The Balkans Scientific Center of the Russian Academy of Natural Sciences

1st International Symposium:

Modern Trends in Agricultural Production and Environmental Protection

PROCEEDINGS

Tivat-Montenegro
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INVITATION PAPERS

THE INFLUENCE OF ANTHROPOGENIC ACTIVITY ON DIVERSITY OF AMPHIPODA (CRUSTACEA) IN MONTENEGRO

(Contribution to the Knowledge of the Amphipoda 310)

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ABSTRACT

The influence of various anthropogenic activity on diversity of Crustacea Amphipoda (except benthic Caprellidea and planctonic Hyperiidea) in Montenegro is presented. Despite relatively small size, biodiversity in Montenegro is very high, including that of Crustacea Amphipoda, thanks to geological, geographical, ecological and other condition of this area.

Amphipoda in continental fresh and brackish waters (epigean and subterranean) are present in Montenegro by over 70 known species belonging to 18 genera and 11 families; in the Adriatic Sea by over 285 distinct species and subspecies belonging to nearly 140 genera and 59 families.

Anthropogenic activities in so small country with high diversity of Amphipoda with numerous endemic species must be very carefully provided within the framework of sustainable development, to preserve the type-localities of numerous endemic species and to protect numerous very different vulnerable ecological niches of single endemic or rare species.

Keywords*: anthropogenic activity, Amphipoda, diversity, endemic taxa, type-locality, Montenegro*

INTRODUCTION

Montenegro (Crna Gora) is relatively very small country, with size of 13.812 km2, sitting on western site of Balkan peninsula, at eastern coast of Adriatic Sea (fig. 1).

The most part of country is covered by numerous mountains: Durmitor, Sinjajevina, Orjen, Lovćen, Prokletije, etc), with piks over 2000 meters height. The karstic rocks are dominant rocks, with developed all kinds of karstic phenomena (numerous caves, subterranean waters, karstic springs, waterfalls, canyons, abysses, etc.).

Montenegro is relatively abundant with annual precipitations, rain with over 1000 l/m2 (maximum in Crkvine, with 5.000 l/m2) and snow. By this way, there are numerous springs, torrents and rivers belonging to Adriatic and Black Sea drainage systems. Morača river with its affluents is the biggest river of Adriatic Sea drainage system, and rivers Tara, Lim and Drina with its affluents belong to Black Sea drainage system.

Numerous glacial lakes appear on the mountains (Plavsko, Pešića, Crno, Bukumirsko, Škrčko, Hridsko, Riblje, Zminje, Modro, Valovito, Šiško, Biogradsko, etc.). In the southern part of Montenegro is situated large karstic Skadar Lake with output Bojana River into Adriatic Sea.

The length of the coast of Adriatic Sea belonging to Montenegro is nearly 293.5 Km, with numerous small gulfs and bays with sandy, stony and rocky coasts, often with mouth of small torrents and numerous springs. The largest gulf is Bay of Boka Kotorska incised nearly 28 km into the coast between the mountains, looks like the fiord of Norway, but originated by submersed river after the last glacial perod. In the front of Montenegro is the deepest part of the Adriatic Sea (maximal depth is 1460 m).

The mediterranean, submediterranean and continental type of clime has a great influence on the biodiversity in Montenegro.

Because of its geological and geographical history and origin, with extremely different ecological niches on relatively small area, the biodiversity in Montenegro is one of the highest in Europe (for example, in Montenegro are present over 500 different species of Gastropoda, with numerous endemic taxa).

With so rich biodiversity and very high percent of endemic species existing on relatively small area, Montenegro becomes very sensitive and vulnerable on every kind of anthropogenic activity [tourism, construction of the roads, pollution of the water and terrains by industry, waste waters and waste-deposits from the cities, exploitation of sand, forests, the epigean and subterranean waters, agriculture activity, etc.

All these factors have great influence on the biodiversity in Montenegro in general, and also on the group of small crustacean animals, Amphipoda (Crustacea, Malacostraca), mentioned in this work.

Order AMPHIPODA

Amphipoda is a group of Invertebrata animals belonging to Class Crustacea, inhabiting all kinds of relatively clean waters (sea, brackish waters, subterranean and surface fresh waters) over the World (fig. 2).

At present, amphipod crustaceans comprise 9,980 species, 1,664 genera, 444 subfamilies, and 221 families (Arfianti et al., 2018), but numerous new species are still describing every year over the World, including Balkan peninsula (G. Karaman, 2016).

In the Mediterranean Sea are present nearly 400 species and subspecies of Gammaridean Amphipoda, belonging to over 170 genera and nearly 70 families [Ruffo, S. (editor), 1998].

In the Adriatic Sea, as a part of the Mediterranean Sea, Amphipoda fauna is only partially investigated (G. Karaman, 2011a), where over 285 distinct species and subspecies are known, belonging to nearly 140 genera and nearly 59 families, and we are expecting discovery in Adriatic Sea numerous additional species, genera and families, already known from the Mediterranean Sea.

In Boka Kotorska Bay are established nearly 25 families, 38 genera and 59 species of Amphipoda (suborder Gammaridea) (G. Karaman, 2019, in press).

The animals belonging to the order Amphipoda in continental fresh and brackish waters (epigean and subterranean) are present in Montenegro by over 70 known species belonging to 18 genera: *Accubogammarus, Bogidiella, Corophium, Cryptorchestia, Echinogammarus, Gammarus, Hadzia, Laurogammarus, Melita, Metohia, Neogammarus, Niphargus, Psammogammarus, Pseudoniphargus, Rhipidogammarus, Salentinella, Synurella, Typhlogammarus* and 11 Families: Bogidiellidae, Corophiidae, Crangonyctidae, Gammaridae, Hadziidae, Melitidae, Niphargidae, Pseudoniphargidae, Salentinellidae, Talitridae, Typhlogammaridae. Among them, there are numerous endemic species for Montenegro or western Balkan.

The intense various anthropogenic activity in Montenegro, create strong pressure on entire biodiversity including Amphipoda, on terrestrial, freshwater and marine environment.

The intense visiting of **numerous caves** in Montenegro settled by rare of endemic Amphipoda, by various visitors and collectors who collect often without control cave animals and study them in their countries, has very negative influence on diversity of Amphipoda (*Niphargus zorae* G. Kar., 1967 in Megara Cave; *Niphargus navotinus* G. Kar., 2014 in Navotina Cave; *Typhlogammarus mrazeki* (Schäferna, 1906) and *Bogidiella montenigrina* G. Kar., 1997 in Lipska pećina Cave, *Niphargus hercegovinensis* S. Kar., 1950 in Jabukov Do Cave, *Accubogammarus algor* (G. Kar., 1973) in Stanina pećina Cave, *Niphagus carcerarius* G. Kar. 1987 in Cave of torrent Šljivanski potok, *Niphargus brevicuspis* Schell., 1937 in Gornja Pokljuka Cave etc.

Many subterranean amphipods have been described from various springs in Montenegro (*Niphagus pulevici* G. Kar.,1967 in spring in Bar; *Niphargus vulgaris* G. Kar., 1968 from spring in Sotonići; *Niphargus occultus* G. Kar., 1998 from spring near Mratinje, *Niphargus podgoricensis* S. Kar., 1934 from spring of Ribnica in Podgorica; *Niphargus kusceri* S. Kar., 1950 from Ljuta spring in Boka Kotorska; *Niphagus abavus* G. Kar., 1911b from spring in Markov Rt, Boka Kotorska; *Niphargus vranjinae* G. Kar.,1967a from springs on Vranjina, Skadar Lake, *Niphargus alatus* G. Kar., 1973b from springs on Veruša Mt., etc., and most of them are endemic for Montenegro.

Some of these springs are destroyed or closed and these waters are transferred in water systems for domestic use. By this way, not only subterranean species, but also epigean species of Amphipoda living in the torrents of these springs disappear together with water. But, some springs remain clean and unpolluted, as spring Ljuta, spring Škurda, etc.

Numerous glacial lakes in various mountains of ,Montenegro are settled by amphipod *Gammarus lacustris* Sars, 1863, north European species who settled these mountain waters during Glacial period. After the end of Glacial period this cold-water species disappear from these regions except as isolated populations in glacial lakes of Montenegro (Crno, Pošćensko, Bukumirsko, Pešića, Riblje, Zminje, Šiško, Škrčko, etc.).

The intense use of these lakes as touristic destinations, made in danger the biodiversity of these lakes and clean water in it, because of possible water pollution. On the other hand, many of these lakes have been settled artificially by various species of fish to create attractive fishing places. Introduction of new species corrupt the biological balance in these lakes and many native endemic species in these lakes disappear. Often, introduced fish species have eaten all amphipods and other water animals, remaining later hungry and poorly developed, with large head and skinny body.

The use of various plant-fertilizers and other chemical agents during the seasonal agricultural work, become very damaging for amphipods and other living beings when during rains arrive these polluted waters into springs, torrents, rivers or lakes. Maybe some of poisoned amphipods survived this pollution, but later, when amphipods are eaten by fishes, the poison from amphipods will be accumulated in the fishes and become harmful for people.

The subterranean amphipods in Montenegro are highly endemic for Montenegro
or western Balkan [*Accubogammarus algor* (G. Kar., 1973) from Stanina Cave, *Bogidiella glacialis cataracta* G. Kar., 2002 from Plav region, *Niphargus asper* G. Kar., 1972 and *Hadzia crispata* G. Kar., 1969 from wells in Podgorica, etc.]. All subterranean species, usually invisible for ordinary people, are very sensitive on pollution and can be good indicator for clean waters. With the introduction of the water systems, many drinking wells have been abandoned or transformed into septic pons, and by this way the subterranean waters have been polluted and endemic subterranean fauna destroyed.

As the subterranean fauna is only partially known, probably many species of amphipods are destroyed before they were discovered. Already some subterranean species of Amphipoda collected in the vicinity of great cities, disappear from the subterranean waters of this region, or maybe become so rare that is is very difficult to discover again (*Hadzia crispata* and *Niphargus asper* in Podgorica, etc.).

On the coasts of the Adriatic Sea in Montenegro we observed the high anthropogenic influence through the creating of large urbanistic structures, hotels, partially developed waste water systems, destruction of natural sea-coast, what conduct to degradation of various ecological niches at the sea coast, inhabiting numerous endemic or rare Amphipoda taxa [*Rhipidogammarus karamani* Stock, 1971, *Pseudoniphargus adriaticus* S. Kar., 1955, *Neogammarus adriaticus* G. Kar., 1973a, etc.].

The cleaning of algue deposits and leaves of *Posidonia* and *Cymodocea* on the beaches, often several meters far from the sea-line, have great negative influence on numerous Amphipoda species eating these half-dried algue and plants, or use them also as refuges: *Talitrus saltator* (Montagu, 1808), *Orchestia mediterranea* Costa, 1853, *Platorchestia platensis* (Kroyer, 1845), species of genera *Hyale, Maera, Melita, Ceraocus, Gammarus, Stenothoe,* etc.), etc.

The presence a large number of customers on the beaches and use of various sunbeds and mechanical pressure on the sandy bottom, have negative influence on populations of epigean and subterranean amphipods in that area.

The tidal zone of the sea coast (in Adriatic Sea only 30-40 cm) is very important for the existence of numerous amphipods regarding its food and habitat. This zone is usually under very strong anthropogenic influence (*Melita palmata* (Montagu, 1804), *Melita hergensis* Reid, 1939, *Maera grossimana* (Montagu, 1808), etc.).

The zone with highest number of rare and endemic species of amphipoda is the coastal brackish-water zone (epigean and subterranean brackish waters) where are present several rare species: semisubterranean *Melita bulla* G. Kar., 1978 (coast of Kamenovo near Budva), *Rhipidogammarus karamani* Stock, 1971 (Buljarica near Petrovac); *Melita valesi* S. Kar., 1955 and *Psammogammarus caecus* S. Kar., 1955 (coast of Bar), *Neogammarus adriaticus* G. Kar. 1973 (coast in Bečići near Budva, , *Pseudoniphargus adriaticus* S. Kar., 1955 (gravel coast in Budva), etc. These zone are with significant anthropogenic activity.

The marine traffic of numerous small and great vessels and kruzers along the coasts of Adriatic Sea, in the ports as well as in Boka Kotorska Bay (fig. 4), has also influence on the pollution of the sea and degradation of various coastal ecological niches inhabiting by Amphipoda also [*Rhipidogammarus karamani*, *Pseudoniphargus adriaticus*, *Neogammarus adriaticus*, etc.].

Intense road traffic near the sea, is additional source of seawater pollution (fig. 3). There are many other influences not mentioned here.

The special attention we must put on the data that numerous Amphipoda have been discovered and described from Montenegro [locus typicus], marine as well as from continental surfacial and subterranean waters.

Some of these species are known from continental freshwaters of Montenegro only, i.e. endemic species for Montenegro [*Laurogammarus scutarensis* (Schäferna, 1922), *Gammarus pljakici* G. Kar., 1964, *Synurella intermedia montenigrina* G. Kar., 1974, *Hadzia crispata* G. Kar., 1969; *Bogidiella glacialis cataracta* G. Kar., 2002, *Bogidiella montenigrina* G. Kar., 1997, *Niphargus carcerarius* G. Kar., 1987, *Niphargus zorae* G. Kar., 1967, *Niphargus occultus* G. Kar., 1998, *Niphargus brevicuspis* Schell., 1937, etc.]. Some other species discovered and described from continental fresh and brackish waters of Montenegro have been later discovered in other parts of Europe [*Neogammarus adriaticus* G. Kar., 1973; *Melita bulla* G. Kar., 1978, etc.]

There are several marine species of Amphipoda discovered and described from the Adriatic coast of Montenegro, and later discovered by other authors in other parts of the Mediterranean Sea [*Seba aloe* G. Kar., 1971; *Degocheirocratus spani* G. Kar., 1985, *Ampelisca dalmatina* G. Kar., 1975, *Leucothoe oboa* G. Kar., 1971, *Autonoe karamani* Myers, 1976, *Leptocheirus mariae* G. Kar., 1973, *Maera sodalis* G. Kar. & Ruffo, 1971; *Paraphoxus lincolni* G. Kar., 1988, etc.].

CONCLUSIONS

The presence of type-localities (locus typicus) of numerous species of Amphipoda on the continental territory of Montenegro and on Adriatic coast of Montenegro represent remarkable data for Montenegro in general, and obliges the state on protection of all type-localities, as place of interest.

During all present anthropogenic activity and development of the country, it is necessary to follow sustainable development, to coordinate the wishes with the possibilities of nature-capacity of Montenegro within the framework of sustainable development.

The development of active monitoring system is necessary for protection of the present biodiversity in all continental waters and Adriatic Sea as one important segment in the sustainable development of Montenegro.

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4 Figures for the manuscript Karaman:

Fig. 1. Map of Montenegro (from Internet)

Fig. 2. *Niphargus* sp. (photo R. Ozimec)

Fig. 3. Road traffic along Bay of Boka Kotorska (orig.)

Fig. 4. Cruise ship in Verige, Bay of Boka Kotorska (orig.)

IS THE MICROBIAL COMMUNITY MANAGEMENT IN ECOSYSTEMS WITH A CYCLE OF THE MICROORGANISMS THE PROTECTING OF THE ENVIRONMENT?

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Keywords: *microbial community, concepts, laws, ecosystems, soil ecosystem, soil health*

ABSTRACT

INTRODUCTION

By the end of the first quarter of the 21st century, a large amount of knowledge has been accumulated in biology and microbial ecology with consistently recurring patterns. This made it possible to recognize in these patterns the status of rules, concepts, or even laws. The recognition was due to the ubiquity, ineluctability and inevitability of the impact of the identified patterns (Zvyagintsev, 1987; Turchin, 2002; Semenov, 2005, 2010, 2011). The significance of rules, concepts and laws for the knowledge of the laws of nature is indisputable and indicates that microbial ecology, as science is becoming mature. Since the main subject of discussion is the microbial community, we give the definition of the microbial community.

The microbial community (MC) should be presented as a set of taxonomically different, but functionally interacting populations of microorganisms that exist for some time in the appropriate place. The components of an MC can be both strongly interconnected, including physically, and the MC can be highly specialized, or weakly interconnected, and the MC can be low specialized ”(Semenov, 1991; 2005; 2010; 2011). Of course, the rules, concepts and even laws are not equivalent in their significance, ubiquity of manifestation and possibility of use. However, among the concepts, there are those that are always significant, although their manifestation is difficult to measure, but, nevertheless, they need to be known and their manifestation taken into account.

One such concept that needs to be known and taken into account everywhere in managing not only man-made, but also natural ecosystems is the cycle of microorganisms (CM) (Semenov et al., 2007; Semenov et al., 2010). The meaning of this concept is that the microorganisms of the microbial community (MC) in the form of individual cells, populations and even communities are subject to **movements** in ecosystems between different successional elements of ecosystems, econiches, ecotopes and other habitats due to various reasons (natural and anthropogenic). The main elements of habitats in cyclic movements are soil and water, plants, animals and their gastrointestinal tracts, animal excrement and plant excreta and again soil and water (Semenov et al., 2007). Microorganisms, by surviving and by virtue of displacement, constantly mastering “new” habitats, adapted to the conditions of habitats and “adapting” these habitats with their presence and their activity. Consequently, the qualitative and quantitative composition of the populations of microorganisms in each element of the ecosystem is not constant, variable.

The question arises how to manage microbial communities and, accordingly, ecosystems, if nature constantly adds, removes, moves and mixes organisms?

The objective of this message is to organize and propose the subordination of known laws and concepts, to discuss examples of the significance of laws and concepts in the management of functioning microbial communities (MC) in ecosystems. What laws and concepts from those already known can give direction to understanding the management of microbial communities and microbial ecosystems in ecological biotechnology (Dukic et al., 2018 a, b). Briefly, consider them.

THE LAWS OF POPULATION BIOLOGY AND MICROBIAL ECOLOGY

It is quite obvious that to control an MC it is necessary to focus primarily on the fundamental laws, and among them to use their subordination. Currently, at the level of the axiom “cellula ex cellula”, the laws of population growth are significant.

The law of exponential growth, that is, the ability to exponentially grow all sorts of populations (and communities), a mathematical expression is: $x= x_o e^{\mu t}$. All populations (and communities) are subject to the phenomenon of “self-limiting” growth or, more precisely, limiting or inhibiting growth, a mathematical expression: $\mu=\mu_{max}*S/K_s+S$ - Mono equation or $\mu = \mu_{max}K_p/K_p+P$ - Jerusalemsky's equation. All populations (and communities under conditions of limiting or inhibiting growth exist and develop in wave-like (as effects of limiting or inhibiting), the law of wave-like growth, mathematical expression: $dX/dt = (\mu(S)-D(S))*X$ (equation growth), $dS/dt = -X*(S)/Y+Kr*X*D(S)+BGF+Input(t)$ (dying off equation), **(**Zelenev et al., 2000**).**

These laws are objective for all living organisms and of course for microorganisms. The significance of these laws is unconditional and their functioning is constantly felt. Turchin P.V. (2002) compared the significance of these laws with the first three laws of dynamics in physics. The listed laws have a mathematical expression that, apparently, distinguishes laws from concepts and rules. Apparently, understanding and feeling the importance of laws and concepts will help their arrangement in a certain hierarchy, and the discussion of significance in a certain sequence, reflecting their ubiquity of functioning.

Universal concepts

The concept that MC is not only the main functional, but also an evolving ecosystem unit (Zavarzin, 1990), and the evolution of microorganisms, MC, occurs in the community and through the community, is presented as universally significant. The individual organism is affected by many factors created by nature and the community. The “selection” (nature selects) of individual mutations, occurs under the conditions (the fittest survives) created by the community. That is, the survival of the “new” population in the “new” habitat, “allows” the community and, of course, the environment.

The concept of the evolution of microorganisms in the community and through the community is associated with the concepts of symbiosis and succession. The concept of “symbiosis” as a global phenomenon is not so much, for example, in mutualism. More globally, its significance lies in the fact that all organisms somehow coexist with each other.

The concept of succession can be equated by law with its significance. If the disturbances or changes in the environment are so large, then the coexistence in the MC is disturbed (destroyed) and some organisms (species) are replaced by others, which is called succession. The interconnected concepts of symbiosis and succession orient theorist and practice to a systematic approach to achieve a result. Focusing on achieving sustainability of the used microbial communities, the path to managing natural microbiological processes. In practice, the effects of succession processes are not rarely negatively manifested, for example, in sewage treatment plants or other technological processes based on the use of natural, and especially artificially created microbial communities (Dukic et al., 2018a,b).

As a detail of the concept of succession, it is necessary to bring the concept of the regulation of the activity of MC in natural and anthropogenic ecosystems. The concept states that the change in the activity of MC is not carried out by changing the activity of a separate component of the MC, but by changing the dominant composition of the community through succession. Consequently, when trying to change the activity of the process carried out by the MC, it is necessary to be prepared for the fact that the composition of the microbial community will necessarily change (Semenov, 2005).

The concept of the dependence and significance of a process in an ecosystem on the number and activity of microorganisms that carry it out or, only that process is significant and noticeable if the organisms that carry it out are many and they are active, have the deepest meaning (Zavarzin, 1989). This concept, echoes the philosophical law of the transition of quantitative changes into qualitative ones.

This concept makes it possible to understand that the discovery of several cells of a saprotrophic microorganism in a certain habitat does not mean the importance of the process, potentially significant in the implementation of its many such microorganisms. A phenomenon actively exploited in the literature, called “quorum sensing”, is merely a manifestation of the above formulated concept.

Each microorganism has its own limits of existence, and the properties and characteristics of the microorganism are reflected in physiological characteristics and constants. Genetic determinism of the properties of microorganisms does not exclude their physiological “flexibility”. In this regard, it seems that the laws of growth and succession should be supplemented with the concept of "the limited adaptive capacity of an organism within the norm of the response of a particular organism to environmental conditions".

The meaning of this concept is that it is impossible to adapt a population of a microorganism to a non-specific inhibitor, but an attempt to adapt MC will only lead to succession in MC, since adaptation occurs in the population and in the community - succession.

Specific concepts

In addition to the general ecological concepts, no less important, but more specific concepts of MS, which reflect the functional properties of microorganisms and ecosystem features, are known (Zvyagintsev, 1987).

The concept of the microbial pool and the redundancy of this pool in the soil ecosystem compared to the availability of the nutritional resource. Its essence is that the viable part of the microbial biota “exceeds” the mass of available nutrients. Consequently, most of the microorganisms in the soil ecosystem are not provided with food and are in a state of starvation, and, consequently, a physiological survival (dormant state, anabiotic state and even viable - but non cultivable). The importance of this concept must be taken into account especially when introducing microorganisms into the natural ecosystem, with some pragmatic goals.

The concept of functional interchangeability, functional parallelism among microorganisms or functional duplication. It is known that bacteria, actinomycetes and fungi, that is, prokaryotes and eukaryotes, can perform the same function in ecosystems, for example, cellulose hydrolysis. Suppression, and even more complete removal of any one functional group, will cause an increase in the activity and number of the functional understudy, the activity of which may be completely undesirable.

The concept of micro zonality of the soil ecosystem as a habitat of microorganisms, along with the concept of redundancy of the microbial pool in the soil, recalls that, for example, the introduced species will be viable if it is located on one side in the microzones, and on the other, the substrate must also be delivered to the microzones.

The concept of the *r* - *K* - continuum in microbial ecology is a reflection of the continuity and discreteness of the properties and diversity of organisms. This concept clarifies the meaning of the most significant concept - the microbial community and justifies the importance of understanding the concept of oligotrophy as a phenomenon. The existence of oligotrophic ecosystems is the mechanism for the emergence, creation and maintenance of healthy ecosystems (and of course the soil). The most impressive example of using the knowledge of the functioning of MS to control microbial communities in ecosystems with a cycle of microorganisms is the discovery and application of the concept of “disturbing influences and wave-like development of microbial populations (MP) and MS” (Semenov et al. 2001). This concept, in essence, is an addition to the law of (third!) General ecology on the wave-like dynamics of population development (Turchin, 2002). In this case, the model of the wavelike dynamics of A. Lotka and V. Volterra (Gause, Witt, 1934) is usually mentioned. However, if in the general ecology the cause of wave-like dynamics is traditionally explained by the relationship between predator and prey, which is realistic, then in the ecology of microorganisms, as we have established, this is the interaction between the substrate and the consumer. The consequence of this is the alternation of the phases of growth and death of the MP and MS (Zelenev et al., 2005). The authors understood the biological essence of the wave-shaped, oscillating dynamics of the existence and development of microorganisms in nature. In this regard, the authors had an idea to apply this knowledge for practical purposes, to determine the parameter of soil health (Semenov et al., 2011a; Semenov et al., 2011b; Semenov, Đukić, 2017). An example of developing a method for determining the soil health parameter in which images of different soils are used, that is, with very different biodiversity and, therefore, different activity is an indicator of the ability to manage microbial communities and natural ecosystems where the microbial cycle is as natural as the cycles of day and night and the seasons.

Conclusion

In the present communication, for the first time, the laws of microbial ecology have been collected and discussed, although already known, but still not generalized, non-subordinated laws. Among the scientists and practitioners long cherished dream and question. How to manage microbial communities and, accordingly, natural ecosystems? The infinitely increasing amount of knowledge in microbial ecology, on the one hand, allows us to comprehend nature more deeply and in more detail, but on the other, an infinite number erodes the significance of concrete achievements. It seems that a multitude of disparate knowledge can be combined and streamlined through laws and concepts. The application of laws and concepts of microbial ecology makes sense of the management of the environment and allows us to bring meaningful management of microbial communities and microbial ecosystems. Coordination and discussion of well-known laws and concepts of microbial ecology, with examples, is the first step towards solving the conscious use in practice of managing microbial communities and, accordingly, natural ecosystems. Of course, not all laws and concepts of microbial ecology have been summarized in this report. There are laws and concepts in force in epidemiology, the importance of which for society is enormous, and their knowledge is obligatory. However, due to the limited diversity and even the number of epidemiologically significant microorganisms, there is enough “gap” in their cycles to control their circulation, for example, the introduction of quarantine measures, vaccination, etc.

