiarities (Table 3).

The difference in cells density in control (100 per cent) and test samples for examined serovariants of leptospira was L. *pomona* – 78.32 per cent; *L. grippotyphosa* – 69.96 per cent; *L. icterohaemorrhagiae* – 50.00 per cent; *L. kabura* – 48.94 per cent; *L. tarassovi* – 39.54 per cent; *L. canicola* – 27.52 per cent. The higher rate of cells in control samples as compared to the test ones, granted the statistically-valid difference proves the inhibitory effect of the *T. latifolia* root diffusates on the pathogenic leptospira. At the same time, only in case with *L. pollonica* the spirochetes' density was higher in the test samples for 21.88 per cent than in the control ones. As the stated difference in the cells density is statistically-valid, this is the proof of the stimulatory impact of the broadleaf cattail's secretions on this stock culture of leptospira.

The populations of pathogenic microorganisms interact with various species of aquatic organisms being a component of fresh water ecosystems. The nature of such interactions determines the current state, existence period of the above mentioned pathogenic microorganisms' populations, and so, the level of danger for both the animals and human being infecting in some parts of water areas to a great extent.

CONCLUSIONS

The performed laboratory tests reported the response of such pathogenic microorganisms, as *L. interrogans* and *E. rhusiopathiae* to the allelopathic effect of the higher plants, in particular, *T. latifolia,* by means of population density changes.

The obtained results afford ground for hypothesis that the broadleaf cattail's thickets provide favorable conditions for *E. rhusiopathiae* pathogenic bacteria's existence. The root diffusates of *T. latifolia* not only enhance the *Erysipelotrixes* survival, but also promote the significant increase in their density, what strengthens the infecting both for the human being and animals.

Decrease in *L. interrogans* cells density in the test samples as compared to the control ones due to effect of the *T. latifolia* root diffusates allows to contemplate that the broadleaf cattail's thickets provide unfavorable conditions for *L. interrogans* (except for *L. pollonica*) existence.

It is quite possible that the character of some "infection focal points" consists in the difference of favorable conditions for existence of various pathogenic microorganisms, what is determined with the peculiarities of vegetation cover and the allelopathic effect of the latter.

Table 3. *L. interrogans* Cells Density In Test and Control Samples Upon Influence of *T. latifolia* Root Diffusates

Leptospira Cells Density, mln/cm^3					
L. grippotyphosa		*L. icterohaemorrhagiae*		*L. tarassovi*	
Test	Control	Test	Control	Test	Control
1,83	5,20	2,56	4,40	1,33	2,15
2,16	5,37	2,14	4,65	1,72	2,33
1,94	5,44	2,47	4,93	1,17	1,98
2,32	4,89	2,30	4,74	1,00	2,00
2,00	5,35	2,28	4,78	1,28	2,29
Arithmetic Average					
2,05	5,25	2,35	4,70	1,30	2,15
t					
21,93		18,32		5,46	

Note. t – Student's coefficient; t_{cr} = 5,04 − critical value of characteristic *t*; P = 0,001.

With due regard to important epidemiological and epizootic significance of the pathogenic microorganisms in the nature, it is necessary to continue the exploration dedicated to determination of the tendencies in their existence and interaction within the environment.

Зміна щільності популяцій патогенних мікроорганізмів у відповідь на алелопатичний вплив *Thypha latifolia*