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MICROBIOLOGICAL ASPECTS OF TECHNICAL DAMAGE OF THE LAND

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Due to the growing trend of industrialization, urbanization, transport and agriculture, before scientists of various specialties, including land microbiologists, there is a problem of optimizing the interaction between man and the natural environment.

Land microorganisms are an important component of terrestrial ecosystems. Their number can reach several 109 / g of land (Zvjagincev, 1987). Therefore, they, like other biological objects, undergo the impact of technogenic pollution (mineral fertilizers, pesticides, heavy metals, liquid manure, polluted waters, pasture, recreational degressions and heavy machinery, oil and its products, nitrification inhibitors, detergents, etc.). We already have a significant experimental material on the influence of chemical agents of anthropogenic origin on microorganisms (Sencova, Maksimov, 1985; Kohlmaier, 1989; Shukla, 1990; Đukić et al., 1999; Đukić, Mandić, 2000; Đukić et al., 2009 , 2014, 2015, 2018a, b, c).

However, the information, which can be found in the literature, does not allow for a sufficiently comprehensive presentation of the changes that occur in the soil microbial system under the influence of pollution.

Therefore, it is not possible, on the one hand, to evaluate the degree of harmful effects of the pollutant, and on the other, to judge the adaptation properties of soil microorganisms to different influences.

Literary data relating to this problem are extremely controversial, and the conclusions are often inconclusive. It is noticed that in a large number of cases one and the same agent can inhibit, stimulate or even show any effect on soil microorganisms (Zvjagincev, 1989). Because of this, different opinions are still given in the scientific literature regarding the possibility of using soil microorganisms for the assessment of the effects of pollutants: from positive ones (Dobrovoljski et al., 1985; Đukić et al., 1994a, b; Đukić, Mandić, 1996; Đukić , Mandic, 1998; Djukic, Mandic, 1999; Djukic et al., 2012; Semenov and Djukic, 2017), to negative (Wainwright, 1980). However, the existing experimental experiences in the field of sanitary and hygienic normative production, which are carried out under strict laboratory conditions, testify to the fact that soil microorganisms react positively to relatively low concentrations of pollutants in the soil (Gončaruk, Sidorenko, 1986; Mandić et al., 1995 Djukic et al., 1999; Djukic and Mandic, 2000; Djukic et al., 2011; Semenov and Djukic, 2019). Due to all this, it is very important to examine the changes of saprotrophic soil microorganisms in technical soil contamination.

The aim of this our work is to elaborate the concept of ecological and microbiological assessment of the state of soil in conditions of technical pollution.

The goal set is achieved by gradually solving the following tasks:

- developed methods of testing saprotrophic microorganisms in contaminated soil;
- generalizing their own experimental data and literary material in the form of a concept,
- which shows the overall legality of changes in the land microbial community in technical pollution;
- proposing ecological and microbiological solutions for some problems of soil protection.

Method of initiated microbial communities

There are objective difficulties that the researcher encounters during the study of the impact of pollution on microbiological processes in the soil. In our opinion, one of the more important is to use the methods of seeding on solid nutrients (borrowed from medical microbiology) that are completely indefinitely characterized by the return reaction of soil microorganisms to technical pollution in solving this problem. These methods are completely suitable for the separation of pure cultures of microorganisms, however, for ecological microbiological soil testing, their application is clearly insufficient. Due to all this, it is still the current search for new methodological approaches, which correspond to the new tasks, which are placed before the land microbiologists.

A practical inclusion of methodological studies is the method of the initiated microbial community (Guzev et al., 1982), intended for laboratory experiments in order to clarify the modifying effects of different factors on the microbial soil system in conditions that resemble the natural ones.

Methodological explanation

The proposed method includes several key operations that require independent explanation. The starting position, which forms its basis, is the opinion of Vinogradski (1952) - that saprophytic microorganisms of the soil should be examined in the conditions of active faction - in the process of mineralization of organic substrates, which arise in the soil. In accordance with this approach, the reaction of soil microorganisms to pollution is studied only after enrichment of the soil with an energy substrate.

Earlier research has found that multi-component substrates, for example, plant residues, initiate the development of a microbial community, which only uses their easy-to-use ingredients (Zajcev etc., 1979). Therefore, for model experiments, the use of individual compounds is more fundamental than substrates. The development of microbial communities initiated with soluble carbohydrate compounds, such as glucose or sucrose, depends to a large extent on their concentration. The same dose of these compounds in different soils can be used in different ways: oxidation, followed by the accumulation of microbial biomass or fermentation, which monitors the enrichment of the soil with toxic products, but without a significant increase in biomass. In the experiment, it is difficult to control the concentration effect (Guzev etc., 1986a, b).

Therefore, it is more advantageous to use insoluble polymers of plant origin. Cellulose is quickly accumulated and broken down by microorganisms in the soil, which can be controlled well. However, the microbial community, initiated by cellulose, has a small biomass and a relatively poor composition of species, which significantly complicates the work. Starch has unique properties for activation. It regularly comes to the soil in the form of plant residues, is quickly colonized and exploited by many land microorganisms. This is evidenced by the practice of application (in soil microbiology) of starch-based substrates, where the development of various systematic groups of microorganisms is always observed.

The significant complexity of the analysis of the impact of technical pollution is conditioned by the large spatial-time variability of the intensity of the microbiological processes in the soil. This phenomenon, firstly, is related to the micro-zonal characteristics of the spread of microorganisms in the soil and the incontinence of nutrient substrates into it (Zvjagincev, 1987). Second, the technogenic change is difficult to distinguish from the natural dynamics of microbiological processes in the soil. Both types of changes are characterized by the shift of one actively functioning microorganism to another. In one case, the change in organisms in the community is conditioned by pollution, and in the other, there is a change in the activity of ecologically-trophic groups of microorganisms with different development strategies (Guzev, Ivanov, 1986, Zvjagincev etc., 1994). At the expense of the natural dynamics of microbiological processes in freshly harvested soil samples, there may be a significant difference in the activity of different groups of microorganisms. In order to compare soil samples with varying degrees of pollution, these differences need to be leveled. By holding soil samples in an air-dry state, with subsequent simultaneous moistening before the experiment, it is possible to selectively synchronize the activation moment of the hydrolytic group of saprophytic microorganisms. In this way, other groups of microorganisms (oligotrophs and cytoropha) are temporarily derived from the active state (Guzev etc., 1986). For this reason, this work presents the research of various soil samples, which were kept in an air-conditioned state until the experiment.

When describing the microbial community, special attention is paid to the populations of microorganisms, which are bulkyly represented in the active group. Three gradations of abundance were used: dominant microorganisms, microorganisms that are often found, and microorganisms that are rarely found. An example is the description of amylolysis microbial communities of a large number of soils (Figure 1). In the picture, a wide variety of bands of different widths highlight the different representation of microorganisms in the land community.

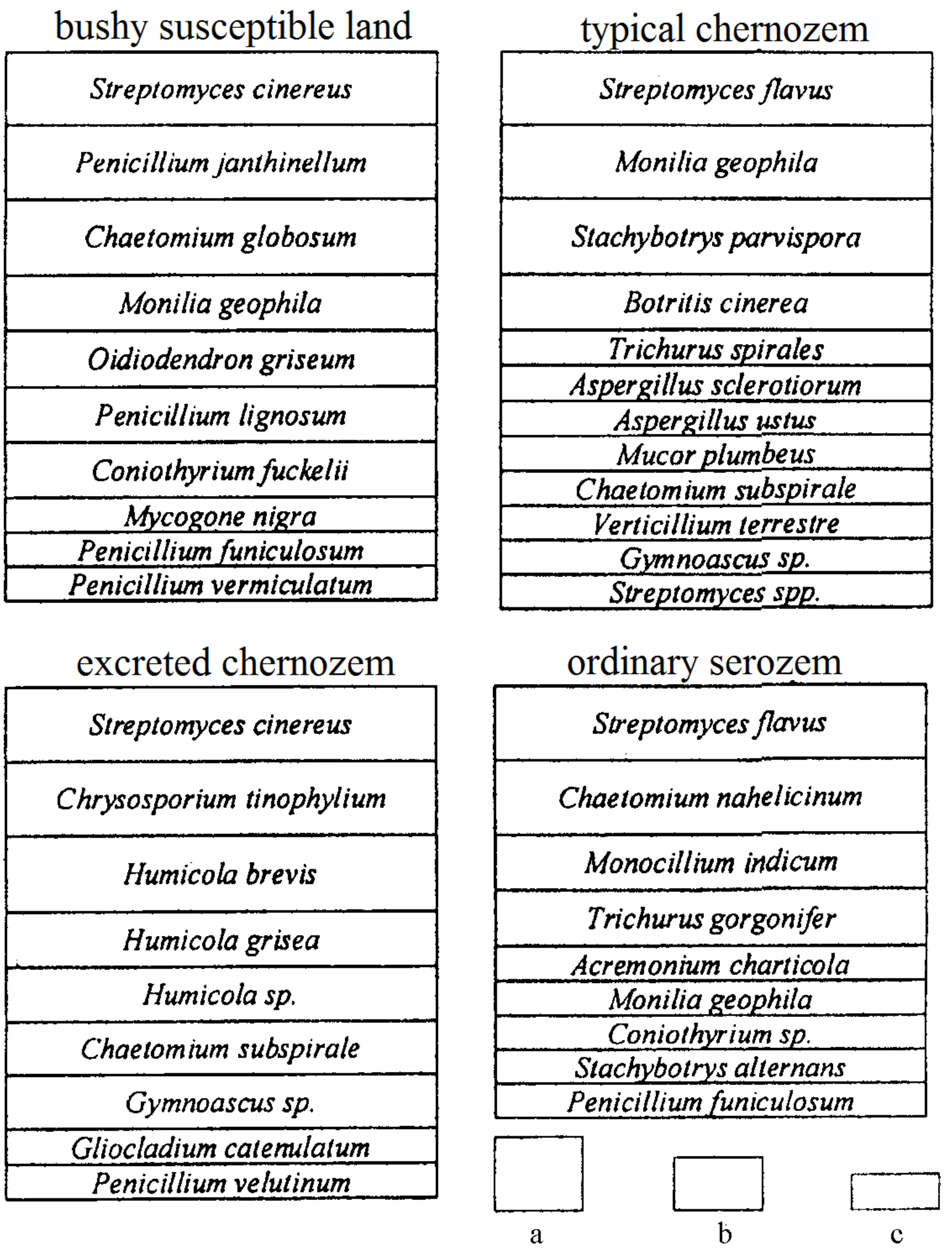
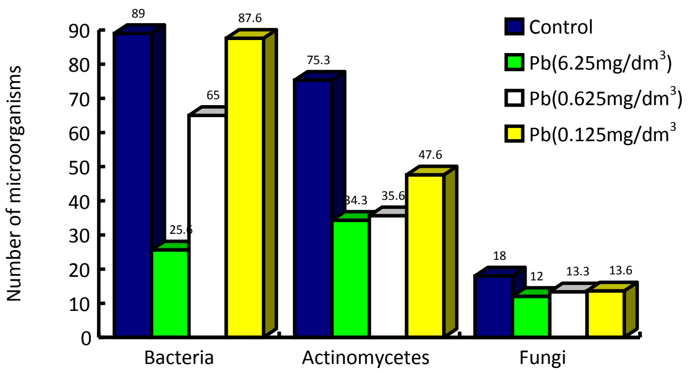
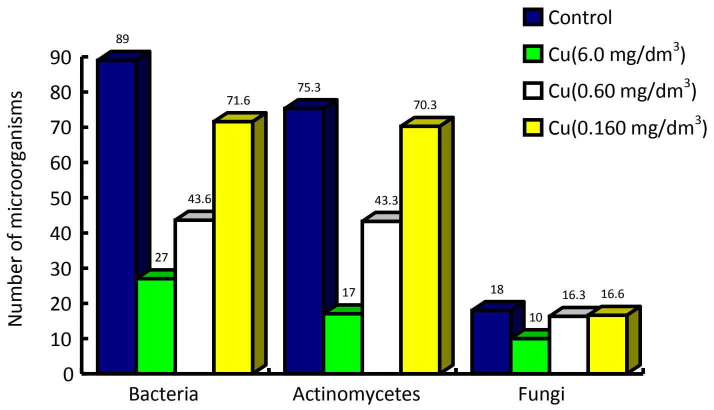


Fig. 1. Composition and organization of amylolytic microbial communities in different soils; a - the dominant level, b - microorganisms that are often found, and c micro-organisms that are rarely found (Guzev, Levin, 2001)

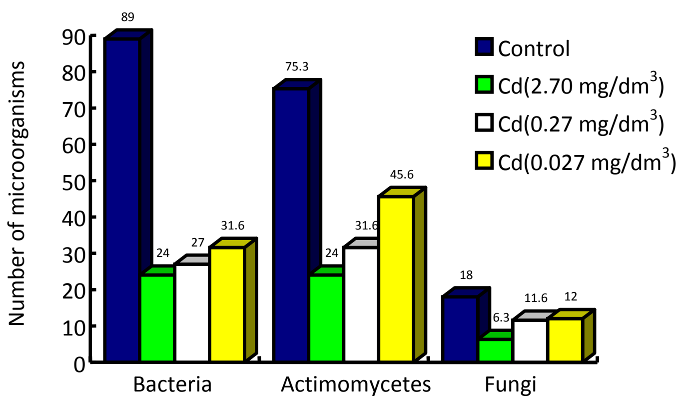
It should be pointed out that using this scale of abundance, very illustrative results are obtained. There are relatively few dominant microorganisms in the communities of different lands, but their composition is significantly different, even in genetically similar soils. The lawfulness of changes occurring in the community of saprotrophic soil microorganisms in technical pollution was studied using the amylolysis microbial community as a real model of the land microbial system. Initially, laboratory experiments determine the tendency of changes in the composition of soil microorganisms under the influence of artificial insemination of pollutants in growing doses. The gradient was the concentration of pollutants from extremely small to extremely large (Đukić, Mandić, 1997; Đukić et al., 1999) - graph. 1,2,3,4. A mandatory requirement for a minimum concentration of any pollutant is the absence of any changes in the microbial community of land compared to control. In addition, during the work on the gradient analysis of biotic communities (Ramenskij, 1938; Witteker, 1980), as a way of presenting test results, a diagnostic diagram is used, with one of the axes representing the concentration of pollutants, and on the other qualitative and quantitative characteristics microbial communities. The reality of legality, expressed in laboratory conditions, is checked by examining samples taken in field trials and in areas that are contaminated due to disasters or chronic disposal of insufficiently purified waste.



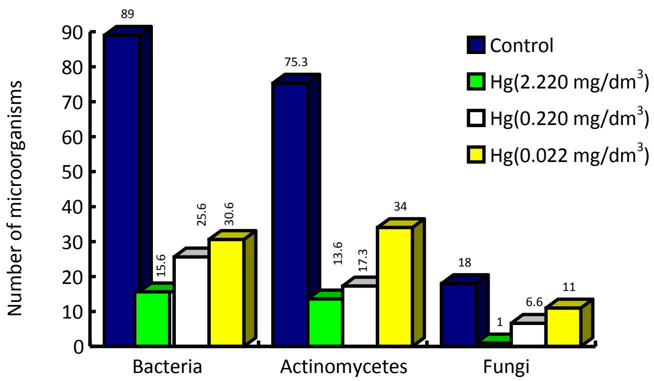
Graph.1. Effect of diverse concentrations of lead on total number bacteria (10^6 g^{-1} abs. dry soil), fungi and actinomycetes (10^5 g^{-1} abs. dry soil)- \uki} i sar., 1999.



Graph. 2. Effect of diverse concentrations of coper on total number bacteria (10^6 g^{-1} abs. dry soil), fungi and actinomycetes (10^5 g^{-1} abs. dry soil)- \uki} i sar., 1999.



Graph. 3. Effect of diverse concentrations of cadmium on total number bacteria (10^6 g^{-1} abs. dry soil), fungi and actinomycetes (10^5 g^{-1} abs. dry soil)- \uki} i sar., 1999.



Graph. 4. Effect of diverse concentrations of mercuty on total number bacteria (10^6 g^{-1} abs. dry soil), fungi and actinomycetes (10^5 g^{-1} abs. dry soil)- \uki} i sar., 1999.

Microorganisms, which colonize a significant part of starch on the surface of the soil, are checked for toxic activity in relation to soil animals and test plants. The selectivity of microbial biomass consumption in soil by microscopic nematodes and ticks is determined, as well as the suppression of germination of seed of the test plant, which is located directly on the biomass of the community. Information on the phytotoxic properties of the dominant microorganisms in the initiated community is used to qualitatively evaluate the changes occurring in the saprotrophic group of soil microorganisms in the pollution process.

It should be pointed out that both the energy substrate, which is used for activation, as well as the conditions for the development of the initiated microbial community, exhibit a certain selective effect. That is, information on the reaction of the microbial community of land to any impact is obtained on the basis of the "answer" of a limited, but rather numerous group of soil microorganisms - amylolithic. Pointing to the existence of a close interaction of microbiological processes in the soil, it can be established that the impact of the technogenic load directly or indirectly involves all parts of the soil microbial system, including the group of amylolysis microorganisms. The experience of experimental work is confirmed by the high indicative properties of this group of microorganisms. This is latter obviously related to the fact that the development of amylolithic, in accordance with the proposed methodology, takes place in non-sterile soil in conditions of natural competition of a large number of populations, capable of using an easily accessible substrate, such as starch.

The basic legitimacy of changes in the microbial soil system under the influence of technogenic pollution

The study of the reaction of microbial communities, which actively functions in soil, to various technogenic pollution, made it possible to formulate the basic setting for the given study: regardless of the nature of the pollutant, the change in soil microorganisms in response to the increasing load, is manifested alternately by four adaptive zones . Under the adaptive zone (Reuters, 1990), a conditional space is defined, limited by the interval of concentrations of the investigated agent, in which a certain entirety of changes in the microbial community, which actively functions in the soil, is observed. Each of the above zones corresponds to a certain level of technogenic excitation.
It turned out that, in the case of various contaminants, it is worthwhile to examine the reaction of soil microorganisms to different levels of technogenic load. On the one hand, this allows studying situations that are approximate to real, because in typical cases pollutants reach the soil at concentrations corresponding to different load levels: heavy metals - from several grams to several kilograms per 1 ha; fertilizers - from a few kilograms to several tons; oil - from thousands to tens of thousands t. On the other hand, the method of exposure of the subject matter allows to comprehensively examine the principally different ecological and microbiological aspects of the problem of optimizing the interaction of man and the natural environment.

Low load level

The first adaptive zone, characterized by a low load level, is the homeostasis zone of the microbial soil system. In the synocular diagram, a low level of loading is illustrated by the example of the action of cadmium on the initiated microbial community of busy-podzolic soil (Figure 2). In this figure, as well as in all subsequent analogue diagrams, the width of each strip (piece of land) bounded by the broken line, conditionally shows the representation of a particular microorganism in the community, depending on the dose of the pollutant. The homeostasis zone is limited to a diaspense concentration of 0 to 7 mg Cd / kg of soil, in which only the summarized biomass of an actively functioning community of microorganisms changes, and its composition and quantitative relationships of species are practically no different from the same in the control soil. Lead acts on the amylolysis microbial community analogously, but the homeostasis zone parameters are expanding - from 0 to 200 mg / kg (Figure 3).

Changing the composition or quantitative relationships of species in the community, in increasing the burden, testify to the transition to the next adaptive zone.

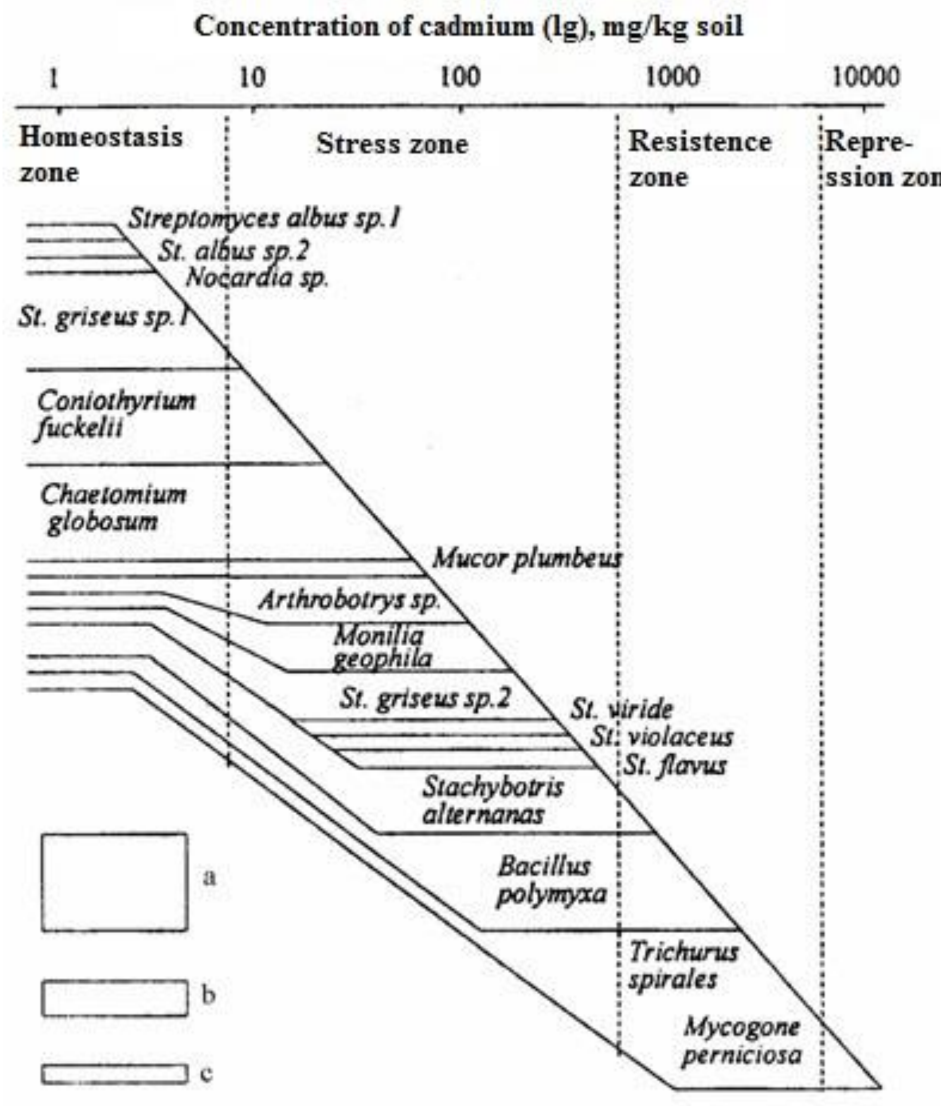


Fig. 2. The influence of the concentration of cadmium (in the form of acetate) on the organization of the amylolytic microbial community of podzolic soil; a- dominant species; b- frequently occurring species; c- rarely occurring species (Guzev., 1985).

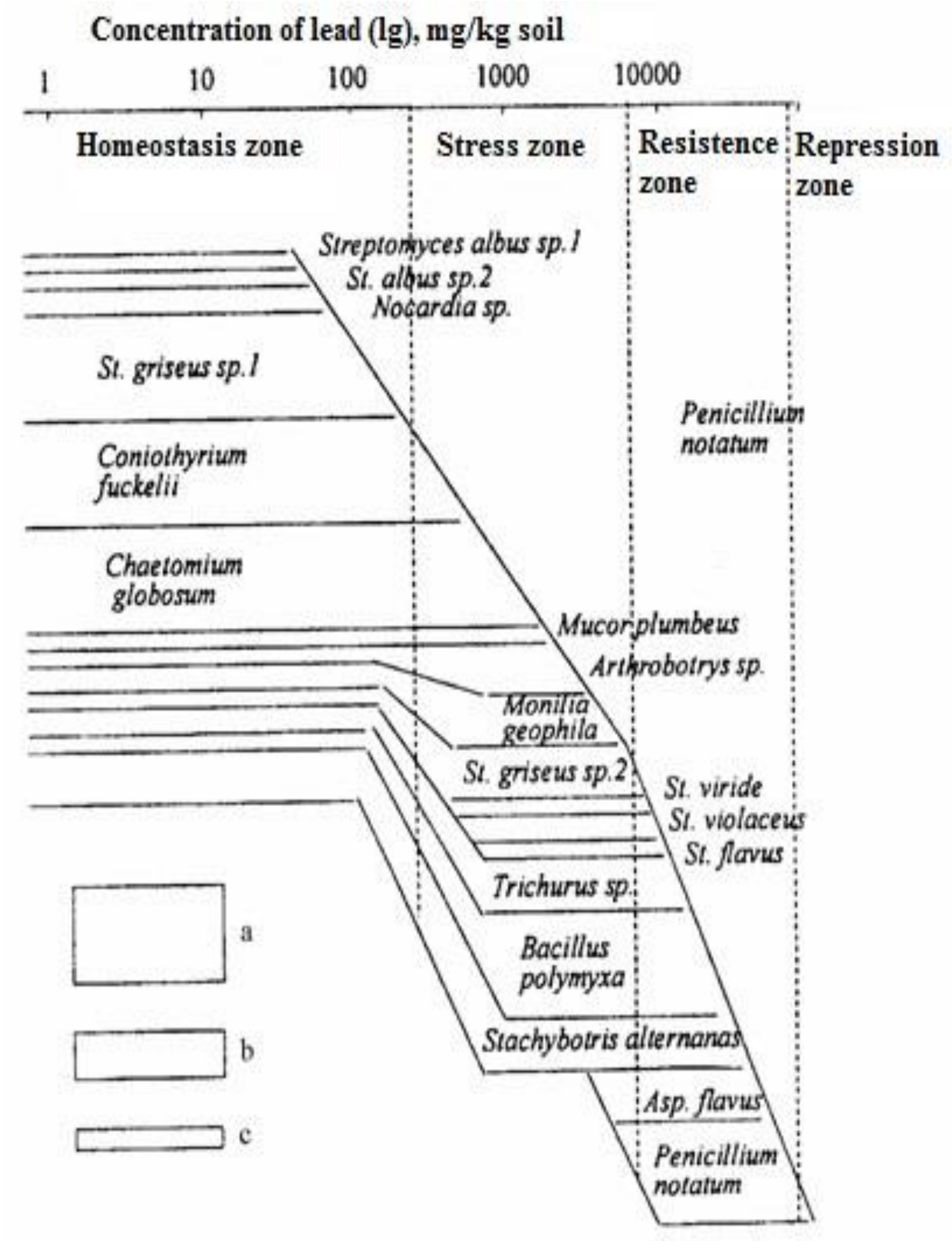


Fig. 3. The influence of the concentration of lead (in the form of acetate) on the organization of the amylolytic microbial community of podzolic soil; a- dominant species; b- frequently occurring species; c- rarely occurring species (Guzev, 1985).

The size of the homeostasis zone quantitatively reflects the comparative toxicity of the various contaminants directly in the soil. Thus, for example, for cadmium (Figure 2) in busy-podzolic soil this characteristic is 30 times smaller than for lead (Figure 3).

Comparison of different heavy metal compounds, according to the size of the homeostasis zone in the particular soil, allows these substances to be placed in a series with decreasing activity: Hg> Cd> Ni> Cu> Pb (tab.1). In the above sequence, the activity of certain compounds, in general, corresponds to their solubility. In this case, practically insoluble oxides of heavy metals in the soil are 2-3 times less toxic than from the well-soluble salts of these elements (Jemcev, Đukić, 2000; Đukić, Mandić, 2000).

Tab. 1. Comparative ecotoxicological activity of a range of heavy metals according to behavior in relation to the microbial system of busy-podzolic soil (Guzev, Levin, 2001)

Active element	**Form of compound**	**Size of the homeostasis zone (mg / kg land)**
mercury	nitrate (well soluble) sulphate (poorly soluble) sulphide (practically insoluble)	2 5 10
Cadmium	acetate (well soluble) oxide (poorly soluble)	7 20
Nikl	nitrate (well soluble)	30
Copper	acetate (well soluble)	70
Lead	acetate (well soluble) oxide (poorly soluble)	200 300

Comparison of the size of the homeostasis zone, as a measure of the potential resistance of soil microorganisms to pollution (tab 1), with other standards accepted in biology and medicine (Table 2), shows that the indicator, proposed by Guzev et al. (1985) in its entirety, corresponds to the concepts of comparative toxicity of heavy metals. It can be seen that, according to each normative, metals form strings that are somewhat different from each other. In doing so, it has been shown that practically in all cases Hg and Cd are the most toxic, while Ni, Cu, and Pb are less toxic. Naturally, the least concentrations accepted as permitted are characteristic of normative nutrients, and the concentrations accepted for the land are the highest. Other norms occupy an intermediate position.

Tab. 2. Comparative toxicity of a range of heavy metals (mg / kg) according to some sanitary and hygienic indicators (Guzev et al., 1985)

Indicator of toxicity	**Hg**	**Cd**	**Ni**	**Cu**	**Pb**	**Literary source**
Background in different land	0.01-1.0	0.01-1.0	5-500	2-100	15-100	*Dobrovoljskij*, 1980 *Rence, Kirstja*, 1986
MDK in the soil	2.1	5.0	35	23	20	*Gončaruk,Sidorenko*,1986 *Ammosova* i dr., 1989
MDK in plants	2.0-5.0	3.0-5.0	100	100	100	*Kloke*, 1979 *Rence,Kirstja*,1986
MDK in food products	0.005-0.7	0.01-0.2	0.4	0.4-30	0.5-3	*Bespamjatnov,Krotov*,1985 *Ribalskij* i dr., 1993
LD_{50}*	18	88	-	43	100	*Bespamjatnov,Krotov*,1985

* lethal dose of reagent, which causes 50% of mortality

The toxic effect of heavy metals on soil microorganisms, determined by the size of the homeostasis zone, is closest to the maximum allowed concentrations (MDKs) of these elements in the soil and is practically always above their minimum basic content. On the basis of all this, it can be considered that the proposed criterion is quite objective.

Medium load level

The second adaptive zone, characterized by the medium load level, is the zone of stress of the microbial soil system. Changes inherent to this zone are observed in experiments with all studied pollutants and are highlighted in all of the above mentioned diagnostic diagrams. The specificities of this zone are considered on the case of the effects of nitrogen fertilizers (Figure 4, Chart 5, 6). Under conditions of laboratory experiment, the stress zone is limited by diapasonic concentration of the agent from 100 to 7000 kg N / ha. In this interval, the concentration of mineral nitrogen, in the active microbial community, comes primarily to the redistribution of populations by the degree of domination. The leading position in the community is gradually occupied by organisms that are rare in the control soil (and in the homeostasis zone), and, on the contrary, the dominant control areas are rarely found. As a result, the composition of the community of stress areas practically does not differ from the control, and the community organization is undergoing significant changes. A further increase in the load leads to a change in the composition of species in the community, which indicates the transition to the next adaptive zone.

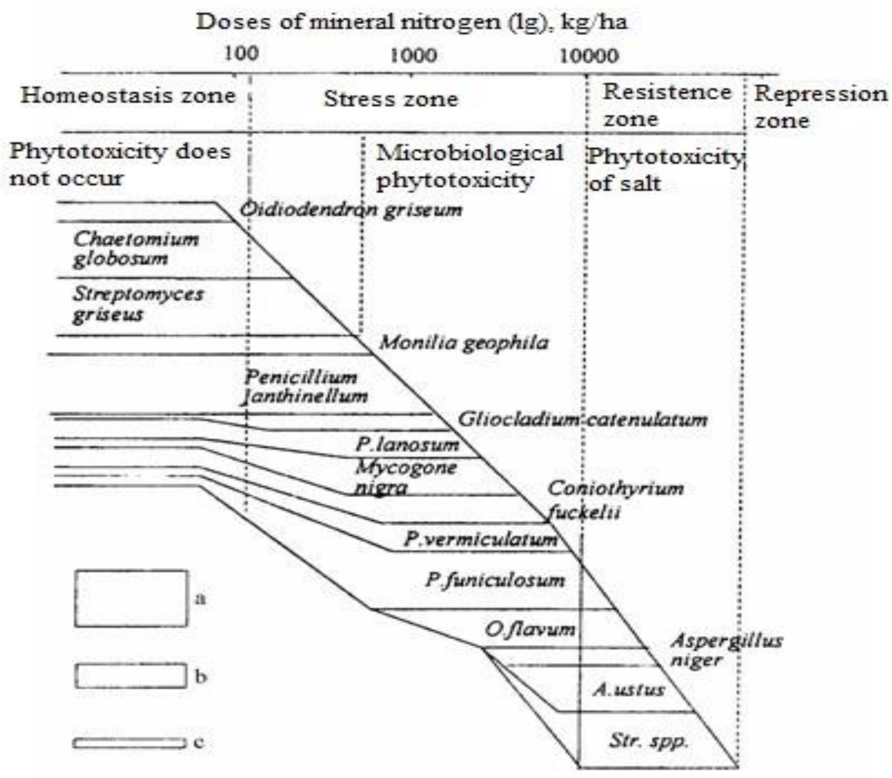
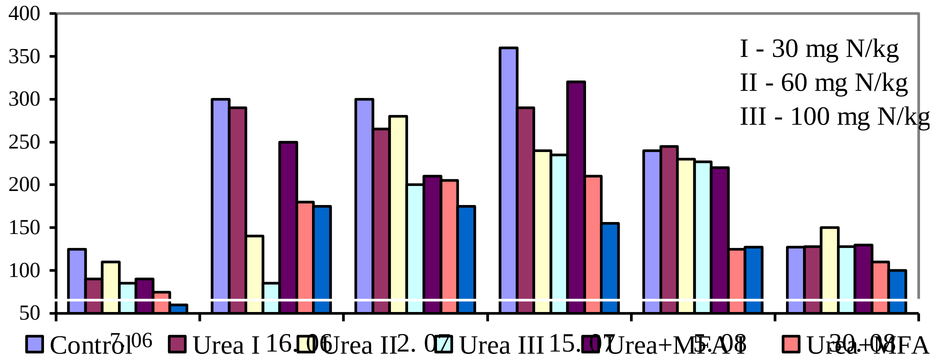
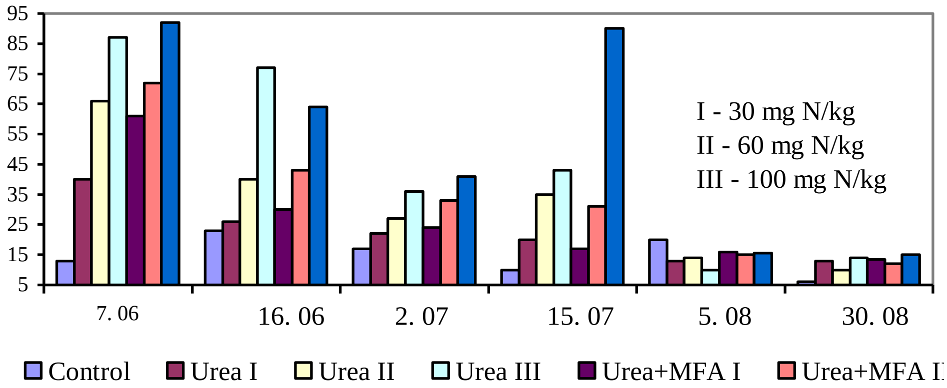


Fig. 4. Effect of mineral nitrogen (in the form of ammonium nitrate) per organization amylolytic microbial communities of podzolic soil; a- dominant species; b- frequently occurring species; c- rarely occurring species



Graph. 5. Changes in potential nitrogen fixing activity in a podzolic soil in the absence of plants under nitrogen fertilisation, x 10^{-3} mg/kg/ha



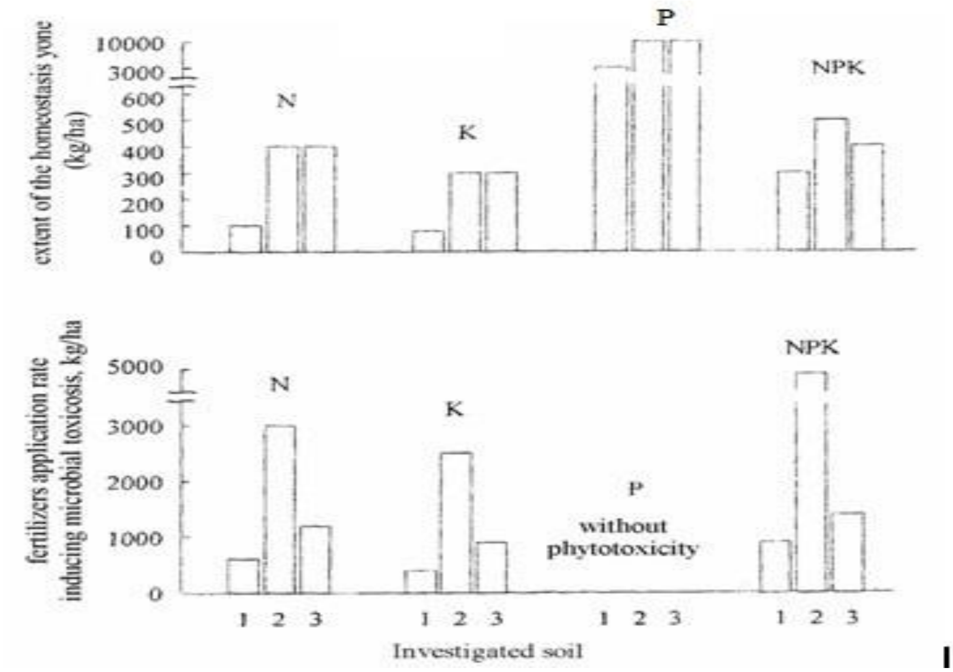
Graph. 6. Changes in potential denitrification activity in a podzolic soil in the absence of plants under nitrogen fertilisation, x 10^{-3}mg/kg/ha

It is very important to emphasize that under the influence of increased doses of nitrogen fertilizers in the community, in addition to structural changes, the physiological activity of soil microorganisms is also changing. For doses of 600 kg N / ha and above, the micromycetes Penicillium funiculosum, P. janthinellum and P. vermiculatum, which dominate the amyloidal community, directly in the soil inhibit seed germination (Figure 4), the development of seed-salad seeds and are not used by microscopic invertebrates - ticks and nematodes.

This phenomenon is called microbial soil toxicity (Guzev et al., 1984a, b). Previously, it was assumed that the leading role in soil toxicity, due to the application of mineral fertilizers, has microorganisms

(Krasiljnikov, 1958, Mandić et al., 2011, 2012; Đukić et al., 2015), however, the soil toxicity is, as a rule, explained by his strong acidification (Mineev, 1990), which directly or indirectly suppresses the plant's activity. However, in the given experiments, the acidity of the soil is slightly changed during the single feeding of mineral nitrogen, within the limits of 0.2-1.0 pH, and only at the highest doses of fertilizer. It should be emphasized that in the doses of mineral fertilizers, which are above the limits of the stress zone, the direct negative effect of these substances on the plants is observed - salt phytotoxicity.

Potassium and a lot of mineral fertilizer in increased doses show the effect of nitrogenous mineral fertilizers on the soil microorganisms (Zvjagincev, 1989; Yemtsev, Djukic, 2000; Djukic et al., 2018a, b), while only various forms of phosphorus fertilizers are not followed by microbial phytotoxicosis of soil Figure 5). In the zonal series, the studied soils differ from one another to the boundary concentrations between the homeostasis zone and the stress, and, in particular, according to the doses of fertilizers that cause microbial phytotoxicity.



Sl. 5. Comparative stability of the microbial systems of busy-podzolic soil (1), derived chernozem (2) and typical serozem (3)) as related to mineral fertilisers (Guzev, Levin, 2001)

The reality of the changes characteristic of the stress zone was shown in field experiments with long-term use of relatively small doses of mineral fertilizers (Figure 6). In the figure of dashed rectangles indicate the composition, and the lines - the quantitative relationship between the species of initiated microbial communities of soil in different variants of the experiment. The amyloidal microbial community of bush-podzolic soil in a variant with thirty years of introduction of 100 kg / ha / year of mineral nitrogen fertilizer is similar to the same in the model experiment with the one-time application of the same fertilizer in the dose range from 600 to 1300 kg / ha (Figure 4). The introduction of nitrogen, potassium and nitrogen - potassium fertilizers has also led to the dominance of the toxinogenic mushrooms of the genus Penicillium (Figure 6). Between the degree of microbial soil toxicity and the yield of agricultural crops, a close correlation was established: in these variants of the Polish experiment yields of all crop cultures decreased by 30-60% of control (Gomonova, 1984). It was further established that, when introducing the nitrification inhibitor (Popov et al., 1990; Đukić et al., 2009), some herbicides and certain heavy metals (Zvjagincev, 1989; Mandić and Đukić, 2010; Đukić et al., 2011) concentrations, corresponding to the stress zone, in the amyloidal community, toxinogenic microorganisms are predominantly developed. This allows for the very wide spread of the occurrence of microbial toxics and is marked as negative (i.e., which deteriorates the natural fertility of the soil) the medium load property.

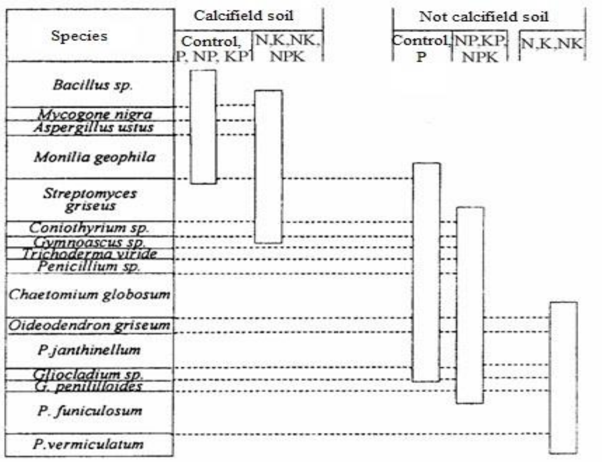


Fig. 6. Organization of the amylolysis microbial union of subsoil soil in field examination in calcium and the application of various mineral fertilizers; dominant level, microorganisms that are often and rarely found are presented with "strips" of different widths (Guzev, Levin, 2001)

The effect of eliminating toxinogenic microorganisms in the community, which is the opposite already described, is determined by the calcium acidification (pH 4.2) of bushy-podzolic soil. In this case, in the conditions of the laboratory experiment, a significant increase in the stability of the microbial soil system is observed on the high doses of mineral fertilizers. This reflects on the increase in the size of the homeostasis zone and the dose of fertilizer, which causes microbial phytotoxicosis (Table 3).

Tab. 3. The influence of calcium on the stability of the microbial system of busen-podzolic soil according to different doses of full mineral fertilizer (Guzev et al., 1985)

Dose of lime, hydrolytic acidity	**Doses of full mineral fertilizer, kg / ha**	
	Homeostasis zone	**Microbial toxicology**
0	300	1000
0.5	400	1300
1.0	500	1500
2.0	500	2500
4.0	600	3000

High load level

Resistance zone (zone of development of resistant forms of microorganisms) is the third adaptive zone of microbial soil system. It is characterized by a high level of loading and is exhibited at high doses of all studied pollutants, regardless of their nature. The characteristics of this zone are demonstrated in the case of oil effects (Figure 7). In the conditions of the laboratory experiment, the resistance zone is approximately "bounded" by the oil concentration range from 10 to 100 dm3 / m2 of soil. In this diapason, the dose of oil revealed radical changes in the composition of the amylolysis microbial community. Its variety of species is rapidly decreasing, but predominantly they do not develop for typical soil, but rather on the given factor of the highly resistant population of microorganisms. In bushy-podzolic soil such microorganisms are presented to the species of micellar mushrooms Aspergillus Ustus and Penicillium Tardum. These micromycetes are not characteristic of the land of a given type, but are more often adapted to the soil, enriched with organic matter. The composition of the amyloidal community, established in the zone of resistance, has nothing in common with the same in unpolluted soil. The total biomass of microorganisms in the community, in this interval, concentration of oil, as well as other pollutants, is, as a rule, stable, though its characteristic is not the same in the action of different reagents. A further increase in the load leads to the complete elimination of growth and the development of microorganisms in the soil. This testifies to the transition to the next (fourth) zone - the zone of repression of the microbial system of land, which is characterized by a catastrophic level of technogenic pollution.

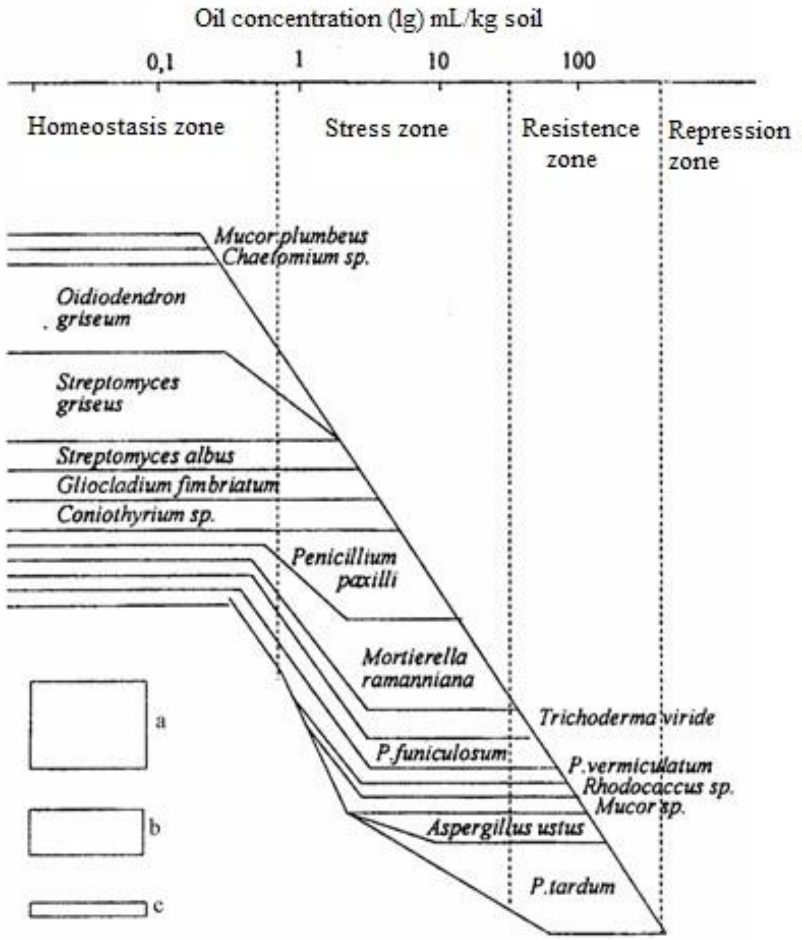


Fig. 7. Influence of the crude oil concentration on the organization of the amylolysis microbial substance of bushy-podzolic soil; a-dominant level, b-micro-organisms that are often found and c-microorganisms that are rarely found)

Gradient analysis of changes in the microbial soil system under the influence of technogenic load

The fundamental feedback of the community to increase doses of pollutants is to gradually inhibit the sensitivity and natural selection of resistant populations. Keeping in the framework of the given scheme Guzev etc. (1985) have been devoted to changing the given model. Their concept consists in the following: the return reaction of the soil microbial system to pollution can be in the basic form characterized by four types of changes in the community of microorganisms, which in the soil function actively. With the gradual increase in the technogenic load, these changes alternate, forming four adaptive zones.

The zone of homeostasis of the microbial soil system comprises a range of active agent concentrations, in which the composition and quantitative relationship of species in the community are unchanged. The total biomass of microorganisms in the community, as well as their activity, can grow somewhat, testifying to the basic stimulative effect of low concentrations of contaminants on microbiological processes in the soil.

The stress zone is a range of concentrations of the active agent, in which the composition of the active microbial community in the soil remains virtually unchanged, while the quantitative relationships of species are subject to significant changes. This is reflected in the redistribution of populations in the community according to degree of domination. The total biomass of microorganisms in the community, which expresses the intensity of microbiological processes, varies greatly in this adaptive zone. In the stress zone, the microbial system responds to changed environmental conditions, through the modification of its organization, in virtually unchanged composition.

The resistance zone is determined by the diapasonic concentration of the acting agent, in which there is a sudden decrease in the variety of species and the change in the composition of the microorganisms that actively function in the soil. Populations of microorganisms that are resistant to high doses of the given substance are developed. The stability of the overall biomass of the community is typical for this zone. The functioning of the microbial system in a given technogenic load interval is shown to be possible only at the expense of the realization of the maximum capabilities of some highly resistant populations, which were previously found in soil in limited quantities.

The zone of repression of the microbial soil system is characterized by complete suppression of growth and development of microorganisms in the soil. This type of change testifies to the complete exhaustion of the reserves of resistance of the living world of the land to the active burden and the inability to function microorganisms in the given conditions.

DIAGNOSTIC PROPERTIES OF DIFFERENT LEVELS OF SOIL CONTAMINATION

The specific properties of the adaptive zones of the microbial soil system, which were singled out by Guzev et al. (1985), have been determined by the action of different pollutants and are experimentally well described. On this basis, they can be proposed for the diagnostic properties of different levels of soil contamination. For the reference point, the basic content of contaminants in virgin lands, land of a specific geochemical region can be taken (Zirin etc., 1985). Such land may be considered unpolluted.

In the concentration of pollutants, which exceed the pollution base several times, only quantitative changes of the intensities of the microbiological processes in the soil can be determined (Panikova, Percovskaja, 1982; Jemcev, Đukić, 2000; Đukić et al., 2018 b, c), without any what qualitative disturbance of the state of the living world in the soil. However, significant spatial and temporal variability of these indicators greatly complicates their use for the diagnosis of pollution (Cairns, 1984; 1986, Zvjagincev, 1989). In addition, the continual character of the change in quantitative parameters does not allow to determine the boundaries between contaminated and unpolluted land. Bearing in mind this and taking into account that the concentration of pollutants in the soil is above the basic, it is suggested that the given load is considered a low level of pollution, which does not exceed allowed. Such a level of pollution has no subsequent effect, so the system easily returns to the starting state in case of elimination of the operation.

With the further increase in pollutant content, microorganisms are redistributed according to degree of domination, as a result of which negative phenomena can develop in soil. On the basis of this last one, one can speak of overcoming the norm, and on this basis, isolate the average level of pollution. As a diagnostic feature of a given level, a certain reduction in the diversity of species of microorganisms in contaminated soil and the increase in the participation of toxinogenic organisms in it can be called (Jevdokimova et al., 1984; Mirčink, 1988). As a supplementary property of this level of pollution, a sharp decrease in the variability of the indicators, characterizing the biological activity of the soil (Zvjagincev, 1989), and the increase in the participation of pigmented forms of microorganisms in soil

(Levin, Babjeva, 1985; Richardson et al., 1985) can be highlighted. These changes are characterized by a clearly expressed subsequent effect after removal of the load.

Changes in the living world in the soil, observed when the concentration of the pollutant exceeds the basic content several times, testifies to the high level of pollution. The diagnostic properties of this level are a sudden decrease in the microbiological activity of the soil, according to many indicators, and the development of a very limited number of resistant forms of microorganism. Land with such concentrations of pollutants is characterized by high total toxicity not only in relation to microorganisms, but also more organisms (Zvjagincev, 1989). And, in the end, practically completely suppressing the activity of soil microorganisms can be emphasized as a diagnostic feature of a catastrophic pollution level.

MICROBIOLOGICAL INDICATION OF SOIL CONTAMINATION WITH EXOGENOUS CHEMICAL COMPOUNDS

For the purpose of bioindication of different levels of soil contamination in real terms, the following is proposed. Low level can be determined on the basis of exceeding the basic concentrations of pollutants, which is determined using chemical analysis methods (Dobrovolsky, 1980; Zirin et al., 1985).