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Мета. З'ясувати реакцію популяцій патогенних мікроорганізмів *Erysipelothrix rhusiopathiae* та *Leptospira interrogans* на вплив кореневих дифузатів рогозу широколистого *(T. latifolia)*. **Методи.** Водні розчини з кореневими дифузатами *T. latifolia* стерилізували фільтрацією під вакуумом через фільтри з діаметром пор 0,2 мкм. Дослідні зразки містили виділення рогозу, стерильну воду та культури *E. rhusiopathiae* або *L. interrogans*. Як контроль використано стерильну воду в об'ємі, аналогічному об'єму у дослідних зразках, в яку додавали таку ж кількість культур мікроорганізмів. Після експозиції визначали щільність клітин у дослідних і контрольних зразках. **Результати.** Кореневі дифузати *T. latifolia* спричиняли зростання щільності клітин у популяціях *E. rhusiopathiae* в усьому діапазоні досліджених розведень (1:10–1:10000). У популяціях спірохет *L. interrogans* шести досліджених серологічних варіантів (*pomona, grippotyphosa*, *copenhageni*, *kabura, tarassovi*, *canicola*) вплив кореневих дифузатів рогозу широколистого призводив до зниження щільності клітин. У дослідних зразках з лептоспірами серологічного варіанта *pollonica* відмічено стимулювальний ефект. **Висновки.** Популяції патогенних мікроорганізмів *E. rhusiopathiae* та *L interrogans* реагують на алелопатичний вплив *T. latifolia* зміною щільності клітин. Одержані результати дають підставу для припущення, що у заростях рогозу широколистого складаються сприятливі умови для існування патогенних бактерій *E. rhusiopathiae*. Зменшення щільності клітин *L. interrogans* у дослідних зразках порівняно з контрольними за дії кореневих дифузатів *T. latifolia* дозволяє припустити, що в заростях рогозу широколистого у водоймах для патогенних лептоспір (за вийнятком *L. pollonica*) умови існування є несприятливими.

Ключові слова: *Erysipelothrix rhusiopathiae*, *Leptospira interrogans*, щільність популяцій, алелопатичний вплив, *Thypha latifolia*.

Изменение плотности популяций патогенных микроорганизмов в ответ на аллелопатическое влияние *Thypha latifolia*

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Цель. Выяснить реакцию популяций патогенных микроорганизмов *Erysipelothrix rhusiopathiae* и *Leptospira interrogans* на влияние корневых диффузатов рогоза широколистного *(T. latifolia)*. **Методы.** Водные растворы с корневыми диффузатами *T. latifolia* стерилизовали фильтрацией под вакуумом через фильтры с диаметром пор 0,2 мкм. Опытные образцы содержали выделения рогоза, стерильную воду и культуры *E. rhusiopathiae* или *L. interrogans*. В качестве контроля использовали стерильную воду в объеме, аналогичном таковому в опытных образцах, в которую добавляли такое же количество культур микроорганизмов. После экспозиции определяли плотность культур в опытных и контрольных образцах. **Результаты.** Корневые диффузаты *T. latifolia* вызывали увеличение плотности клеток в популяциях *E. rhusiopathiae* во всем диапазоне исследованных разведений (1:10–1:10000). В популяциях спирохет *L. interrogans* шести исследованных серологических вариантов (*pomona, grippotyphosa*, *copenhageni*, *kabura, tarassovi*, *canicola*) действие корневых диффузатов рогоза широколистного приводило к снижению плотности клеток. В опытных образцах с лептоспирами серологического варианта *pollonica* отмечен стимулирующий эффект. **Выводы.** Популяции патогенных микроорганизмов *E. rhusiopathiae* и *L interrogans* реагируют изменением плотности клеток на аллелопатическое влияние *T. latifolia*. Полученные результаты дают основание предположить, что в зарослях рогоза широколистного для существования патогенных бактерий *E. rhusiopathiae* создаются благоприятные условия. Уменьшение плотности клеток *L. interrogans* в опытных образцах по сравнению с контрольными при действии корневых диффузатов *T. latifolia* позволяет предположить, что в зарослях рогоза широколистного в водоемах для патогенных лептоспир (за исключением *L. pollonica*) условия существования являются неблагоприятными.

Ключевые слова: *Erysipelothrix rhusiopathiae*, *Leptospira interrogans*, плотность популяций, аллелопатическое влияние, *Thypha latifolia*.

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