Microbiological indicators are the most appropriate for the indication of the mean level of pollution, among which it is considered the most important - the change of the dominoes between the microorganisms, which are active in the soil. It is therefore necessary to experimentally determine the given phenomenon, as well as its immediate connection with the operation of the investigated pollutant. In order to solve this problem, a method of indication was developed based on the reaction of the amylolysis microbial community to the soil of the "stress" dose of the pollutant. This latter equals approximately the doubled concentration, which determines the homeostasis zone. Injecting such a dose is necessary for the microbial community of unpolluted soil to be translated from the homeostasis zone to the stress zone. If there is no change in the domains in the initiated community after the dosing of the stress dose, then their redistribution should be considered in real conditions. Thus, it can be considered that the soil studied is characterized by a medium load level.

This method was examined in field and vegetation trials and enabled the realization of (biosecurity) of the medium level of soil contamination by heavy metals and pesticides. We consider that adequate graphical presentation of the results obtained by the given method is presented in

Figures 9 and 10. In the first case, the pollution of the soil by the mercury is registered (Figure 9), and in the second - different combinations of three pesticides: simazine, SSS and benomy). As an additional indicator of the medium pollution level, the reduction in the variability of soil biochemical process indicators, the reduction in the richness of species and the diversity of soil microorganism complexes, and the increase in the proportion of toxinogenic microorganisms in it.

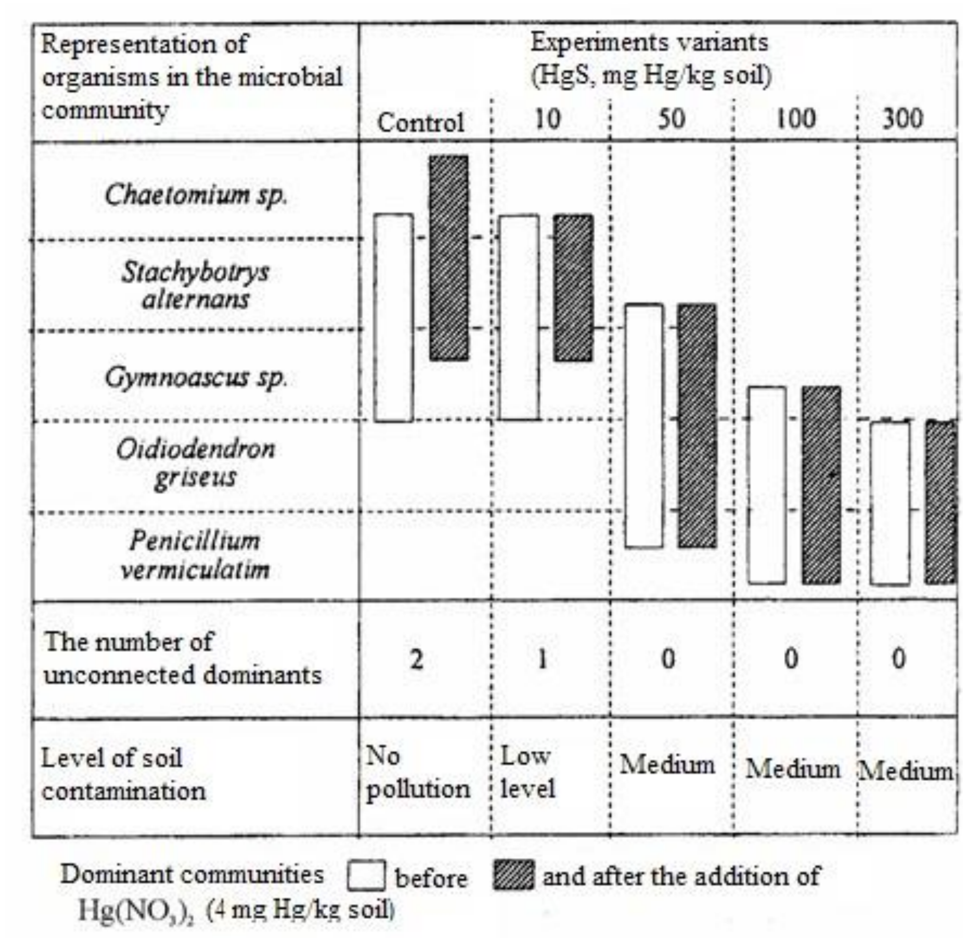


Fig. 9. Change of the dominant composition of the amylolysis microbial joint of bushy-podzolic soil in response to the supplemental introduction of HgS into the soil (Guzev etc., 1985)

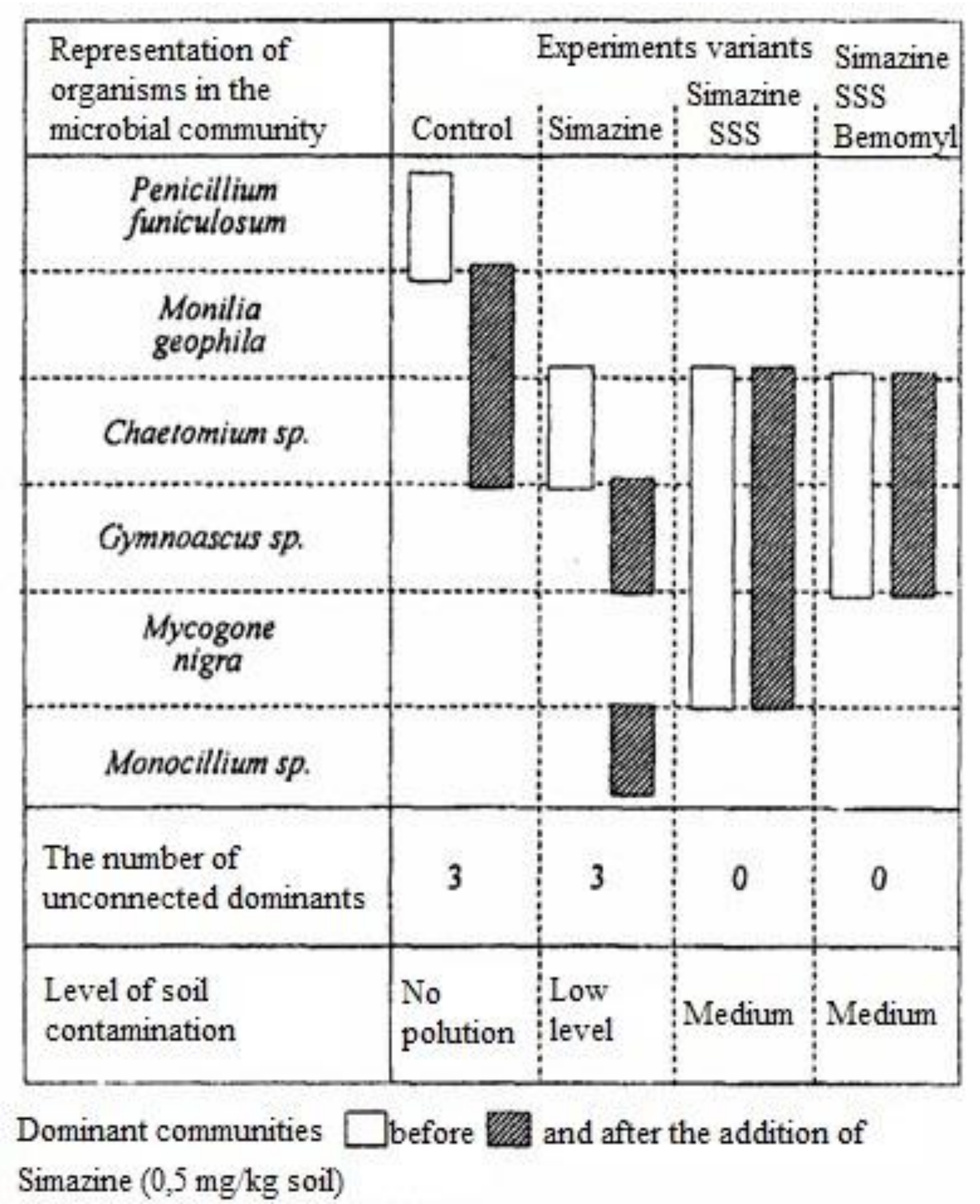


Fig. 10. Modification of the dominant composition of the amylolysis microbial joint of bushy-podzolic soil in response to the additional introduction of simazines into the soil (Guzev etc., 1985)

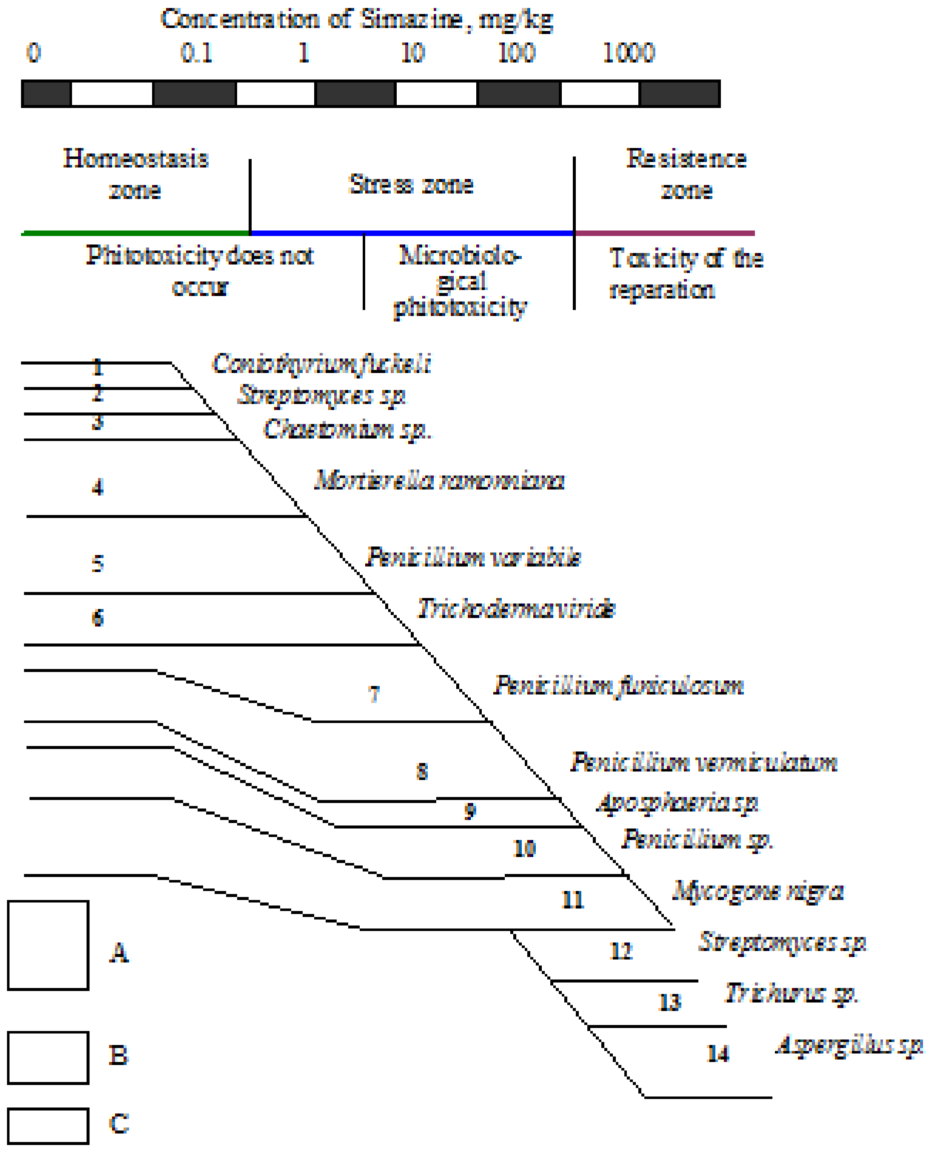


Fig. 11. The effect of the simasin on the composition and organization of the amylolysis microbial community

A-dominant species; B - species that are often found; C - a rare type

For the indication of a high level of soil contamination, many microbiological indicators are appropriate, for example, the widespread distribution of highly resistant microorganisms in the microbial soil community (Marfenina, 1981). However, we believe it is more appropriate to use other tests for it. Since soil at a given level of pollution is characterized by high toxicity, it is easier to determine the existence of that level using the reaction of higher organisms, for example, plants (Dobrovolsky, 1980; Zirin et al., 1985; Ilyin, 1986).

In this way, the proposed microbiological method of indicating contamination allows for a fairly reasonable determination of the mean level of burden of the living world in the soil, whereby the concealed negative consequences of the technical impacts on the soil can be determined.

CONCLUSION

Constant control of the state of the environment must rely on fundamental law, but in real conditions it is necessary to use, first of all, simple procedures and met . This latest request corresponds to today's most developed concept of maximum permissible concentrations (MDK) of pollutants in the soil. This norm is applied in the following way: one of the modern expression methods measures the content of the pollutant in real land, then it is compared with the value of MDK and on the basis of this comparison, a conclusion is drawn on the degree of contamination of the given land. In such a maximally formalized approach, the assessment of the state of the soil consists of its obvious advantage. However, it seems to be apparent (Dobrovolsky etc., 1985).

On the one hand, MDK pollutants in the soil are being developed, modeling experimental conditions and using a large group of different indicators and test objects, and then the strength of the law is added to that norm. However, for its application in real conditions it is necessary to introduce corrective coefficients, which take into account the properties of concrete land (Goncharuk, Sidorenko, 1986). Determining these coefficients requires a lot of severe regional irradiation, which, obviously, are not less extensive and versatile than in determining the MDK itself. In addition, the need to determine the impact of the plethora of exogenous substances that enter the soil, and which interact in different land-climatic zones, significantly complicates both the process of standardization and control.

On the other hand, the concept of MDK causes serious criticism and essence. From the aspect of global (basic) monitoring, it seems that this norm is unreasonably overestimated, since the concentrations corresponding to them often approach the threshold and sometimes critical values of pollution

(Ausmus, 1984; Krivoluckij etc., 1987; Stepanov, 1988). In this case, the basic MDK function is not fulfilled: to guarantee an acceptable (allowed) entry into the surrounding environment of a certain amount of exogenous matter, without any negative consequences for the biosphere. From the point of view of local monitoring, MDK is, to the contrary, too low, because on this basis it can be considered that most of the modern land is overcrowded (Zirin etc., 1985). This significantly impedes the focus of efforts on the implementation of concrete measures for nature protection and the prevention of local critical situations, as the abandonment of any kind of production appears as a uniquely acceptable alternative to any technical impact.

For local monitoring, it seems that a more constructive idea is the comparative expertise of several alternative technologies in order to choose the one that best preserves nature (SCOPE 5, 1979; Smathers et al., 1983). It is assumed that ecological expertise will be performed by specialists of different profiles who are obliged to evaluate the impact of different factors on the environment, health and well-being of people, and also predict the possible consequences of technogenic load by prince of analogous situations. In doing so, each specialist expert will evaluate the reaction of "your" object to a certain effect, with the help of government structures to make a qualified choice between proposed technological variants.

The proposed concept of ecological and microbiological assessment of the state of the land, on the one hand, was elaborated on the basis of the examination of the fundamental laws of changing the living world in soil under conditions of technogenic pollution (Guzev etc., 1985); on the other, contrary to the methodical characteristics of its realization, it can be used for the expertise of the state of the land. The proposed method of microbiological indication does not imply sharp requirements in relation to control samples, since the absolute, but relative, differences in the feedback reaction of the microbial community to the supplementary introduction of the pollutant are not compared. To perform the indication, there is no need for a high level of qualification in the field of the microorganism systematics, because in the microbial community it is sufficient to distinguish the dominant forms, which have clear morphological features. In addition, their identification to a species is often not needed: for practical purposes, it can be operated with mofrological types, which, as a rule, appear as different types. Based on this, it can be concluded that the proposed approach and the ways of its realization are quite simple, accessible for practical application, and therefore can be used in solving the basic task - optimizing the interaction between man and the natural environment.

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PARAMISTOMIDAE SPECIES IN SMALL RIMINSNTS IN SERBIA

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ABSTRACT

Paramphistomatidae are the most important trematods of rumen and reticulum in ruminants. They elapse their maturity period in rumen and reticulum and have no changes in these organs and risk for host. But there are larval forms of these trematods in small intestine while generate pathologic changes there. During study about helminthes of small ruminant at Serbia were performed in period between 2009 and 2019. After histological determination, we concluded that occurred paramphistomided belonging to the species Paramphistomum cervi and Paramphistomum microbotrium P.cervi has been found in sheep and cattle in Serbia, but during our examination P.cervi and P.microbotrium was first time occurred at goats.

Key words: paeramphistomidae, small ruminants, Paramphistomum cervi, Paramphistomum microbotrium

INTRODUCTION

Way of breeding of sheep and goats had prerequisite to a lot of infections especially parasitoses. They are usually kept under extensive conditions and graze or brows on any land that is not being cultivated. Pasture breeding make possible contact within small ruminants and eggs, larvae stages and intermediate host of parasites. In pasture breed condition helminth infection are common especially during late spring and autumn months (Pavlović et al.2012). Research of goats and sheep parasites were made sistematic during last 20 years in Serbia, especially in period between

2011 and 2019, and we have more new data about them. During that study investigations performed in the hilly areas of Serbia (Šar Planina, Stara Planina) (Pavlović et al.1995, 2015a), south, south east and west part of Serbia, (Pavlović et al.2013a,b, 2018a), at Timok District (Ilić,1990, Ilić i sar.1991Pavlović et al.2011), Belgrade area (Pavlović et al.2011,2012) Vojvodina (Pavlović et al.2018b) and South Kosova (Milanović et al.2018).
In our paper we presented results of occurrence of platyhelminth infection with specially care to platyhelminth of genus *Paramphistomidae*.

MATERIALS AND METHODS

During study we collected fecal samples and examinations were performed using standard coprological technique with saturated NaCl solution and sedimentation (Euzeby,1981, Pavlović i Rogožarski,2017). Eggs per gram count (EPC) and degree of infection we assessed by McMaster technique where EPC of 50-700 eggs we treated like low rate of infection, to 1100 like moderate and up 1100 like high. At same time we performed post-mortem examination of dead or slaughtered animals. Found parasits we collected, preservated and determinate by morphological characteristic. Determination of parasites and parasites eggs we performed by keys given by Euzeby (1981).

Found paramphistomidae parasites were fixed in 10% buffered formalin and prepare to histological examination. Selected parasites were embedded in paraffin, sectioned medio-sagittal to 5-6 micrometers, and stained with hematoxylin-eosin. Identification of these flukes was originally based on morphological criteria established by Näsmark (1937). These criteria were later revised by Eduardo (1982a). Identification is based o the morphology of the acetabulum, pharynx, terminal genitalium, tegumental papillae, and internal organs of flukes.

Determination of species we performed based the morphological characteristic as observed of acetabulum and the genital atrium at histological cuts of parasites. The acetabulum was examined for determination of genera and the genital atrium and acetabulum for determination of Paramphistomidae species. The dorsal part of the acetabulum was characteristic. The dorsal circular muscle was divided into two parts, the dorsal exterior circular muscle series 1 and the dorsal exterior circular muscle series 2. These circular muscle layers are used for the determination of the genus *Paramphistomum*. The ventral exterior circular muscle series, the ventral interior circular muscle series, the radial muscle fibers, the external longitudinal and median circular muscle series of the acetabulum specifically identified the parasites species (Vujić 1965, Vishnyakov 1980).

RESULTS AND DISCUSSION

During our examination *Paramphistomum* species were occurred at 11.24% of examined goats and 12.48% sheep. Two paramphistomum species were established *P.cervi* and *P. microbothrium. P.cervi* was most abundant then *P. microbothrium.* Number of *P.cervi* found in sheep was average 197.96 ± 521.850 and 73.31± 281.612 in goats. Number of *P. microbothrium* found in sheep was average 201.96 ± 321.850 and 59.31± 281.612 in goats. The highest infection in the sheep infected with Paramphistomum spp. was found during the summer (July to August) (6.7, 2 %) and followed by the autumn seasons (November to October).

According to Eduardo (1982a) the body surface of *P. cervi* is lacking tegumental papillae, the genital opening of *P. cervi* is of gracile type. According to the literature, the genital atrium of *P. cervi* is located at the level of the posterior part of the oesophagus (Willmoth, 1950), which is more posterior than in flukes studied by us. The genital atrium of *P. cervi* is located at the level of the posterior part of the oesophagus and the absence of tegumental papillae observed in *P. cervi* is just a normal morphological variation seen in one species. These entire morphological characteristic we occurred during our determination of occurred paramphistomides to concluded that was *Paramphismoumu cervi.*

At *P. microbothrium* dorsal part of the acetabulum was characteristic. The dorsal circular muscle was divided into two parts, the dorsal exterior circular muscle series 1 and the dorsal exterior circular muscle series 2. These circular muscle layers are used for the determination of the genus *Paramphistomum* . The ventral exterior circular muscle series, the ventral interior circular muscle series, the radial muscle fibers, the external longitudinal and median circular muscle series of the acetabulum specifically identified the parasites as *P. microbothrium* (Vishnyakov, 1980; Samnaliev, 1981).

At numerous infected animals during necropsy (or post mrtal examination at slaughter house) we found visible lesions include muscular atrophy, subcutaneous edema, accumulation of fluid in body cavities and duodenal mucosa superior portion thickening, Bloody mucus in intestinal and sometimes, ulcer and hemorrhage have been recorded in the bowel mucosa. The largest number of adult parasites of both species was found in the rumen and, to a lesser extent in the omasum and reticulum. At the the rumen - primary site of infestation, we occurred destruction of the papillae as well as hyperplasia of the epithelium and inflammatory reaction with the lymphocytes, similar to that described by Singh et al (1984), Pavlović et al. (2007) and Seck et al. (2007).

Young parasites were found attached just distal to the pylorus, with the wall and folds so thickened that the intestinal lumen was almost completely occluded. In those places they looked like brownish-pink cluster in the mucosa of the duodenum. In the mucosa we had a visible erosions and minor haemorrhages and the intestinal content was discoloured red. In those places serosa was reddened, blood vessels enlarged and prominent. Within the pale areas there were irregular patches up to 1mm in diameter.

Although infections with trematodes are less frequent, related to gastrointestinal helminths, they can also cause serious health problems, including fasciolosis and distomatosis (Pavlovic et al., 2007). Paramphistomiasis is a seldom-reported platyhelminth infection in ruminants (Horak 1971, Silvestre et al.2000) . The development of *Paramphistomum* sp. includes an intermediate host – a snail of the genus *Bulinus* (Soulsby 1977) After the ingestion of the metacercaria by the final host, the development is completed after the passage through the rumen, abomasum, and small intestine (Vujić, 1965). The prepatent period is 8 week in cattle and 10 week in sheep (Rangel-Ruiz et al., 2003) and under normal conditions, the complete infection cycle takes 3-4 month. The disease is characterized by sporadic epizootics with acute parasitic gastroenteritis, followed by high morbidity and mortality of predominantly young animals (Seck et al., 2007).

Infections of paramphistomidae are worldwide spread, especially at Africa countries and East Asia (Sissay et al.2007, Seck et al.2007). In Southern and Eastern Europe, the species *Paramphistomum microbothrium, P.cervi* and *P. ichikawai* (Horak, 1971, Kotrlá and Kotrlý 1982, Vishnyakov, 1980, Silvestre et al.2000) have been recorded in domestic and wild ruminant. In Serbia, *P.cervi* has been found in sheep and cattle and *P.microbothrium* has been found in sheep and cattle as well as in deer and red deer (Vujić and Petrović, 1971, Pavlović et al., 2007,2012a).

CONCLUSIONS

During our study performed in 2009-2019 were occurred two *Paramphistomum* species were occurred at 11.24% of examined goats and 12.48% sheep. *P.cervi* was most abundant then *P. microbothrium.* In Serbia, *P.cervi* and *P. microbothrium* has been found in sheep and cattle, but during our examination both species of paramphistomidae was first time occurred at goats.

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EVALUATION METABOLIC STATUS IN HOLSTEIN HERD IN CENTRAL SERBIA DURING TRANSITION PERIOD AND FULL LACTATION

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ABSTRACT

The objective of the present study was to evaluate metabolic status in late pregnant (n = 12) early lactation (n = 12) and full lactation (n = 12) Holstein dairy cows on the basis changes of blood concentrations of selected biochemical indicators. The various blood metabolites and serum enzyme activities were determined by spectrophotometric methods. The lipomobilization indicator, serum beta-hydroxybutyrate (BHB), and non-esterified fatty acids (NEFA) concentrations were significantly increased (P <0.05) in early lactation cows than in late pregnant and mid lactation cows. The significantly lower (P <0.01) blood triglyceride (TG) and total *cholesterol (tChol.) levels in the postpartum cows indicated their accumulation in liver cells. The lower blood levels of glucose ($P < 0.05$), total protein (TP) ($P > 0.05$), albumin ($P > 0.05$) and urea ($P<0.05$) were recorded in the postpartum cows, which suggested the reduced synthetic capacity of liver cells. Some cellular lesions as evidenced by significant increases ($P < 0.01$) in the serum total bilirubin (tBIL.) concentrations and enzyme activities of aspartate-transaminase (AST), gamma-glutamyltransferase (GGT), alanine-aminotransferase (ALT) in early and full lactation cows. This study suggests that early lactation cows showed physiological adaptive changes, which were associated with subclinical ketosis and mild fat infiltration of liver cells .*

Keywords *- holstein dairy cows, transition period, full lactation, blood metabolites, blood enzymes.*

INTRODUCTION

Ketosis and fatty liver are closely linked and responsible for severe economic losses in dairy herd due to declining milk production and reproductive performance, and increasing culling rates. A metabolic profile, as a series of specific blood analytical tests, is routinely used to estimate metabolic disorders in dairy cows. It includes collecting blood samples from dairy cows at 4 time periods relative to calving (late pregnancy, early lactation, full lactation and mid lactation) and estimating selected blood indicators (Oetzel, 2004; Gross *et al.*, 2011). Clinical ketosis in dairy cows usually occurs between the second and seventh week of lactation. Nevertheless, most of the dairy cows in this period of lactation may have subclinical ketosis defined as increased blood ketone bodies, without any other symptoms, with decrease in milk yield and other metabolic problems (Duffield, 2000). Dairy cows have negative energy balance (NEB) during the first week of lactation due to energy expenditure associated with milk production and limited feed intake, resulting in NEB, a high mobilization of lipids from body fat reserve, and hypoglycaemia in early lactation (Veenhuizen *et al.*,1991; Overton and Waldron, 2004; Gonzales *et al*, 2011; Djokovic *et al.*, 2015). The main blood indicators of lipomobilization in dairy cows are BHB, the most important ketone body, and NEFA (Oetzel, 2004; Gonzales *et al*, 2011; Civelek et al.,2011). NEFA are accumulated as TG in the liver, primarily because of a decrease in the very low density lipoproteins (VLDL) synthesis by liver cells (Herdt et al., 1983; Sevinc *et al.*, 2003). However, under lipidosis conditions, endogenous liver synthesis decreased, resulting in reduced levels of blood glucose, TP, albumin, globulin, tChol., TG and urea (Sevinc *et al.,* 2003; Bobe,2004; Djoković *et al.,* 2011).

Furthermore, the excretory capacity of hepatocytes is decreased and, accordingly, the blood concentrations of some metabolites such as tBIL, ammonia and bile acids are generally increased (West 1990; Sevinc *et al.*, 2003; Bobe,2004; Djoković *et al.,* 2011). Fatty liver infiltration and hepatocyte degeneration involve cell membrane damage and hepatocyte destruction coupled with the release of cytoplasm enzymes and significantly increase in the circulating activities (Pechova *et al.,* 1997; Lubojacka *et al.*, 2005; Stojevic *et al.*,2005).

The objective of the present study was to evaluate metabolic status in transitional and mid lactations dairy cows on the basis changes of blood concentrations of selected biochemical indicators.

MATERIALS AND METHODS

A total of 36 dairy cows were selected from the same Holstein herd containing 445 cows (FARM: Sarulja, Kragujevac, Central Serbia). Three groups of clinically healthy cows were chosen from the herd. Group 1 consisted of late pregnant cows (n = 12) from 30 to 1 day (20 ± 15) to partus; Group 2 comprised early lactation cows (n = 12) in the first month of lactation (18 ± 12 days), and Group 3 included full-lactation cows (n = 15) between 60 to 90 days of lactation (80 ± 25 days). The cows were high-yielding with a preceding lactation of about 8500 l (late pregnant cows: 8325 ± 795 l, early lactation cows 8458 ± 920 l, and full-lactation cows: 8677 ± 1055 l). The experimental cows were housed in open-stall barns. Diet and housing facilities were adapted to research purposes, with diet suited to the energy requirement of late-pregnant, early lactation and mid-lactation cows. Diet for late-pregnant cows included 7 kg grass hay, 5 kg corn silage (30% Dry Matter, DM), 4 kg sweet corn silage, 6 kg beet noodle silage, 5kg straw, 1 kg concentrate (18% crude protein, CP). Diet for early lactation cows consisted of 4 kg grass hay, 10 kg corn silage (30% Dry Matter, DM), 20 kg sweet corn silage, 12 kg beet noodle silage, 4 kg concentrate (18% crude protein, CP) and 1 kg molasses. Diet for full-lactation cows contained 4.5 kg alfalfa hay, 19 kg corn silage (30% Dry Matter, DM), 16 kg beet noodle silage, 9 kg concentrate (18 % crude protein, CP) and 1.2 kg soybean expeller.

The chemical composition of total mixed rations offered to late-pregnant, early lactation and full-lactation dairy cows is given in Table 1.

Table 1. Chemical composition of total mixed rations offered to late pregnant, early lactation and full lactation dairy cows.

	Late pregnancy	Early lactation	Full lactation
Dry Matter (DM) (kg)	12.85	15.60	19.58
Net Energy of Lactation (NEL) (MJ)	60.94	95.52	128.65
Crude Protein (CP) (% of DM)	8.25	11.31	16.88
Rumen undegradable protein (RUP) (% of CP)	28.86	33.91	26.33
Fat (% of DM)	2.41	3.47	4.68
Fiber (% of DM)	34.16	22.17	18.85

The blood samples were collected after morning milking and feeding, by puncture of the jugular vein into sterile disposable test tubes without anticoagulant. After clotting for 3 hours at 4 °C and centrifugation (1500 g, 10 minutes, 4° C), sera were carefully harvested and stored at -20 °C until analysis. Blood samples collected on fluoride were immediately centrifuged according to the same modalities and plasmas were assessed for glucose concentrations. The beta-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), triglyceride (TG), total cholesterol (tChol), glucose, total protein (TP), albumin, urea, total bilirubin (tBIL) and serum aspartate transaminase (AST) gamma-glutamyl transferase (GGT) and alanine aminotransferase (ALT) were measured in the biochemical laboratory OXUS (Kragujevac, Serbia) by spectrophotometric techniques using a BT 1000 spectrophotometer (Biotehnica Italia) and the corresponding commercial kits (DIALAB, YUNICOM).

The data obtained from various blood metabolites and serum enzyme activities in each group were analyzed by the method of analysis of variance and the Pearson test was performed for evidencing significant correlations. For this purpose was used statistic software (Statgraphic Centurion, Statpoint Technologies Inc.Warrenton, Va, Virginia, USA) .

Results and Discussion

Blood biochemical indicator in the transition and mid-lactation dairy cows were compared in this study (Table 2).

Table 2. Blood metabolites in late pregnant (Group 1), early lactation (Group 2) and full-lactation (Group 3) dairy cows (n=12 per group). Results are expressed as mean ± standard deviation (SD).

Variables	Group 1	Group 2	Group 3
Glucose (mmol/ l)	3.17 ±0.32 ^{a}	2.63±0.53 ^{b}	3.00±0.28 ^{a}
BHB (mmol/ l)	1.10± 0.32 ^{a}	1.50±0.25 ^{b}	0.87±0.20 ^{a}
NEFA (mmol / l)	0.16± 0.07 ^{a}	0.36±0.20^{b}	0.12±0.05 ^{a}
TG (mmol /l)	0.13 ±0.05 ^{A}	0.03±0.03 ^{B}	0.04±0.04 ^{B}
tChol. (mmol /l)	3.63±1.83 ^{A}	2.93 ±0.59 ^{A}	5.93±1.17 ^{B}
TP(g /l)	73.10 ±6.23 ^{a}	68.36±7.62 ^{a}	72.36±5.5 ^{a}
Albumin(g/ l)	35.09± 2.25^{a}	32.64±4.46 ^{a}	34.63±2.61 ^{a}
Urea (mmol/ l)	4.81±1.34 ^{a}	3.67 ±0.71 ^{b}	4.70±1.09 ^{a}
tBIL. (µmol/ l)	3,14 ±0.77 ^{A}	7.27± 5.24 ^{B}	3.20± 1.70 ^{A}

AST (IU/ l)	59.72± 10.95 [A]	90.81±21.98 [B]	84.18±16.19 [B]
GGT (IU/ l)	15.82 ± 2.4 [A]	23.45± 7.8 [B]	26.54 ±6.21 [B]
ALT (IU /l)	28.54 ±3.96 [A]	28.00±8.46 [A]	36.45±9.62 [B]

Legend: Mean values within a row with no common superscript differ significantly; values followed by small letters differ significantly (P < 0.05); values marked with capital letters differ highly significantly (P < 0.01).

The blood glucose concentrations in the late pregnant and lactating groups of cows were within the physiological values, from 2.5 to 4.2 mmol/l (Radostits et al., 2000). But, blood glucose levels were statistically significantly lower (P<0.05) in the early lactation cows, which suggested an increased glucose uptake by the mammary gland and decreased gluconeogenesis in the liver. (Veenhuizen *et al.*,1991; Drackley *et al.*, 2005; Dann *et al.* 2005; Jozwik *et al.*, 2012). The blood concentration of NEFA as the best indicator of negative energy balance and the lipomobilisation during the transition period (Jorritsma *et al.*, 2001; Oetzel, 2004; Civelek *et al.*,2011) was significantly increased (P < 0.05) in the group of cows in early lactation compared to the groups of late pregnant and full lactation cows. The serum BHB concentration is another indicator of energy metabolism in the early lactating cows showed also significantly higher (P<0.05) concentrations than the pregnant and full lactation cows, suggesting a intensive mobilisation of fat stores. Subclinical ketosis may be diagnosed when serum BHB concentrations are above 1.2 mmol/l, and clinical ketosis with blood BHB level above 2.6 mmol/ l (Duffield, 2000; Oetzel, 2004). The early lactation cows had the indicative values of the BHB (1.50 ± 0.25 mmol/ l) but did not show any clinical signs, which means that they had a typical subclinical condition. On the other hand, significant decreases (P < 0.01) in serum TG and tChol. concentrations were observed in groups of lactating cows compared to late-pregnant cows and other biochemical parameters synthesized in the liver, such as glucose (P < 0.05), albumin (P >0.05), urea (P <0.05) and TP (P >0.05), were also decreased during the puerperal period. These results suggested an increased accumulation of TG and tChol. in hepatocytes in puerperal cows, which was probably associated with decreased liver synthesis of VLDLs, and can confirm the reduction of liver synthesis induced by the development of fatty infiltration in the liver (Herdt et al., 1983; Sevinc *et al.*, 2003; Jorritsma *et al.*, 2001; Bobe *et al.*, 2004).

By contrast, liver damage induces an increase in the serum tBIL and the haemic compound is considered as a sensitive indicator for liver injury (West 1990; Bobe *et al.,* 2004; Djoković *et al.,* 2011). In this study, the mean tBIL. concentration was significantly and markedly increased ($P < 0.01$) in the puerperal cows compared to the late pregnant and full lactation dairy cows. As tBIL. concentrations, high serum activities of some enzymes highly expressed in liver in dairy cows such as AST, ALT and GGT are observed in liver injury and highly contribute to evaluate the degree of tissue damage (Pechova *et al.,* 1997; Lubojacka *et al.,* 2005; Stojevic *et al.,*2005; Djoković *et al.,* 2011). In the present study, the serum AST, GGT and ALT activities were significantly higher ($P < 0.01$) in early and full lactation cows than in late pregnant cows, suggested that the process of lipomobilization was sufficient to cause mild fat infiltration of liver cells in of the lactating cows and release these enzymes in circulation.Correlation between biochemical indicators calculated for all cows were estimated in this study (Table 3).

Table 3. Correlation coefficients for biochemical indicators in blood calculated for all cows in the present study.

	BHB	NEFA	TG	tChol.	TP	albumin	urea	tBIL	AST	GGT	ALT
glucose	-0.23	**-0.40**	0.22	0.30	-0.14	0.20	0.26	**-0,41**	**-0.38**	0.19	**-0.37**
BHB		**0.47**	**-0.42**	-0.15	0.25	0.14	**-0.34**	**0.38**	**0.45**	**0.53**	0.26
NEFA			**-0.35**	-0.13	0.15	0.12	-0.18	**0.56**	**0.52**	**0.46**	**0.35**
TG				**0.53**	**0.38**	0.15	-0.15	0.05	-0.26	**-0.54**	-0.18
tChol.					0.03	0.02	0.09	**-0.48**	0.11	**-0.77**	**-0.70**
TP						0.17	0.10	-0.03	-0.18	0.21	-0.01
album							**0.4**	-	0.1	-	0.2

in							**0**	0.02	4	0.02	1
urea								**-0.46**	**-0.78**	-0.14	0.19
tBIL									**0.50**	**0.34**	**0.36**
AST										0.24	0.19
GGT											**0.61**

Legend: Significant correlations (P < 0.05).

Indicators of lipomobilisation (NEFA or BHB concentrations) were found highly and positively correlated (P < 0.05) together in the current study. The serum BHB and NEFA concentrations in puerperal cows clearly indicated that the intense lipomobilisation in the post-partum period has induced ketogenesis and lipid infiltration in the liver. (Jorritsma *et al.,* 2001; Oetzel *et al.,* 2004; Civelek *et al.*,2011; Djokovic *et al.*,2015). It was also conifirmed a positive significant (P < 0.05) associations between in liver sintetised indicators: urea with albumin and TG with tChol and TP in this study. West (1990) reported a positive and significant correlation between the lipid amounts in the liver and the serum tBIL concentrations. Additionally, tBIL. concentrations significantly and positively correlated (P < 0.05) with the NEFA and BHB concentrations and serum activites of AST, GGT and ALT in this study. Moreover, according to Pechova *et al.* (1997), the blood activities of liver enzymes are correlated with the degree of fatty infiltration in the liver. In this study a positive correlation between AST GGT and ALT activities and lipomobilization indicators (NEFA and BHB values) were observed by the significant coefficient (P < 0.05). These results are in the accordance with previously studies (Bobe *et al.,* 2004; Overton and Waldron, 2004; Drackley et al., 2005; Oetzel, 2004; Dann *et al.*,2005; Gonzales *et al*, 2011; Djokovic *et al.*,2011) and suggested that the process of lipomobilization was enough to cause liver lesions in the early and full lactating cows. This statement confirmed a significant negative (P < 0.05) correlation between blood NEFA and BHB with blood TG in current study. This study has shown a possibility of the development a fat infiltration of the liver in lactation cows which was confirmed by a significant correlation between biochemical indicators.

CONCLUSIONS

These results demonstrate that mild fatty liver physiologically occurred during post-partum period inducing some cellular lesions and a weak function impairment of the liver. On the basis of changes and relationships of blood biochemical indicators, this study suggests that early lactation cows showed physiological adaptive changes, which were associated with subclinical ketosis and mild fat infiltration of liver cells

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THE NEED FOR CHANGING THE OPERATING RULES OF THE EXISTING HYDRO SYSTEMS IN THE AREA OF WATER SUPPLY AND DRAINAGE OF URBAN WASTE WATER

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ABSTRACT

Water supply systems are a constant movement of development and monitoring of the development of urban areas that are supplied with drinking water and the needs of the population and the economy. The existing practices in the operational management of large urban water supply systems are already outdated and inefficient. Therefore, a new approach is needed in the rules for operational system management. In the new operational rules of management, special emphasis should be given to preserving the water resources of the water resources, as well as to the rational use of water in water supply systems. More control over distilled water in systems, as well as analysis of invoiced water and greater control of water losses is needed.

Key words:*Water sources, Water supply norm, Invoiced water, Water losses, Water production.*

1. INTRODUCTION IN THE PROBLEM

Hydrosystems are water management facilities that are composed of a group of hydraulic structures connected to each other with the conditions of joint work and location. The complex of hydro-technical objects that are united in several hydroelectric power plants and are connected with common purpose is called water management system or hydrosystem. Hydrosystems can generally be divided into the following four categories:

1. Energy Hydrosystems.
2. Meliorative hydro systems.
3. Traffic hydraulic systems.
4. Communal (sanitary) hydro systems.

In this paper, the Komunalni hidrosistemi for water supply and drainage of urban wastewater will be considered in detail. Apart from theoretical assumptions in the paper, the long-term operation of JP Vodovod and Kanalizacija Skopje will be analyzed, as an example of which the pros and cons of the operation can be seen, both from a technical aspect and from an organizational aspect. These two aspects of the operation of the company are crucial for making strategic decisions in the operation of the company, as well as for correct long-term analysis of the development of the enterprise itself and thus the quality of the level of services provided by the public enterprise towards the citizens.

Water supply systems in their operation are always regularly connected with sewage systems that are integral part of water supply systems. The water used by the water service users at the same time should be taken away from the place where the water is used. This transformation of used water is carried out in the sanitary facilities where drinking water and other needs of the citizens are simultaneously used, and at the same time the used water is passed to the recipient through the sewage system.

Water-supply systems are generally composed of the following hydro-technical facilities without which there is virtually no water supply system.

1. Water source for water supply. Underground or overhead sources. (Wells, hills, rivers).
2. Pumped stations, which provide the necessary pressure in the supply pipelines, the drinking water to be distributed to all covered and registered water users.
3. Water treatment plants for the treatment of drinking water or only systems for disinfection of drinking water.
4. Pumping pipelines that distribute water from the source to the water tanks and at the same time distribute
    a. drinking water to the water supply network.
    b. Water tanks. These facilities have the role of storing water and doing a daily leveling of water needs in populated areas. According to their location, they can be reservoirs in front of a populated area, reservoirs outside the settlement, as well as a combination of the two previous cases.
5. Secondary water supply network that distributes drinking water to all consumers in the consumer area.
6. Water supply network facilities. Closures, air valves, exhaust valves, firefighting hydrants, pressure regulators, water gauges.

All of these elements that are listed are a typical water supply system that needs to respond to the needs of the population that is being supplied by the system.

1. Period from 1907 to 1918 This is the period when the first well was built beside the river Vardar, a pumped diesel station and the first reservoir on the top of Skopje Fortress. During this period, the old water supply system of the City of Skopje, which supplied drinking water from the famous Aqueduct, was no longer used, since in 1915, as a consequence of the military actions, the Aqueduct was no longer in operation. During this period the water supply system of the City of Skopje developed exclusively on the left side of the river Vardar.
2. Period from 1918 to 1945 This is a period when the new water supply of the City of Skopje is being put into use with water, which is the source of Rasche. From 1936 to 1938, the first substrate Rasche was built with a diameter of 400 mm from cast iron pipes and a new reservoir in the Skopje Fortress. Also in this period the water supply network is spreading on the right bank of the river Vardar.
3. Period from 1945 to 1963 This is a period when the water supply system is expanding rapidly and new pipelines are being built especially for the new settlements Avtokomanda, Michiru and Prolet.
4. Period from 1963 to the present. This is a period when the water supply system is experiencing expansion and new water intakes are being built. In 1964, the Rashte 1 cassava, in 1982, the rashce 2 cassava, in 1986, the Nerazi boulevard with 4 wells and in 1992, the well-known Nerezi region with 3 wells. During this period, many new pumping stations and tanks are being built and the length of the water supply network reaches a length of L = 1330 km.

For this development of the city of Skopje, that is, for the development of the Skopje water supply system, the most important are the sources of clean drinking water without which the City of Skopje could never be developed and expanded.

The main and unique sources of clean drinking water for the city of Skopje are the spring area of Rasce, the Rasche 1 and the Rashte 2 cascade, and the Neresi-Lepenec well with a total of 7 tubular wells. [6].

The soda of the water sources of the City of Skopje is given in the following review:

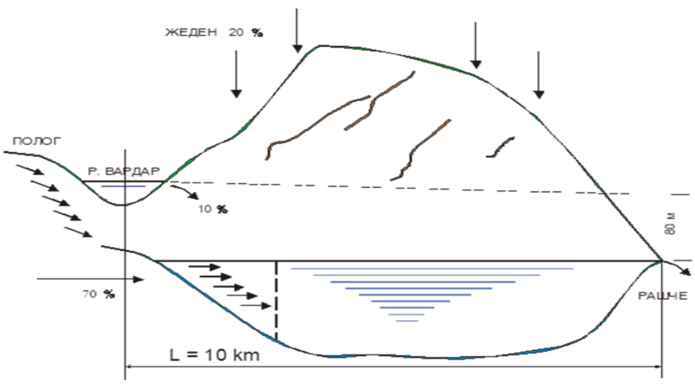
1. Rasche 1
   - Qsr/god=3.09 (m^3/sec).
   - Qmin/god=2.30 (m^3/sec).
   - Qmax/god=5.12 (m^3/sec).

2. Rasche 2
   - Qsr/god=0.818 (m^3/sec).
   - Qmin/god=0.635 (m^3/sec).
   - Qmax/god=1.190 (m^3/sec).

3. Total quantities of water from the two basins
   - Qsr/god=4.15 (m^3/sec).
   - Qmin/god=2.96 (m^3/sec).
   - Qmax/god=7.02 (m^3/sec).

These quantities of water give high reliability in the stable water supply of the city of Skopje, without the danger of possible restrictions on water in individual parts of the city. Figure 1 gives a schematic representation of the feeding of the underground acipher to the source Rasche. [1].

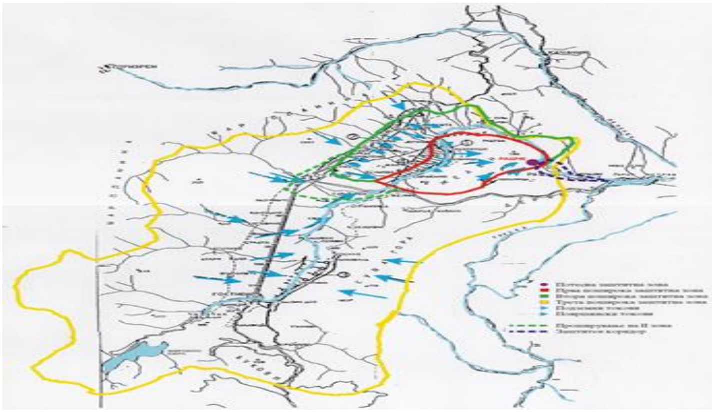
Figure 1. Schematic presentation of feeding water at the spring Rasche



Source: Protecting the Source Raspberry from pollution [1]

For the protection of groundwater that flows to the source Rasche from pollution, three protective zones are defined that provide a high degree of safe protection of drinking water for the city of Skopje. Figure 2 shows the schematic representation of the coverage of the protective zones at the source Rasche.

Figure 2. Schematic representation of the coverage of the protective zones at the Rasce spring



Source: Protecting the Source Raspberry from pollution [1]

As spare quantities of water that can be immediately put into the water supply system of Skopje are the wells Nerezi-Lepenec with the following fruits:

1. Draw well Nerezi
   - Б1=135 (l/sec).
   - Б2=230 (l/sec).
   - Б3=230 (l/sec).
   - Б4=135 (l/sec).
2. Draw well Nerezi
   - 3*230=690 (l/sec).

The total amount of water that is obtained from the wells is 730 + 690 = 1420 (l/sec) or Q = 1.42 (m^3/sec). All these quantities of water provide enough water supply for the city of Skopje with clean and healthy drinking water.

2. METHODS AND MATERIALS OF THE RESEARCH

The annual capacity of the source Rasche allows water production up to 150,000,000 (m3 / year), [1], which practically covers all water needs for the population and for the economy.

The waters from the wells NereziLepenc are used only when needed when the water consumption is increased by 20-25% of the average amount of water that is spent in the water supply system of the city of Skopje.

Table 1. gives the annual production of water and water consumption in the City of Skopje. From this table you can see some characteristic data that will be used in further analysis.

Table 1. Produced and invoiced water in the Skopje water supply system

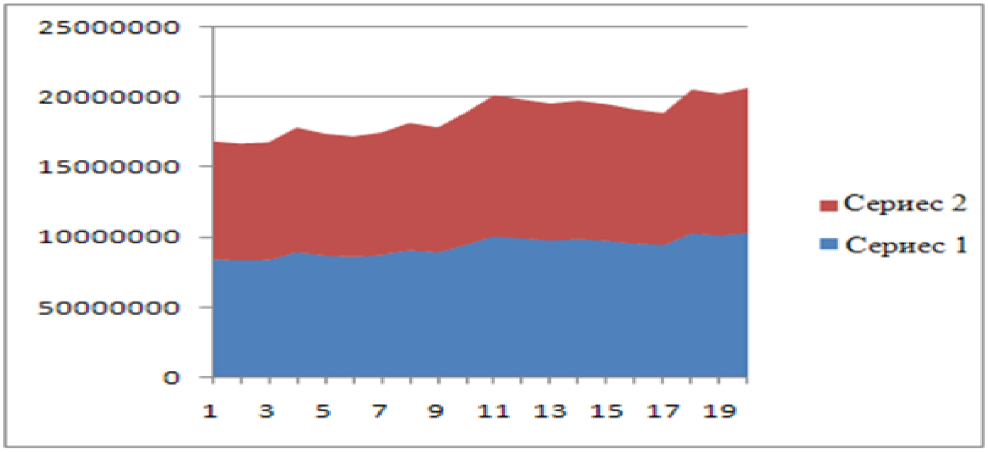
Year	Production of water	Invoiced households	Invoiced - property	Invoiced water (m^3)
1993	84.341.840	23.167.417	18.449.037	41.616.454
1994	83.498.506	26.028.502	18.226.269	44.254.771
1995	83.919.338	24.546.153	16.911.684	41.457.837
1996	89.247.164	24.976.242	16.531.106	41.507.348
1997	87.032.828	24.820.683	16.721.556	41.542.239
1998	86.050.867	26.645.323	16.894.935	43.540.258
1999	87.457.010	27.874.913	16.670.655	44.545.568
2000	90.844.370	28.733.934	16.707.760	45.441.694
2001	89.238.224	29.590.247	15.336.153	44.926.400
2002	94.671.785	29.070.237	15.172.964	44.243.201
2003	100.740.677	30.198.013	14.072.031	44.270.044
2004	99.234.707	29.356.319	13.225.445	42.581.764
2005	97.804.992	28.988.494	12.771.561	41.760.055
2006	98.800.832	29.193.511	12.195.451	41.388.962
2007	97.515.677	28.552.632	11.242.323	39.794.955
2008	95.618.935	27.758.052	10.414.680	38.172.732
2009	94.419.410	27.313.252	10.302.029	37.615.281
2010	102.812.616	27.670.870	9.939.755	37.610.625
2011	101.280.847	27.717.637	9.683.159	37.400.796
2012	103.343.837	27.302.819	9.584.720	36.887.539
2013	102.700.170	27.151.494	9.017.416	36.168.910
2014	100.467.113	27.278.166	8.586.233	35.864.399
2015	102.076.381	29.677.357	9.711.365	39.388.722
2016	103.377.070	31.806.376	9.330.090	41.136.869
2017	107.263.501	30.048.650	9.940.493	39.989.143
2018	103.670.229	28.815.209	9.613.033	38.428.242
Secondary	**95.670.343**	**27.854.713**	**12.971.227**	**40.921.863**

Source: The author under r. 2 until 2014, and from 2015 to 2018 the data were taken by Public companyWater and Sewerage Skopje [2]

Bearing in mind that the annual production of water ranges far below the maximum possibilities of the spring Rasche, it follows from Table 1. that the spring waters from Rashece will continue to be the bearers of the water supply of the City of Skopje without the possibility of their replacement , with additional water from the wells Nerezi Lepenec in periods of bad hydrological conditions, ie the appearance of drought years.

Chart 1.gives the distribution of the volume of water and invoiced water in the Skopje water supply system.

Chart 1. Schedule the volume of produced and invoiced wate r



Source: The author under r. 2 until 2014, and from 2015 to 2018 the data were taken by Public companyWater and Sewerage Skopje [3]

Table 2. Water Supply Standards

Year	Water production	Invoiced households	Invoiced economy	Qo1	Qo2	Qo3	Qo4
1993	84.341.840	23.167.417	18.449.037	498,08	245,77	136,82	108,95
1994	83.498.506	26.028.502	18.226.269	488,15	258,73	152,17	106,56
1995	83.919.338	24.546.153	16.911.684	486,61	240,4	142,33	98,063
1996	89.247.164	24.976.242	16.531.106	536,47	249,5	150,13	99,369

1997	87.032.828	24.820.683	16.721.556	497,71	237,56	141,94	95,624
1998	86.050.867	26.645.323	16.894.935	488,92	247,39	151,39	95,993
1999	87.457.010	27.874.913	16.670.655	494,13	251,68	157,49	94,19
2000	90.844.370	28.733.934	16.707.760	505,62	252,92	159,93	92,991
2001	89.238.224	29.590.247	15.336.153	489,38	246,38	162,27	84,103
2002	94.671.785	29.070.237	15.172.964	511,66	239,12	157,11	82,003
2003	100.740.677	30.198.013	14.072.031	543,45	238,49	162,58	75,913
2004	99.234.707	29.356.319	13.225.445	534,34	229,28	158,07	71,213
2005	97.804.992	28.988.494	12.771.561	515,54	220,12	152,80	67,32
2006	98.800.832	29.193.511	12.195.451	518,37	217,15	153,17	63,985
2007	97.515.677	28.552.632	11.242.323	509,40	207,88	149,15	58,728
2008	95.618.935	27.758.052	10.414.680	497,36	198,55	144,38	54,172
2009	94.419.410	27.313.252	10.302.029	488,96	194,79	141,44	53,35
2010	102.812.616	27.670.870	9.939.755	530,02	193,89	142,65	51,242
2011	101.280.847	27.717.637	9.683.159	519,78	191,94	142,25	49,69
2012	103.343.837	27.302.819	9.584.720	556.78	188,03	139.17	48.85
2013	102.700.170	27.151.494	9.017.416	520.90	183.45	137.71	45.73
2014	100.467.113	27.278.166	8.586.233	507.04	181.00	137.67	43.33
2015	102.076.381	29.677.357	9.711.365	508.48	196.21	147.83	48.38
2016	103.377.070	31.806.376	9.330.090	493.81	196.50	151.93	44.60
2017	107.263.501	30.048.650	9.940.493	475.48	177.27	133.20	44.10
2018	103.670.229	28.815.209	9.613.033	443.79	164.5	123.35	41.15
Secondary	**95.670.343**	**27.854.713**	**12.971.227**	**510.67**	**219.75**	**148.85**	**70.91**

Source: The author under r. 2 until 2014, and from 2015 to 2018 the data were taken by Public companyWater and Sewerage Skopje [2]-[3].

Table 2. gives an overview of the values of the water supply norms obtained as a product of the annual volumes of produced and invoiced volumes of water and the number of water users that has been increasing over the years.

The following water supply norms can be defined from Table 2.

1. Water supply norm according to total produced water, Qo1
2. Water supply norm according to total invoiced water, Qo2

3. Water supply norm according to invoiced household water, Qo3
4. Water supply standard according to invoiced water from the economy, Qo4

From Table 2. it can be immediately concluded that the value of the Household Supply Chain is constant over the time series of 26 years, while the Water Supply Standard for the Economy has a constant decline as the number of economic entities in the City of Skopje decreases, water consumption for the economy. The increased production of water from year to year does not improve the water supply in the city of Skopje, but on the contrary creates large water losses that are a problem in the regular operation of the water supply system. Table 3. gives an overview of the increase in water losses as a result of increased water production in the system.

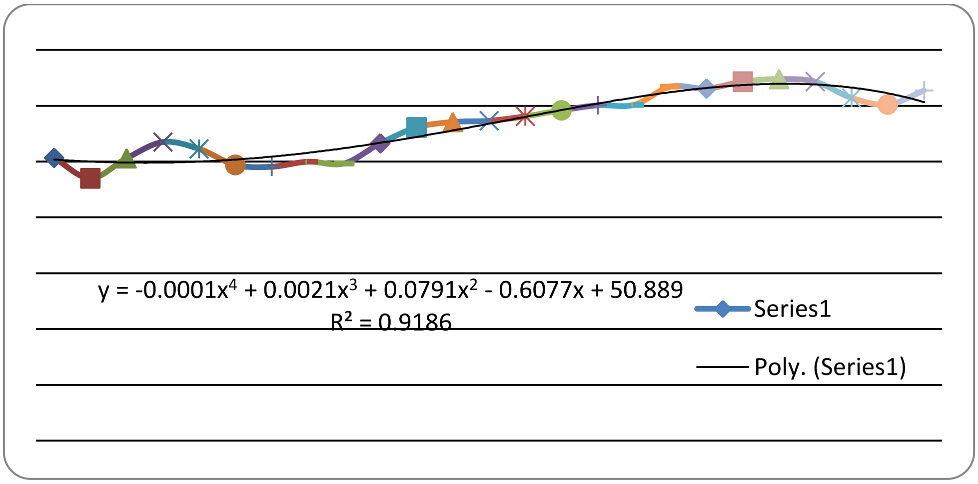
Table 2. Percentages of water loss in the supply system

Year	Produced water (m^3)	Invoiced water (m^3)	Lost water (m^3)	Qo1 in total	Qo2 necessary	Losses P %
1993	84.341.840	41.616.454	42.725.386	498.08	245.77	50.66
1994	83.498.506	44.254.771	39.243.735	488.15	258.73	46.99
1995	83.919.338	41.457.837	42.461.501	486.61	240.40	50.60
1996	89.247.164	41.507.348	47.739.816	536.47	249.50	53.49
1997	87.032.828	41.542.239	45.490.589	497.71	237.56	52.27
1998	86.050.867	43.540.258	42.510.609	488.92	247.39	49.40
1999	87.457.010	44.545.568	42.911.442	494.13	251.68	49.06
2000	90.844.370	45.441.694	45.402.676	505.62	252.92	49.97
2001	89.238.224	44.926.400	44.311.824	489.38	246.38	49.66
2002	94.671.785	44.243.201	50.428.584	511.66	239.12	53.27
2003	100.740.677	44.270.044	56.470.633	543.45	238.49	53.06
2004	99.234.707	42.581.764	56.652.943	534.34	229.28	57.09
2005	97.804.992	41.760.055	56.044.937	515.54	220.12	57.30

2006	98.800.832	41.388.962	57.411.870	518.37	217.15	58.10
2007	97.515.677	39.794.955	57.720.722	509.40	207.88	59.19
2008	95.618.935	38.172.732	57.446.203	497.36	198.55	60.01
2009	94.419.410	37.615.281	56.804.129	488.96	194.79	60.16
2010	102.812.616	37.610.625	65.201.991	530.02	193.89	63.42
2011	101.280.847	37.400.796	63.880.051	519.78	191.94	63.07
2012	103.343.837	36.887.539	66.456.298	526.78	188.03	64.31
2013	102.700.170	36.168.910	66.531.260	520.40	183.45	64.78
2014	100.467.113	35.864.399	64.602.714	507.04	181.0	64.30
2015	102.076.381	39.388.722	62.687.689	508.48	196.21	61.41
2016	103.377.070	41.136.869	62.240.201	493.81	196.50	60.21
2017	107.263.501	39.989.143	67.274.358	475.48	177.27	62.72
2018	103.670.229	38.428.242	65.241.987	443.79	164.5	62.93
Secondary	**95.670.343**	**40.825.940**	**54.484.260**	**510.67**	**219.75**	**56.95**

Source: The author under r. 2 until 2014, and from 2015 to 2018 the data were taken by Public companyWater and Sewerage Skopje [2].

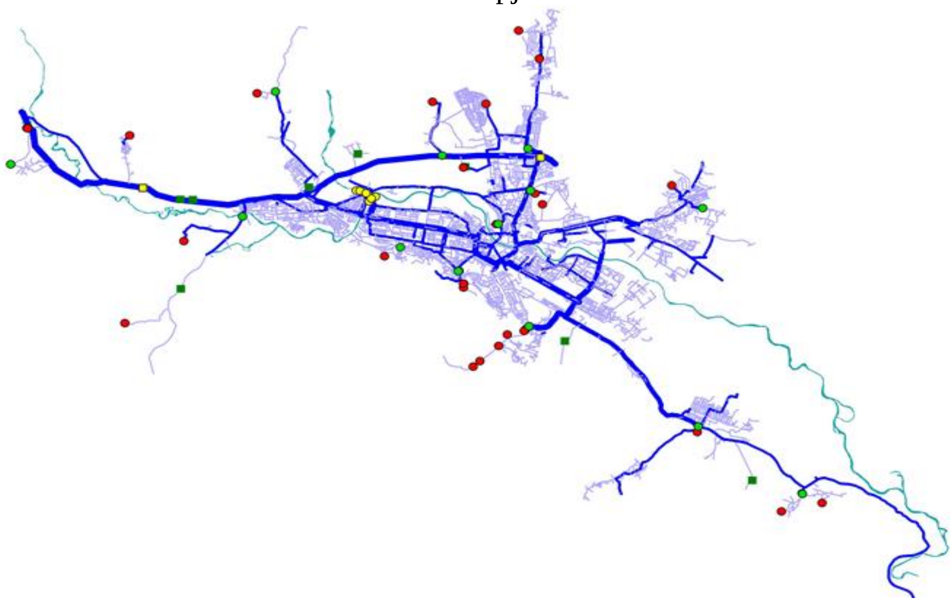
Chart 2. Mathematical dependence of water losses



Source: The graph gives the mathematical dependence of water losses in the city water supply system[2]

Figure 3, gives a schematic diagram of the branching of the water supply network in the Skopje water supply system, in order to develop a visual representation of the complex system in question.

Figure 3. Schematic representation of the water supply network of the city of Skopje



Source: Impact of the Water supply norm in reducing the water losses in the water supply networks Skopje 2015[4]

The average daily water consumption is Q=3401.3 (l/sec), ie the daily volume of water supplied is W=293.872,6 (m^3/day). This average quantity of water is higher by 12.97% of the minimum value of the spring Rasche from Q = 2.96 (m^3/sec). If the daily consumption is compared with the average daily yield of the source Rasche from Q = 4150 (l/sec) then the average amount of water consumed in the City of Skopje is 18.04% lower than the average daily yield of the spring Rasche.

This indicates the need for rational use and wastage of spring waters, especially if it is taken into account that the actual amounts (invoiced) of water are average W = 111,851.90 (m3/day) or Q = 1294.58 (l/sec). This is a daily amount of water consumed, which is 61.94% less than the daily average distributed water quantity in the system. This ratio, ie the percentage of the difference between the consumed and distilled water is almost equal to the percentage of water losses in the water supply system of the city of Skopje for the analyzed time series of 26 years. [6].

From the previous calculations it can be concluded that the only way to preserve the high degree of confidentiality of the Skopje water supply system is to reduce the large water production at annual level, which over the last ten years exceeds 100,000,000 (m^3/year). These large quantities are unavailable and unsustainable for a longer period of time, especially taking into account the following facts. [7]

1. The projections for increasing the number of inhabitants in the city of Skopje have long overcome the most optimistic predictions, yet the increased number of inhabitants does not cause problems in the regular water supply. The number of inhabitants by 2020 was projected to increase to 640,000 inhabitants, but the number of inhabitants in the City of Skopje in 2015 was 811,045. According to the records of Public company Water and Sewerage Skopje Skopje, more than 703,000 residents used water services. In line with the projections for the daily water consumption in Skopje prepared in 2000, it was assumed that in 2020 the total water consumption would be Q = 5.5 (m^3/sec), while the total real needs of water without loss of water in 2018 Q = 1.29 (m^3/sec), and according to the total production of water, the demand for water amounted to Q = 3.29 (m^3/sec).

2. Economic or industrial facilities that were active until 1990 are largely out of the question, and therefore the water supply for industrial processes has been reduced considerably. Of the 14 large factories on the territory of the city of Skopje that once spent up to Q = 544.56 (l/sec), there are now only 5 active factories that consume a reduced amount of water. This is a decrease in industrial capacities by 64.28%.

3. The new industrial plants are being built in industrial zones that are not connected to the water supply system of the city of Skopje. Regardless of the increased capacity of the new industrial plants, the city's water supply capacities are not burdened with new quantities of water, as new facilities are not connected to the existing water supply system at all.

4. The megalomaniac projects that were prepared in the past as an alternative and supplementing the quantities of water in the Skopje water supply system for the increased number of inhabitants and the increased industrial capacities have not been realized at all.

The main projects that were to be realized are.

- The Kadina Reka-Paligrad hydropower plant from which Q = 1.8 (m^3/sec) should be used, of which Q = 0.8 (m^3/sec) for the population and Q = 1.0 (m^3/sec) for industrial facilities primarily for OHIS and Working UnitSkopje.

- Accumulation from the accumulation of Matka II, ieSvetaPetka with Q = 5.0 (m^3/sec) along with a pipeline known as the Southern Canal is also not realized.

- The construction of 40 wells in the Skopje valley that would provide technical water for greenery and light industry is also not realized.

- Efforts by the Vardar River to provide technical water for greenery and light industry are also not realized.

Constant water capacities in the Skopje water supply system are provided in the 4th development period of the City of Skopje after the 1963 earthquake, when two steel pipelines with a profile of 1600 mm were built, and large water intakes such as Kaptazata Rasce 1 and Rasce 2, together with the well-known Lepenec Nerezi.

The main problem that occurs in the estimates of water consumption in the City of Skopje is the excessive value of the water supply norm which, according to all previous calculations, is taken to be Qo = 500 (l/day/h). From Tables 1,2 and 3 it was stated that the real needs of water for households and businesses total Qo = 220 (l/day/h), of which only for water supply of population Qo = 150 (l/day/h), and needs for the economy are Q = 70 (l/day/h) [4].

If the new water supply capacities (pumping stations, pipelines and tanks) are started to be dimensioned according to the actual water supply norms, then conditions will be provided for drastically reducing the large annual production of water, and if the process for digital monitoring of work is also fully started of the water supply system through the SCADA system, then we can expect a complete revitalization of the Skopje water supply system and an extension of its service life for a longer period.

Certainly, at the end of the rationalization of water consumption, measures need to be taken to increase the utilization of technical water through new wells that will be used to flood green areas in the city, as well as for the technological processes in the light industry.

Regarding the change in the operating rules of the sewage, it should be emphasized that the Skopje sewerage system is separated ie divided into faecal and atmospheric sewers.

Fecal sewerage covers the City of Skopje with 80%, which means that more engagement in the part of the construction of the fecal sewer is needed, which should be 100% built and fully monitor the development of the water supply network in the city. The new operational rules should be mandatory in the direction of equal access to development both on the water network and to the development of the sewer network

The atmospheric sewage system covers only 35% of the territory of the city of Skopje, which does not cover the basic standards for protection of the flooding of urban areas from the rainwater. The new operational rules must necessarily take into account climate change, which should find its reflection in the new input parameters for the design of new atmospheric lines.

3. CONCLUSIONS FROM THE CONDUCTED ANALYSIS

The Skopje water supply system, as one of the oldest organized water supply systems in the territory of Northern Macedonia, should at all costs undergo substantial changes in the existing operational rules for managing water resources at its disposal. The need for the change in the operational rules is perceived in the fact that the Skopje water supply system has a large water production that is unjustified and unnecessary, and on the other hand the system faces a large percentage of water losses, where in the last ten years the percentage of losses is higher of 60%. The following conclusions can be highlighted as concrete conclusions from the analysis carried out.

1. The percentage of total invoiced water for households and businesses is only 43.05% as the average value of the time series of 26 years. This percentage of invoiced water annually is small and does not meet the criteria for cost-effective operation of the water supply system.

2. The percentage of invoiced water for households and the economy in the last ten years when an increased number of inhabitants in the City of Skopje has been registered is 38%, although in this period water production exceeds 100,000,000 (m^3/year) per year. This indicates a bad organization in the operation and many failures in the current operation of the water supply system operator.

3. The percentage of losses in water in the system is 62.48%, which is a very high percentage of losses. This high level of water loss is problematic in the optimal functioning of the system.

4. It is necessary to redefine the Water Supply Standards from the current total Qo = 500 (l/day/h), to the total water supply standard of Qo = 220 (l/day/h), of which the water supply norm for households Qo = 150 (l/day/h) and the water supply norm for the economy Qo = 70 (l/day/h).
5. To achieve these water supply standards, a complete automation of the system is needed with the aim of controlled water production and controlled water distribution.

6. Introducing new measurement regulatory equipment that will directly enable rational production and rational flow of water resources.

7. The sewage system of the City of Skopje should monitor the development of the water system and create opportunities for efficient drainage of waste water, communal wastewater and rainwater.

All these conclusions and recommendations should find a suitable place in the application of the new Operating Rules in the operation of existing hydro systems for water supply and drainage of municipal wastewater. Only by periphering of new methods and techniques in operational management can the expected results in the operation of communal hydrosystems be achieved.

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ENVIRONMENTAL PROTECTION

ЯВЛЯЕТСЯ ЛИ УПРАВЛЕНИЕ МИКРОБНЫМИ СООБЩЕСТВАМИ В ЭКОСИСТЕМАХ С ЦИКЛОМ МИКРООРГАНИЗМОВ ЗАЩИТОЙ ОКРУЖАЮЩЕЙ СРЕДЫ?

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Ключевые слова: *микробное сообщество, концепции, законы, экосистемы, почвенная экосистема, здоровье почвы*

АБСТРАКТ

В настоящем сообщении, предпринята попытка, создания иерархической системы среди известных законов и концепций, используемых в микробного экологии и экологической биотехнологии. Рассмотрен научный материал из области популяционной биологии и микробной экологии, который по своей значимости, уже получил статус правил, концепций или законов. Поведено упорядочивание и установление соподчинение среди законов и концепций в соответствии с их всеобщностью. Рассмотрены законы, имеющие математическое выражение и концепции, сгруппированные в концепции всеобщие или специфические. Обсуждены пути упорядочивания известных, новых и будущих научных результатов. Приведены примеры решения теоретических и биотехнологических проблем и задач, на основании законов и концепций в микробной экологии.

ВВЕДЕНИЕ

К концу первой четверти XXI века в биологии и микробной экологии накоплен большой объем знаний с устойчиво повторяющимися закономерностями. Это позволило признать в этих закономерностях статус правил, концепций или даже законов. Признание произошло из-за повсеместности, неизбежности и неотвратимости воздействия выявленных закономерностей (Звягинцев, 1987; Турчин, 2002; Семенов, 2005; 2010; 2011). Значимость правил, концепций и законов для познания законов природы – бесспорна и указывает, что микробная экология, как наука обретает зрелость. Так как основным объектом обсуждения является микробное сообщество, приведем определение микробного сообщества. Микробное сообщество (МС) следует, представлять как некую совокупность таксономически разных, но функционально взаимодействующих популяций микроорганизмов, существующих некоторое время в соответствующем месте. Компоненты МС могут быть, как сильно взаимосвязанными между собой, в том числе и физически, а МС – может быть высоко специализированным, так и слабо взаимосвязанными, а МС - низко специализированным" (Семенов, 2005; 2010; 2011; Semenov, 1991).

Конечно, правила, концепции и даже законы не равноценны по своей значимости, повсеместности проявления и возможности использования. Однако среди концепций, есть такие, которые значимы всегда, хотя их проявление трудно измерить, но, тем не менее, их нужно знать и учитывать их проявление. Одна из таких концепций, которую нужно знать и учитывать повсеместно при управлении не только антропогенными, но и природными экосистемами - цикл микроорганизмов (ЦМ) (Семенов и др., 2007; Semenov et al., 2010).

Смысл этой концепции в том, что микроорганизмы микробного сообщества (МС) в виде отдельных клеток, популяций и даже сообществ в силу разных причин (природных и антропогенных) подвержены перемещениям в экосистемах, между разными сукцессионными элементами экосистем, эконишами, экотопами и другими местообитаниями. Основными элементами местообитаний в циклических перемещения, являются почва и вода, растения, животные и их желудочно-кишечные тракты, экскременты животных и экскреты растений и снова почва, и вода (Семенов и др., 2007). Микроорганизмы, выживая и в силу перемещения постоянно осваивая "новые" местообитания, приспосабливалась к условиям местообитаний и "приспосабливая" эти местообитания своим присутствием и своей активностью. Следовательно, качественный и количественный состав популяций микроорганизмов в каждом элементе экосистемы не постоянен, изменчив.

Возникает вопрос, как управлять микробными сообществами и соответственно экосистемами, если природа постоянно добавляет, удаляет, передвигает и перемешивает организмы.

Задача настоящего сообщения, провести упорядочивание и предложить соподчинение известных законов и концепций, обсудить примеры значимости законов и концепций в управлении функционирующими микробными сообществами (МС) в экосистемах. Какие законы и концепции из уже известных, могут дать направление к пониманию управления микробными сообществами и микробными экосистемами в экологической биотехнологии (Дукич и др., 2018 а, в). Кратко, рассмотрим их.

Законы популяционной биологии и микробной экологии

Вполне очевидно, что для управления МС необходимо ориентироваться в первую очередь на фундаментальные законы, а среди них использовать их соподчиненность. На текущий момент на уровне аксиомы “cellula ex cellula” имеют значимость законы роста популяций.

Закон экспоненциального роста, то есть, способность к экспоненциальному росту всяких популяций (и сообществ), математическое выражение: $x= x_o e^{\mu t}$. =
Все популяции (и сообщества) подвержены явлению “самоограничения” роста или точнее - лимитированию или ингибированию роста, математическое выражение: $\mu=\mu_{max} \cdot S/K_s+S$ - уравнение Моно или $\mu = \mu_{max}K_p/K_p+P$ - уравнение Иерусалимского. Все популяции (и сообщества) в условиях лимитирования или ингибирования роста существуют и развиваются волнообразно (как последствия лимитирования или ингибирования), закон волнообразного роста, математическое выражение: $dX/dt = (\mu(S) - D(S)) *X$ (уравнение роста), $dS/dt = -X*\mu (S) /Y + K_r * X*D (S) + BGF + Input (t)$; (уравнение отмирания) (Zelenev et al., 2000).

Эти законы объективны для всех живых организмов и конечно для микроорганизмов. Значимость этих законов безусловна и их функционирование ощущается постоянно. Турчин П.В. (2002) сравнил значимость этих законов с первыми тремя законами динамики в физике. Перечисленные законы имеют математическое выражение, что, по-видимому, отличает законы от концепций и привил. По-видимому, понять и прочувствовать важность законов и концепций поможет расположение их в некой соподчиненности, а обсуждение значимости в определенной последовательности, отражающей их повсеместность функционирования.

Концепции всеобщие

Концепция о том, что МС является не только основной функциональной, но и эволюционирующей единицей экосистем (Заварзин, 1990 а), а эволюция микроорганизмов, МС, происходит в сообществе и через сообщество, представляется как всеобще значимой. На индивидуальный организм действуют множество факторов, создаваемых природой и сообществом. “Отбор” (природа отбирает) индивидуальных мутаций, происходит в условиях (выживает наиболее приспособленный), создаваемых именно сообществом. То есть, выживание “новой” популяции в “новом” местообитании, “позволяет” сообщество и, конечно, окружающая среда.

Концепция об эволюционировании микроорганизмов в сообществе и через сообщество связана с концепциями симбиоза и сукцессии. Концепция “симбиоза” как глобальное явление заключается не столько, например, в мутуализме. Более глобально, ее значимость в том, что все организмы как-то сосуществуют друг с другом.

Концепцию сукцессии по своей значимости можно приравнять закону. Если нарушения или изменения в среде столь велики, то сосуществование в МС нарушается (разрушается) и происходит замещение (смена) одних организмов (видов) другими, что называется - сукцессией. Эти взаимосвязанные концепции, ориентирует теоретика и практика на системный подход для достижения результата. Ориентация на достижение –устойчивости используемых микробных сообществ, путь к управлению природными микробиологическими процессами. В практике последствия сукцессионных процессов не редко негативно проявляются, например, в очистных сооружениях или других технологических процессах, основанных на использовании природных, а тем более искусственно созданных микробных сообществах (Дукич и др., 2018 а, б).

Как детализацию концепции сукцессии необходимо привести концепцию о регуляции активности МС в природных и антропогенных экосистемах. Концепция констатирует, что изменение активности МС осуществляется не за счет изменения активности отдельного компонента МС, а за счет изменения доминирующего состава сообщества – через сукцессию. Следовательно, при попытке изменения активности процесса, осуществляемого МС нужно быть готовым к тому, что произойдет и изменение состава микробного сообщества (Семенов, 2005).

Концепция зависимости и значимости процесса в экосистеме от численности и активности микроорганизмов его, осуществляющих или, только тот процесс значим и заметен, если организмов его осуществляющих - много и они активны (Заварзин, 1989), обладает глубочайшим смыслом. Эта концепция, перекликается с философским законом перехода количественных изменений - в качественные. Эта концепция позволяет понять, что обнаружение нескольких клеток какого-то сапротрофного микроорганизма в каком-то местообитании не означает важности процесса, потенциально значимого при осуществлении его множеством таких микроорганизмов. Феномен, активно эксплуатируемый в литературе, называемый “quorum sensing”, всего лишь проявление выше сформулированной концепции.

Каждый микроорганизм имеет свои границы существования, а свойства и особенности микроорганизма отражаются в физиологических характеристиках и константах. Генетическая детерминированность свойств микроорганизмов, не исключает их физиологическую “гибкость”. В этой связи, по-видимому, законы роста и сукцессии следовало бы дополнить концепцией об "ограниченности адаптационной способности организма пределами нормы реакции конкретного организма к условиям окружающей среды". Смысл этой концепции в том, нельзя адаптировать популяцию микроорганизма к не специфическому ингибитору, а попытка адаптации МС, приведет только к сукцессии в МС, так как адаптация происходит в популяции, а в сообществе - сукцессия.

Концепции специфические

Помимо обще экологических концепций, известны не менее важные, но более специфические концепции МС, отражающие функциональные свойства микроорганизмов и особенности экосистем (Звягинцев, 1987).

Концепция микробного пула и избыточности этого пула в почвенной экосистеме по сравнению с доступностью питательного ресурса. Суть ее в том, что жизнеспособная часть микробной биоты “превосходит” массу доступных питательных веществ. Следовательно, большая часть микроорганизмов в почвенной экосистеме не обеспечена питанием и находится в состоянии голодания, а, следовательно, физиологического выживания (dormant state, anabiotic state and even viable – but non cultivable). Значимость этой концепции нужно учитывать особенно при интродукции микроорганизмов в природную экосистему, с какими либо прагматические целями.

Концепция функциональной взаимозаменяемости, функционального параллелизма среди микроорганизмов или функционального дублирования. Известно, что одну и ту же функцию в экосистемах, например, гидролиз целлюлозы, могут выполнять, бактерии, актиномицеты и грибы, т.е., прокариоты и эвкариоты. Подавление, а тем более полное удаление какой-то одной функциональной группы, вызовет увеличение активности и численности функционального дублера, активность которого может быть совсем нежелательна.

Концепция микро зональности почвенной экосистемы, как среды обитания микроорганизмов наряду с концепцией избыточности микробного пула в почве напоминает о том, что, например, интродуцент будет жизнеспособен, если будет находиться, с одной стороны в микрозонах, а с другой, субстрат должен быть доставлен также в микрозоны.

Концепция *r* - *K* - континуума в микробной экологии - это отражение непрерывности и дискретности свойств и разнообразия организмов. Эта концепция проясняет смысл самой значимой концепции – микробного сообщества и обосновывает важность понимания концепции олиготрофии как феномена. Существование олиготрофных экосистем, это механизм возникновения, создания и поддержания здоровых экосистем (конечно и почв).

Наиболее впечатляющим примером использования познаний функционирования МС для управления микробными сообществами в экосистемах с циклом микроорганизмов, является открытие и применение концепции “нарушающие воздействия и волнообразное развитие микробных популяций (МП) и МС” (Семенов и др., 2001). Эта концепция, по существу, является дополнением закона (третьего) общей экологии о волнообразной динамике развития популяций (Турчин, 2002). При этом обычно упоминается модель волнообразной динамики А. Лотка и В. Вольтерра (Гаузе, Витт, 1934). Однако если в общей экологии причиной волнообразной динамики традиционно объясняют взаимоотношением хищника и жертвы, что реалистично, то в экологии микроорганизмов, как мы установили - это взаимодействие субстрата и потребителя. Следствием этого является чередование фаз роста и отмирания МП и МС (Zelenev et al., 2005). Авторы, поняли биологическую сущность волнообразной, осциллирующей динамики существования и развития микроорганизмов в природе. Возникла идея, применить эти познания для практических целей, для определения параметра здоровья почвы (Семенов, и др., 2011а; Семенов и др., 2011в; Semenov, Đukić, 2017).

Пример разработки метода определения параметра здоровья почв в котором, используются образы разных почв, т.е., с очень разным биоразнообразием и, следовательно, разной активностью является показателем возможности управления микробными сообществами и природными экосистемами, где микробный цикл также естественен, как циклы дня и ночи и времен года.

Заключение

В настоящем сообщении по существу впервые, собраны и обсуждены хотя уже известные, но до сих пор не обобщенные, не субординированные законы микробной экологии. Среди ученых и практиков, конечно, давно витает заветная мечта и вопрос, как управлять микробными сообществами и соответственно природными экосистемами? Бесконечно возрастающий объем знаний в микробной экологии, с одной стороны, позволяет глубже и детальнее постигать природу, но с другой, своим множеством, размывает значимость конкретных достижений. Представляется, что множество разрозненных знаний можно объединить и упорядочить через законы и концепции. Применение законов и концепций микробной экологии, вносит осмысление в управление окружающей средой и позволяет приблизить осмысленное управление микробными сообществами и микробными экосистемами. Проведение соподчинения и обсуждение известных законов и концепций микробной экологии, с примерами, представляется первым шагом к решению осознанного использования в практике управление микробными сообществами и соответственно природными экосистемами. Конечно, далеко не все законы и концепции микробной экологии были обобщены в данном сообщении. Известны законы и концепции, действующие в эпидемиологии, значимость которых для социума колоссальна, а знание их обязательно. Однако из-за ограниченности разнообразия и даже численности эпидемиологически значимых микроорганизмов для контроля их циркуляции бывает достаточно “разрыва” в их циклах, например, введением карантинных мероприятий, вакцинации и др.

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HETEROTROPHIC PLATE COUNT AND ENZYME ACTIVITY IN MONITORING OF MICROBIOLOGICAL QUALITY OF GROUNDWATER

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ABSTRACT

The paper presents results of microbiological quality of the groundwater at three important sites providing drinking water for the city of Novi Sad, affected by human activity and accidental oil spills. The research was performed by means of the cultivation method, monitoring relevant group of organic pollution indicator bacteria (heterotrophs/organotrophs). The media used in the research were high nutrient agar (MPA), the same medium diluted ten times (MPA 1:10) and low-nutrient R2A medium. Phosphatase activity, a sensitive enzyme method, was used as an indicator of organic biodegradable water load. The results indicate that the data obtained by using a high nutrient medium, do not correlate with enzyme activity. On the basis of organotroph number on MPA most of the samples examined can be classified after Kohl into I category of water, while according to index of phosphatase activity quality of water ranged from polluted to very polluted. The results of phosphatase activity was much more in correlation with the number of bacteria cultivated on the low nutrient R2A medium, which was 10 to 1000 times higher comparing to the other two media. Monitoring proved sensitivity of the enzyme method, whereas in cultivation method, it emphasized the importance of low nutrient media for a reliable assessment of water quality.

Keywords: *Groundwater, Low-nutrient medium, Enzyme activity, Microbiological quality.*

INTRODUCTION

Clean groundwaters represent an extraordinary and essential resource, which is why the protection and preservation of their quality is of great importance. These waters have advantages over the surface waters because they are better protected, although all sources for water supply are equally susceptible to pollution. The quality of groundwaters can be compromised, mostly by human negligence or accidental situations.

Supply of drinking water by groundwater capture is a century-old tradition in Vojvodina (Stojšić, 1995; Dimkić, 2007). The water supply system of Novi Sad has only one water treatment plant – Štrand (capacity of 1,500 l/s). Raw water arrives at the plant from the three active sources located in the building area of the city: Štrand, Ratno ostrvo and Petrovaradinska ada. Since Novi Sad waterworks relies on the sources on Danube riverside area, the quality of groundwaters is under the influence of river water quality, the degree of hydraulic connection between groundwaters and surface waters as well as the self-purifying possibilities of aquatic environments.

The microbiological quality of groundwater in different areas of the territory of Vojvodina varies from one to the other aquifers, as the quality of water in these aquifers is subject to variation. In addition to the physico-chemical characteristics (the content of natural organic matter, iron, manganese, various salts and gases), the microbiological properties of groundwater (which are basically caused by the water chemistry) also play a significant role. Groundwater from deep and well-protected aquifers is usually free from pathogenic microorganisms and most often with a low total bacterial count. However, if the source is not adequately protected or there is the possibility of secondary contamination, the situation is different. For these reasons, continuous monitoring of microbiological quality is necessary, whereby the total number of aerobic organotrophic bacteria is most often used as a general indicator.

The quantification of bacteria in an environment by isolation on culture media (cultivation method) is still a fundamental method in microbiological assays. The method does not detect the total count of bacteria in an environment, but the representation of certain groups of bacteria that have identical or similar metabolic pathways and specific ecological and physiological characteristics. The fact that the cultivation methods do not provide a realistic estimate of the number of all bacteria in an environment (all metabolic types), does not diminish the importance of their use and numerous techniques for the isolation and cultivation of various bacterial species have been developed and many nutrient media have been formulated. These indirect methods are important because they enable the enumeration of viable members of a particular group of bacteria by adjusting the cultivation conditions (abiotic factors and the composition of the culture medium). As a general indicator of the total bacterial count in an environment, an estimate of viable, aerobic heterotrophic bacteria is used, since by the type of metabolism (organotrophy) they are the most widespread and most prominent group of bacteria in the nature. The parameter is denoted as *heterotrophic plate count* (**HPC**) or *standard plate count* and represents the counting of the *colony forming units* (CFU).

The monitoring of the quality of groundwater sources was carried out with the aim of assessing the vulnerability of sources considering the possibility of organic pollution and the accidental oil spill at one of the sources. In monitoring, the special attention was paid to low-nutrient media and enzymatic activity in assessing the microbiological quality of waters.

MATERIAL AND METHODS

The testing of the microbiological quality of groundwater was carried out at three drinking water sources of the city of Novi Sad (Fig. 1). During the four-year period, the sources Štrand and Petrovaradinska ada were tested every three months and a source Ratno ostrvo was tested every month.

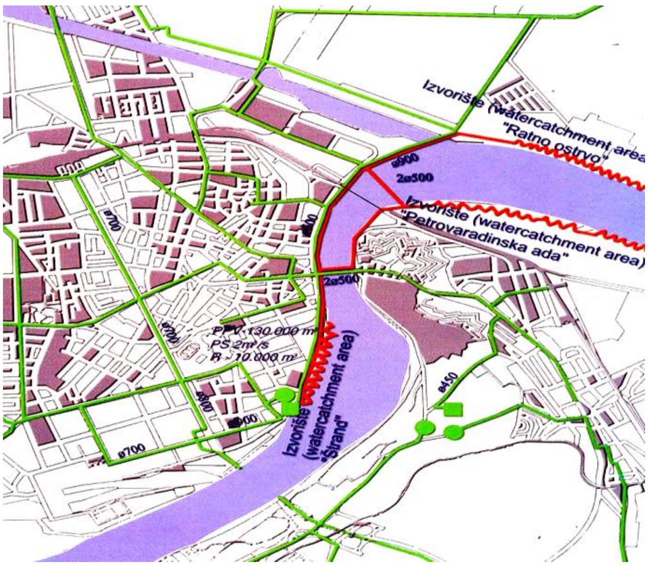

Figure 1. Sources of Novi Sad (Štrand, Petrovaradinska ada and Ratno ostrvo)

The source of Štrand is the oldest source of Novi Sad. It is located next to a water treatment plant. In the area of Štrand six wells with horizontal drains and six vertical wells were built, as well as a series of piezometers (exploration wells).

The source of Petrovaradinska ada is the only source on the right bank of the Danube. In this territory, the groundwater of eight piezometers was examined. Activities that jeopardize this source include the wild garbage depot and the settlement near the source, the sand deposit, the restaurant, the international anchoring of ships, and the accidental pouring a large amount of oil into the Danube.

Ratno ostrvo is the most powerful source of Novi Sad. It extends along the left bank of the Danube, from the mouth of the DTD channel downstream. After accidental oil pollution (the destruction of the rafinery 1999), in this area, in the demarcation zone of the rafinery complex and the source zone, a hydraulic barrier was built as a protection measure. The barrier consists of twelve wells in a row with a distance of 50-100 m. The wells are of a protective character and next to them investigating piezometers designed for monitoring the occurence of pollution were built.

Sampling of the water in piezometers was carried out with special pumps. At the source of Štrand the water of eight piezometers (S 1-8) was tested, as well as at Petrovaradinska ada (PA 1-8). On Ratno ostrvo the testing included water of wells in hydraulic barrier (twelve wells in a row, BHZ 1-12).

Microbiological analyses were performed using standard methods. They included parameters relevant to organic pollution of water - organotrophic bacteria and enzymatic activity of water. The number of organotrophs was determined on three media of different nutritive values. The standard nutrient agar, meat-peptone agar (MPA) was used, and according to the representation of the organotrophs on that medium, the water classification according to Kohl to the classes I-IV (Kohl, 1975) was performed. Organotroph abundance was also determined on the nutrient agar diluted ten times - MPA 1:10 (Petrović et al., 1998). The study also included the count of organotrophs on the low-nutrient R2A medium (Reasoner and Geldreich, 1985), which increasingly finds application in microbiological practice (Ćirić and Petrović, 2017). Incubation for all media was at 26 ºC for 5-7 days. Abundance was expressed as the number of bacteria per ml of water.

The enzymatic, phosphatase activity (Matavulj et al., 1990***),*** expressed as the index of phosphatase activity IFA (µmol/s/dm^3 pNP) was determined as the indicator of eco-physiological state of water. The level of this enzymatic activity reflects the state of total organic load of aqueous systems. According to the load of water by organic pollutants, and based on IFA, categorization of water to the classes I-A to IV-B, ie from a maximum clean to a maximum dirty (Petrović et al., 1998) was done.

RESULTS AND DISCUSSION

The presence of organotrophic bacteria was observed in all samples of groundwater of the investigated sources by cultivation method on all used media. The count of bacteria varied in a very wide range of only a few cells per ml of water, over a dozen, and most often a hundred cells, to high values of several thousand cells per ml of water (Tab. 1-3). In some water samples, very high numbers were found, which were also several tens of thousands of bacterial cells per ml. These differences were not so much caused by the location of the sampled groundwater of a particular source, as they were evidently related to the applied cultivation medium.

The groundwater of Štrand source had approximately the same quality in all piezometers according to the presence of organotrophic bacterial population in the period of investigation. Only piezometers S-7 and S-8 showed far greater count of organotrophs on all media, especially on low-nutrient R2A medium (Tab. 1). The minimum number of organotrophs at the Štrand source was recorded on the MPA medium in the water of piezometer S-3 (only 5 cells/ml), and the maximum on the R2A medium in the piezometer S-8 (68,000 cells /ml). The average representation of organotrophs on the standard nutrient medium (MPA) was such that, according to the categorization of Kohl, the water of the greatest number of piezometers belonged to the first class (Fig. 2). The exceptions were piezometers S-7 and S-8 which belonged to the I-II class of waters due to the high number of organotrophs. Enzymatic activity (Fig. 3) indicated that water quality in piezometers was worse and the most frequent belonged to the III-B category of waters, ie highly contaminated waters (S-2, 3, 5, 8) and even to the IV-A class (maximum dirty water) in the piezometer S-7 (Matavulj et al., 1990; Petrović et al., 1998).

Table 1. The count of organotrophs at the source of Štrand (CFU/ml)

Piezometer	Value	Medium		
		R2A	*MPA 1:10*	*MPA*
S-1	min	650	158	60
	max	11900	3600	2560
	mean	**6333**	**895**	**805**
S-2	min	475	55	40
	max	5275	1265	447
	mean	**2256**	**663**	**268**
S-3	min	10	12	5
	max	3057	877	625
	mean	**1088**	**309**	**237**
S-4	min	45	14	7
	max	2985	725	377
	mean	**720**	**223**	**66**
S-5	min	105	17	7
	max	5000	995	575
	mean	**1491**	**239**	**204**
S-6	min	312	68	7
	max	3900	1127	580
	mean	**1146**	**345**	**124**
S-7	min	2715	544	369
	max	7080	2850	1690
	mean	**4898**	**1476**	**817**
S-8	min	1900	216	101
	max	68000	8000	1509
	mean	**15400**	**2463**	**656**

Table 2. The count of organotrophs at the source of Petrovaradinska ada (CFU/ml)

Piezometer	Value	Medium		
		R2A	*MPA 1:10*	*MPA*
PA-1	min	75	25	12
	max	22600	1354	2450
	mean	**2132**	**291**	**231**
PA-2	min	45	31	17
	max	6650	804	180
	mean	**1207**	**232**	**64**
PA-3	min	75	17	4
	max	7150	1650	1510
	mean	**1073**	**341**	**188**
PA-4	min	82	8	11
	max	1902	415	169
	mean	**695**	**115**	**36**
PA-5	min	70	19	5
	max	3342	4860	564
	mean	**1221**	**1344**	**116**
PA-6	min	30	9	3
	max	31000	892	3000
	mean	**3443**	**215**	**254**
PA-7	min	140	5	4
	max	587	87	72
	mean	**237**	**46**	**40**
PA-8	min	20	15	3
	max	7970	1390	310
	mean	**1653**	**214**	**68**

At the Petrovaradinska ada source (Tab. 2), the organotroph presence varied also within the broad limits of only few cells (MPA; PA-3, PA-5, PA-7 and PA-8), up to several thousand cells per ml of water (R2A; PA-1 and PA-6). The average count of bacteria during a four-year period of investigation did not show as much variation between the different piezometers as regards the MPA and MPA 1:10 media, but there were noticeable differences between these two and the low-nutrient R2A medium. The average representation on the R2A medium was generally several hundred to more than three thousand cells per ml (Tab. 2), and the differences were particularly large in the piezometers PA-1, PA-2, PA-6 and PA-8. According to the average representation of the organotrophs on the

MPA medium, the water of all piezometers at the Petrovaradinska ada source can also be classified in the first class after Kohl (Fig. 4). At this source, as well as at Štrand, enzymatic activity indicated the poorer water quality (Fig. 5), compared to the MPA organotrophic count. In most cases water was classified to the III-A class (contaminated waters) or III-IV class (dirty waters), according to IFA. This picture of enzymatic activity (Fig. 5) was closer to the assessment of the groundwater quality at the Petrovaradinska ada source based on the presence of the bacterial population on the R2A medium, rather than the assessment of the quality on the basis of the bacterial count on the other two media.

Table 3. The count of organotrophs at the source of Ratno ostrvo (CFU/ml)

Well	Value	Medium		
		R2A	*MPA 1:10*	*MPA*
BHZ-1	min	977	70	38
	max	49700	3880	1370
	mean	**12890**	**810**	**247**
BHZ-2	min	300	131	40
	max	61640	4482	3290
	mean	**11351**	**1960**	**664**
BHZ-3	min	1037	154	40
	max	40150	3220	895
	mean	**6617**	**871**	**312**
BHZ-4	min	250	44	15
	max	9670	2480	135
	mean	**2458**	**599**	**69**
BHZ-5	min	322	130	36
	max	42250	1208	710
	mean	**5817**	**567**	**155**
BHZ-6	min	395	97	15
	max	17410	6540	4640
	mean	**5862**	**1297**	**564**
BHZ-7	min	145	65	9
	max	47600	1450	256
	mean	**6817**	**398**	**73**

BHZ-8	min	97	24	11
	max	38320	2240	467
	mean	**6054**	**550**	**197**
BHZ-9	min	417	143	56
	max	30200	1695	1110
	mean	**4804**	**867**	**218**
BHZ-10	min	1275	444	60
	max	55000	5680	5500
	mean	**12107**	**2701**	**2200**
BHZ-11	min	1340	173	55
	max	50000	2840	825
	mean	**10567**	**993**	**242**
BHZ-12	min	900	161	37
	max	6750	2860	1454
	mean	**2237**	**685**	**390**

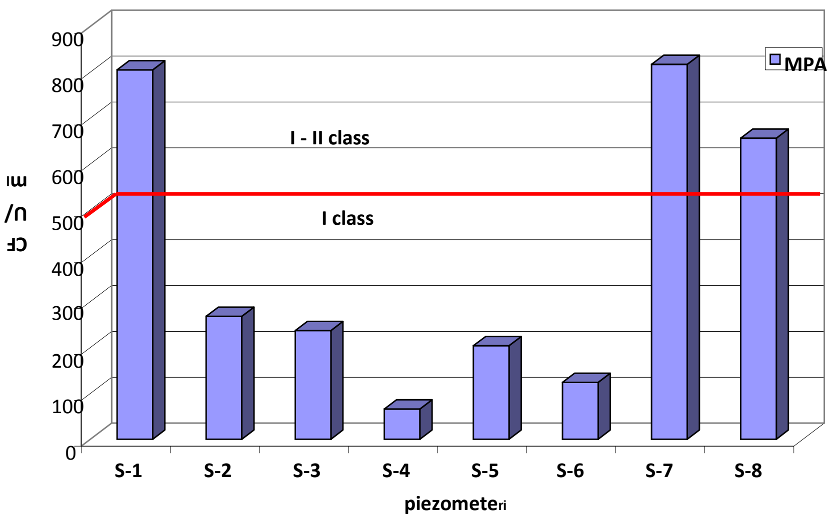

Figure 2. Štrand – representation of organotrophs on nutrient agar and categorization of water according to Kohl

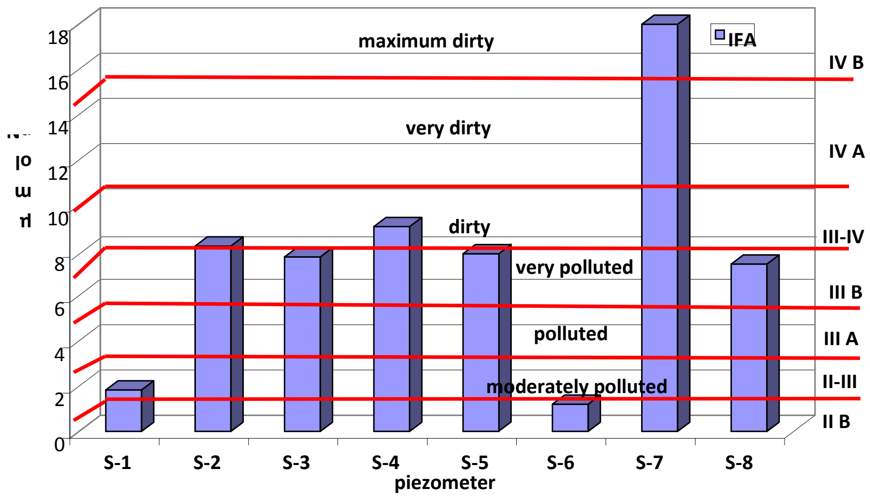

Figure 3. Štrand - enzymatic activity of water (IFA)

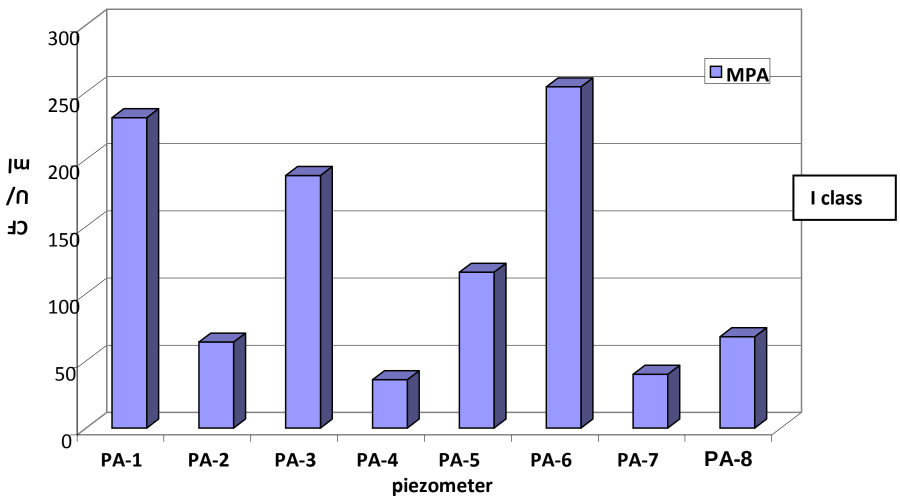

Figure 4. Petrovaradinska ada – representation of organotrophs on nutrient agar and categorization of water according to Kohl

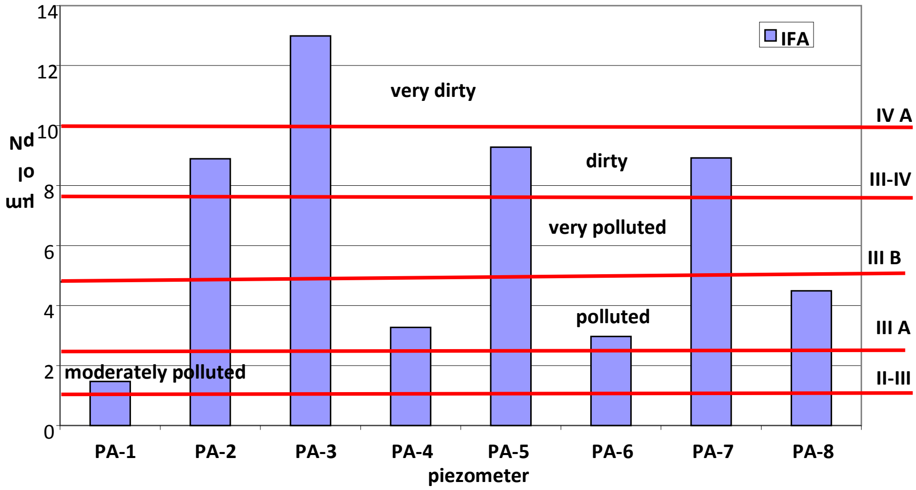

Figure 5. Petrovaradinska ada - enzymatic activity of water (IFA)

Groundwater in the hydraulic barrier wells (Tab. 3) also showed great variations in the representation of organotrophic bacterial population, with a wide range of the lowest and highest recorded values. Based on the average representation of organotrophs, it was noticed that there was not much difference in water quality among wells, with the exception of BHZ-10 which showed slightly higher values. The lowest number of organotrophs were recorded on the MPA medium, and somewhat higher on the diluted medium MPA 1:10, but with similar dynamics of varying. According to Kohl, based on the number of organotrophs on the MPA medium, most of the water samples could be classified in the first class (Fig. 6). The exception was BHZ-10 which belonged to the second class. In the same water samples, using R2A medium, the obtained values gave a completely different picture. First of all, there were great differences in the number of bacteria detected on this medium compared to the other two, and there were differences between the wells. BHZ-10 remained with the highest presence of bacterial population, but BHZ-1, BHZ-2 and BHZ-11 had also high bacterial count on R2A medium. Enzymatic phosphatase activity of water in wells of hydraulic barriers (Fig. 7) showed that water could be categorized as III-A class (contaminated waters) or even III-B class (highly contaminated waters). Results of the presence of organotrophic bacterial population on R2A medium were closer to the results of enzymatic activity and showed a more reliable picture of the tested water quality.

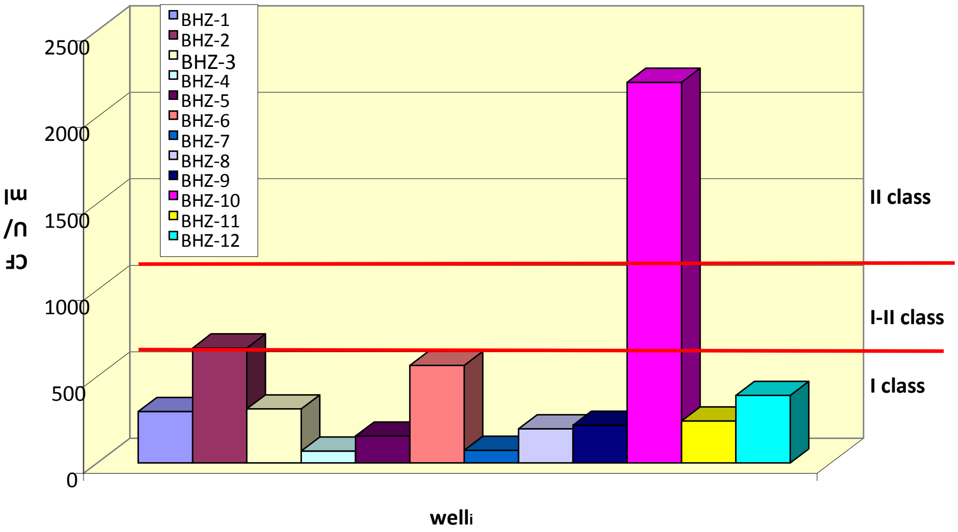

Figure 6. Ratno ostrvo – representation of organotrophs on nutrient agar and categorization of water according to Kohl

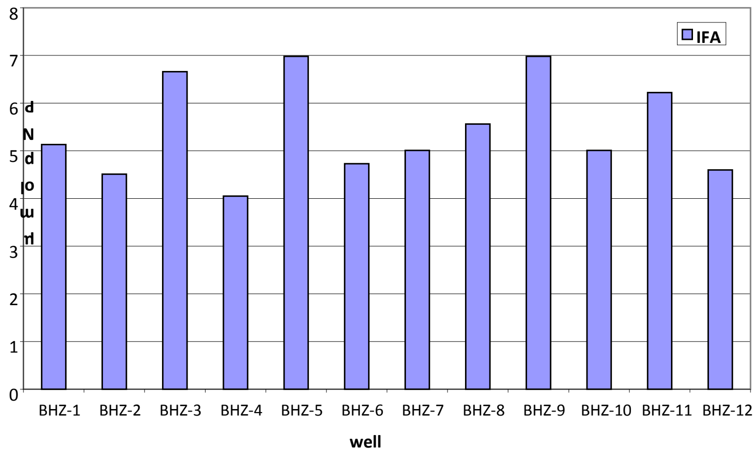

Figure 7. Ratno ostrvo – enzymatic activity of water (IFA)

During the entire research period in the same water samples using three different media for determining the number of organotrophs, the same conclusion was made that the standard nutrient medium showed as the least reliable for assessing water quality. This is clearly evident not only from the average values but also from individual cases.

The average count of organotrophic bacteria in the groundwaters of Novi Sad sources (Fig. 8) showed that if the results obtained on the standard and diluted nutrient agar were considered, there were fewer differences between the sources and the number of bacteria was usually less than 1,000 per ml of water. However, the data on the number of organotrophs on the R2A medium indicated, above all, that the presence of bacterioflora was much higher than estimated by the use of other media and that the sources differ from one another. The average enzymatic activity of groundwaters (Fig. 9) showed that they were polluted to very polluted waters, which was much closer to data on the presence of bacterioflora on the low-nutrient R2A medium.

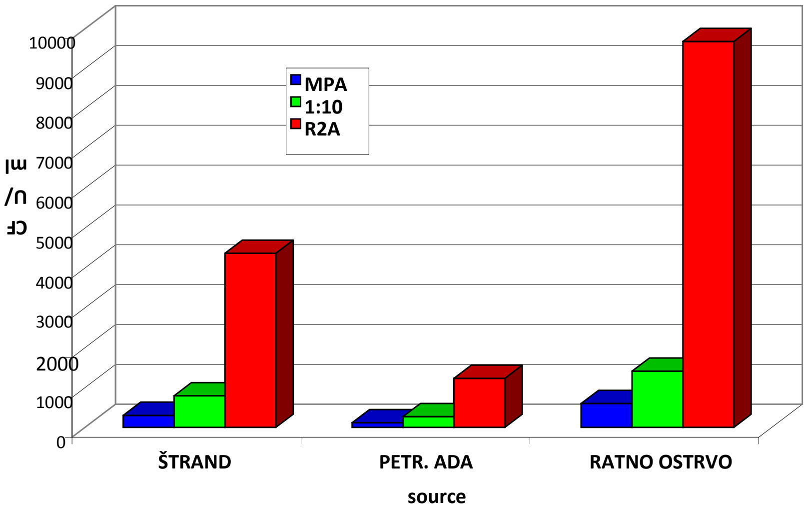

Figure 8. Average representation of organotrophs in water sources during the research period on three used media

In this research, the number of organotrophs on the standard nutrient medium (MPA) which is most commonly used in cultivation methods for estimating the number of bacteria in a certain environments, in the groundwater of all sources was the lowest. Due to such small values, according to the categorization of Kohl, these groundwaters could be classified in the first class of quality. Some of the higher values in the same samples were recorded on this medium in which the concentrations of nutrients were decreased ten times (MPA 1:10).

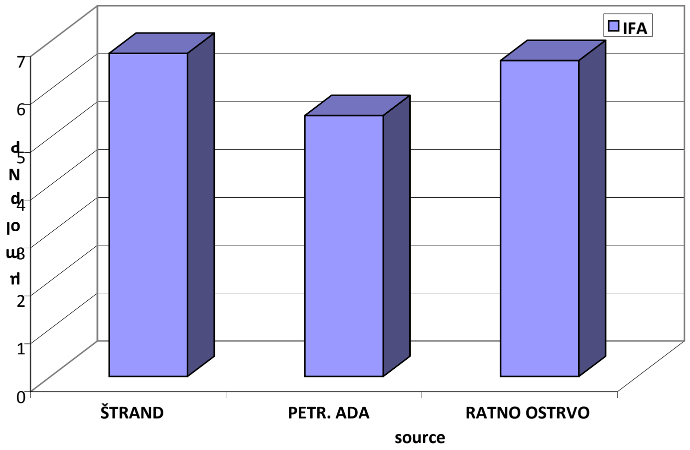

Figure 9. Enzymatic activity of groundwater during the research period

In ecological studies of ambient waters **(**Gajin et al., 1990; Gajin et al., 1992**)**, diluted MPA medium has been used as this nutritionally poorer medium has been found to be more appropriate. The reason is the fact that the dominant microflora of unpolluted waters is an oligotrophic microflora, for which conventional bacterial cultivation techniques on a nutrient-rich medium are insufficiently sensitive. This rule was confirmed in this paper, in the case of groundwaters, because in all samples, regardless of the source, a greater number of present bacterioflora were detected on MPA 1:10 medium compared to the standard nutrient medium. The largest number of bacteria in all groundwater samples was detected on the low-nutrient R2A medium, especially compared to the classic MPA medium.

In addition, the average number of organotrophs obtained on MPA medium in 23 sources (82%) exceeded the legally prescribed maximum number of these bacteria in closed water sources - 100 CFU/ml (Official Gazette of FRY, 42/98). This limit was exceeded by all results obtained on diluted MPA medium, as well as all results obtained on R2A medium. The number recorded on the diluted MPA exceeded the legal limit 1.2-25 times, and the number on the R2A was on average 7-154 times higher than the legally prescribed.

It is known that the established bacterial count can not always be taken as a criterion for assessing their activity in water, and for this purpose, methods of measuring various metabolic processes are used today, such as determining the enzymatic activity of water. One of such relatively fast and precise methods is the measurement of phosphatase activity of water, which reflects the level of organic load and gives a more realistic picture of the state of water (Matavulj, 1986).

Unlike water quality data obtained on the basis of the number of organotrophs on the classical nutrient agar and the Kohl classification, the enzymatic activity of the examined waters showed a different picture. Phosphatase activity of the water was high in most cases at all sources, and according to the index of phosphatase activity water often ranged from II-III to III-IV class, ie from slightly polluted to highly polluted waters. Enzymatic activity data additionally confirmed the fact that for a more rational assessment of water quality based on organotrophic count it is more convinient to use the R2A medium.

CONCLUSION

Based on the obtained results, as well as on the basis on legal norms, it can be concluded that the water of the investigated sources was mostly of unsatisfactory quality. Judging by the high numbers of organotrophs and relatively high enzymatic activities, the groundwater of these sources is vulnerable to organic, biodegradable pollution, which is why the protection of the sources should be taken into account. In order to obtain a realistic picture of water quality, it is advisible to use sensitive enzymatic methods in addition to cultivation. To make a more reliable assessment of the quality and potential vulnerability of groundwater, more sensitive, low-nutrient media should be included in the analysis. The results particularly indicate the importance of applying the R2A medium in the monitoring of groundwater quality.

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ВЛИЯНИЕ ТЕХНОЛОГИИ “NO-TILL” на ЧИСЛЕННОСТЬ И ТАКСОНОМИЧЕСКИЙ СОСТАВ МИКРОСКОПИЧЕСКИХ ГРИБОВ В АГРОЧЕРНОЗЕМах

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Охарактеризованы сообщества культивируемых микроскопических грибов агрочерноземной почвы опытного хозяйства СНИИСХ (Россия, Ставропольский край). В исследованных образцах по численности и обилию преобладают олиготрофные, сапротрофные и целлюлозолитические микромицеты. Наибольшей численностью (8×10^3 КОЕ/г почвы) и таксономическим разнообразием (до 8 видов) характеризовался род Penicillium. Высокая численность целлюлозолитиков (до 4×10^4 КОЕ/г почвы для образца) выявлена в почвах с традиционной обработкой под соей, озимой пшеницей и «no-till» с кукурузой. Тип обработки почвы не влиял на численность фитопатогенных грибов. Численность эккрисотрофной группы была незначительна (от 6×10^1 до 5×10^2 КОЕ/г почвы). Основными эпифитами в составе сообщества микромицетов являлись виды Trichotecium roseum и Aureobasidium pullulans, численность которых достигала 2×10^3 КОЕ/г почвы. Численность и разнообразие условно-патогенных микромицетов заметно больше в почвах с традиционной обработкой (до 4×10^3 КОЕ/г почвы и до 11 видов), по сравнению с вариантами «no-till» (до 8×10^2 КОЕ/г почвы и до 4 видов). Таксономическое разнообразие микроскопических грибов было, преимущественно, больше в почвах с удобрениями (от 10 до 30 видов), чем без них (от 7 до 27 видов).

***Ключевые слова**: микромицеты, КОЕ, таксономическое разнообразие, нулевая обработка почвы, «no-till», традиционная обработка почвы.*

ВВЕДЕНИЕ

В настоящее время черноземы подвергаются усиленной агрогенной нагрузке (Замотаев и др., 2016). Интенсивное воздействие орудиями землепользования, в первую очередь, в процессе традиционной обработки, уменьшает плодородие почв и приводит к ухудшению многих агрономических свойств (Пыхтин, Гостев, 2012; Кирюшин, 2014; Лебедева и др., 2016). При воздействии тяжелых механизмов на почву возрастает ее плотность, разрушаются агрономически ценные агрегаты, увеличивается эрозия. Интенсивная обработка почвы приводит к уменьшению запасов органического углерода, качества и доступности органического вещества почвы (Семенов и др., 2015; Tsiafouli et al., 2015). Также снижается численность и разнообразие микробиоты (Semenov et al., 2018), что негативно сказывается на супрессивной активностью почв по отношению к фитопатогенам (Van Agtmaal et al., 2018). Таким образом, следствием длительного применения традиционной обработки почвы является спад урожайности полей и рост частоты заболеваний растений (Пыхтин, Гостев, 2012; Ma et al., 2012; Ju, 2014; Stewart, Globig, 2016). Недостатки традиционной обработки почвы привели к распространению альтернативных технологий с минимальным воздействием на почву, в частности, «no-till» (Кирюшин, 2014). Ее суть заключается в сокращении числа и глубины механических обработок почвы, использовании относительно легких агрегатов для уменьшения физического давления на почву и покрывание ее поверхности растительной мульчей (Rainbow, Derpsch, 2011; Кирюшин, 2014). Ввиду того, что поверхностный слой почвы остается уплотненным по сравнению с традиционной обработкой, «no-till» в значительной мере препятствует водной и ветровой эрозии, и помогает сохранить большие запасы почвенной влаги (Gras et al., 2016). Поэтому «no-till» рационально применять для почв в условиях недостаточного увлажнения, в том числе и для черноземов. Эти почвы, ввиду хорошей оструктуренности и богатства гумусом, имеют благоприятные свойства для культивирования растений и не требуют интенсивной механической обработки, что облегчает внедрение на поля с этой почвой технологии «no-till» (Лебедева и др., 2016). К достоинствам данной технологии также относятся снижение механической нагрузки на почву и сохранение, как ее структуры, так и запасов органического вещества (Drechsel et al., 2005; Трусов, 2012; Кирюшин, 2014). Минимальная обработка почвы имеет и ряд недостатков, главными из которых являются повышенная засоренность посевов сорными растениями и фитопатогенами (Anderson, 2008; Кирюшин, 2013; Турусов и др., 2017; Железова и др., 2017), а также увеличение пестицидных нагрузок (Харалгина, Рзаева, 2007). Кроме того, «no-till» неэффективно использовать для переувлажненных территорий и полей со сложным рельефом (Anderson, 2008; Трусов, 2012; Кирюшин, 2013). Также нулевая обработка почв из-за недостаточного рыхления приводит к снижению аэрации, что усиливает денитрификацию и приводит к потерям азота из почвы (Yuan et al., 2018). Однако, в большинстве хозяйств черноземной полосы минимальная обработка почвы продолжает оставаться предпочтительным способом земледелия ввиду значительного сокращения энергозатрат и использования дополнительной техники (Кирюшин, 2013).

Микроорганизмы являются наиболее быстро реагирующей биотой почв на разнообразные воздействия, в том числе и на сельскохозяйственную обработку поля (Кузнецова и др., 2016; Wang et al., 2017; Semenov et al., 2018) изменения в численном и таксономическом составе могут служить индикатором заболеваний растений ввиду того, что большинство фитопатогенов являются грибами (Stewart, Globig, 2016), мы уделили внимание в исследовании именно этой группе почвенной микробиоты. Влияние «no-till» на микробиологические свойства почв и, в частности, на сообщества микроскопических грибов, изучены недостаточно полно (Wang et al., 2017; Schmidt et al., 2019). Однако микромицеты в значительной степени определяют здоровье и плодородие почвы (Schmidt et al., 2019), а деятельность сапротрофных микроскопических грибов приводит к улучшению свойств почвы, связанных с ее плодородием (Irawan et al., 2017). Высокое содержание остатков мульчи выращиваемых сельскохозяйственных культур, характерное при технологии «no-till», приводит к неконтролируемым вспышкам заболеваний, вызываемых фитопатогенными грибами (Rainbow, Derpsch, 2011; Stewart, Globig, 2016; Schlatter et al., 2017; Железова и др., 2017). Проведено мало исследований по сравнению численности и таксономической структуры сообществ микроскопических грибов даже для наиболее изученных почв – черноземов – с применением технологии «no-till» (Кутовая и др., 2018; Мельничук и др., 2018). Не известно, приводит ли использование этой технологии к увеличению численности микромицетов, условно-патогенных для человека и животных. Не ясно, как меняется соотношение других экологических групп микромицетов в почвах полей с нулевой сельскохозяйственной обработкой. Важным вопросом в исследовании стала проверка гипотезы о возможности возвращения микробиологических и агрономических свойств пашни чернозема к целине, после длительного применения на полях технологии «no-till».

Цель работы – оценка численности и таксономической структуры культивируемых микромицетов в агрочерноземных почвах Ставропольского научно-исследовательского сельского хозяйства (СНИИСХ), где длительное время применяли технологию обработки почвы «no-till».

ОБЪЕКТЫ И МЕТОДЫ

П о ч в а. Исследования проводили с образцами агрочерноземной почвы, взятыми на территории опытного хозяйства Ставропольского края (СНИИСХ). Образцы почв отбирали в 3-х кратной повторности в октябре 2018. Опытные сельскохозяйственные культуры – кукуруза, соя, подсолнечник и озимая пшеницы. Отбор почвы производили в междурядье (10-15 см от растения). Контролем служила залежная черноземная почва недалеко от СНИИСХ (табл. 1). Почву для микробиологических анализов отбирали с возможными мерами по предотвращению контаминаций. До анализов образцы хранились в холодильной камере при температуре +4°C. Для определения химических свойств почв использовали воздушно-сухие образцы.

Таблица 1. Описание исследуемых объектов.

<table>
<tr><th>Образец/обозначение</th><th>обработка</th><th>Культура</th><th>удобрения</th></tr>
<tr><td>NTSO</td><td rowspan="8">no-till</td><td rowspan="4">Соя</td><td rowspan="2">нет</td></tr>
<tr><td>NTSO2</td></tr>
<tr><td>NTSOF</td><td rowspan="2">есть</td></tr>
<tr><td>NTSOF2</td></tr>
<tr><td>NTC</td><td rowspan="4">Кукуруза</td><td rowspan="2">нет</td></tr>
<tr><td>NTC2</td></tr>
<tr><td>NTCF</td><td rowspan="2">есть</td></tr>
<tr><td>NTCF2</td></tr>
</table>

NTSU			
NTSU2			нет
NTSUF			
NTSUF2		Подсолнечник	есть
NTW			
NTW2			нет
NTWF			
NTWF2		Озимаяпшеница	есть
TRSO			
TRSO2			нет
TRSOF			
TRSOF2		Соя	есть
TRC			
TRC2			нет
TRCF			
TRCF2	trad	Кукуруза	есть
TRSU			
TRSU2			нет
TRSUF			
TRSUF2		Подсолнечник	есть
TRW			
TRW2			нет
TRWF			
TRWF2		Озимая пшеница	есть
REDE1			
REDE2	reference	Залежь	нет

Культивирование на питательных средах. Таксономический состав культивируемых микроскопических грибов определяли методом микробиологического посева на агаризованные среды (Методы…, 1991). В стерильных пластиковых пробирках готовили суспензии исследуемых образцов с разведениями 1:1000, 1:10000, 1:100000 для среды Чапека и с разведениями 1:10, 1:100, 1:1000 для среды Гетчинсона с целлюлозой. Десорбцию грибных пропагул от почвенных частиц осуществляли путем обработки почвенной суспензии на вортексе «MSV-3500» (Латвия) при скорости 3500 об/мин в течение 5 минут. Аликвоту почвенной суспензии объемом 100 мкл помещали на поверхность стерильных агаризованных сред в чашках Петри диаметром 90 мм. Повторность каждого варианта посевов 6-кратная. Для подавления роста бактерий в питательные среды добавляли стрептомицин (100 мг/л). Из посевов в чистые культуры выделяли морфотипы по макро- и микрокультуральным признакам с помощью микроскопа «Биомед-5». Идентификацию штаммов проводили по определителям для разных групп грибов (Ellis, 1971; Domsch et al., 2007). Таксономическую принадлежность отдельных штаммов дополнительно проверяли по анализу участков ITS рДНК. Выделение ДНК осуществляли по методике (Глушакова и др., 2011). Полимеразную цепную реакцию (ПЦР) осуществляли с использованием ПЦР-смеси «ScreenMix» с праймерами ITSf1 и NL4 («Евроген», Россия). ПЦР-продукты были очищены и секвенированы по методу Сэнгера компанией «Синтол» (Москва, Россия) с использованием набора реактивов BigDye Terminator V3.1 Cycle Sequencing Kit («Applied Biosystems», USA). Анализ продуктов реакции проводили на секвенаторе Applied Biosystems 3130l Genetic Analyzer с использованием праймера ITS1f.

РЕЗУЛЬТАТЫ И ИХ ОБСУЖДЕНИЕ

Численность и таксономический состав микромицетов.
Общая численность культивируемых микромицетов в посевах (для индивидуального образца) составляла от 2.0×10^3 до 2.7×10^4 КОЕ/г почвы (рис. 1, 2). Наибольшее количество КОЕ отмечено в образцах полей под традиционной обработкой и соей (TRSOF и TRSO2) на среде Чапека, что может быть связано с повышенным содержанием азота из-за длительного выращивания бобовых. Наименьшая численность микроскопических грибов выявлена в образцах полей под нулевой обработкой и культурой озимой пшеницы (NTWF 2 и TRWF) для среды Чапека. Такая закономерность может быть обусловлена, тем, что пшеница значительно обедняет почву минеральными элементами питания. Численность микромицетов несколько выше для большинства образцов на среде Гетчинсона (в среднем, от 1.37×10^4 /г почвы), чем на среде Чапека (в среднем, 0.88×10^4 КОЕ/г почвы). Это доказывает, что доля целлюлозолитиков чрезвычайно велика для полей вне зависимости от технологии земледелия.

Из исследованных почв СНИИСХа выделено 63 вида грибов, которые относятся к 35 родам из 2 отделов (рис. 1, 2). Отдел Mucoromycota представлен родами *Mortierella, Mucor, Rhizopus, Umbelopsis, Zygorrhynhus.* Отдел Ascomycota – 2 телеоморфными

(*Chaetomium, Eurotium* и *Talaromyces*) и 28 анаморфными родами. Также было выделено 2 типа изолятов, представленных стерильными мицелиями неопределенного систематического положения. Наибольшим видовым разнообразием характеризовались роды: *Penicillium* (8 видов), *Aspergillus* (8 видов), *Acremonium* (3 вида), *Trichoderma* (3 вида), *Cladosporium* (3 вида). Они являются типичными представителями сапротофного блока почвенной микобиоты в умеренном климате (Мирчинк, 1988; Domsch et al., 2007). В тоже время, многие представители данных родов, в первую очередь *Trichoderma harzianum* и *Cladosporium herbarum*, часто являются активными целлюлозолитиками (Ellis, 1971; Domsch et al., 2007). Такая их деятельность может привести к накоплению в почве веществ, увеличивающих ее плодородие (Irawan et al., 2017). Известно, что целлюлозолитическая активность почв, обрабатываемых по технологии «no-till», гораздо больше, нежели почв с традиционной обработкой (Кузнецова и др., 2016). Максимальная встречаемость (в посевах хотя бы на одной из использованных сред) выявлена у видов: *Penicillium janthinellum* (в 44 образцах из 46); *Acremonium strictum* (в 38 образцах); *Fusarium solani* (в 31 образцах); *Aspergillus fumigatus* (в 31 образцах); *Clonostachys rosea* (в 29 образцах); *Penicillium aurantiogriseum* (в 29 образцах); *Penicillium verrucosum* (в 28 образцах); *Acremonium* sp. (в 27 образцах); *Trichoderma harzianum* (в 27 образцах); *Cladosporium cladosporioides* (в 25 образцах). Ввиду обильной численности штаммов этих видов на среде Гетчинсона, можем предварительно заключить, что они обладают ярко выраженной целлюлозолитической активностью. Фитопатогенными свойствами из данного списка, судя по литературным данным (Domsch et al., 2007), обладают лишь *Fusarium solani* и *Cladosporium cladosporioides*; с другой стороны, все виды списка, кроме *Acremonium* sp., входят в перечень условно-патогенных микромицетов для человека (Domsch et al., 2007). Количество выделенных видов микроскопических грибов в исследуемых образцах почвы колебалось от 7 до 30 на образец. Такая широкая вариабельность связана с разнонаправленным влиянием эколого-трофических факторов в вариантах опыта, в том числе с большим количеством сельскохозяйственных культур и разнообразием обработок почвы.

В исследованных образцах СНИИСХ по обилию, численности и таксономическому разнообразию преобладают целлюлозолитические (представители родов *Chaetomium, Doratomyces, Humicola, Oidiodendron, Rhizopus, Sarocladium, Trichoderma, Zygorrhynchus*) и олиготрофные/сапротрофные (виды родов *Aspergillus, Cuninghamella, Eurotium, Paecilomyces, Penicillium, Pseudogymnoascus, Talaromyces,* *Umbellopsis*) микромицеты (рис. 1, 2). Значительно меньше по обилию фитопатогенных микромицетов (виды родов *Acremonium, Alternaria, Cadophora, Cladosporium, Cochliobolus, Coniothyrium, Fusarium, Phoma, Verticillium*). Наибольшей численностью (10^3-10^3 КОЕ/г почвы и видовым разнообразием (до 8 видов во всех образцах) характеризовался род *Penicillium* в образцах полей с традиционной обработкой под культурами сои и подсолнечника (TRSO, TRSOF, TRSU). Относительно высокая численность (10^2-10^3 КОЕ/г почвы) целлюлозолитиков выявлена в образцах с традиционной обработкой под соей TRSO2 (преобладал род *Sarocladium*), с традиционной обработкой под пшеницей TRWF2 (преобладал *Zygorrhynchus molieri*) и с нулевой обработкой под кукурузой NTC3 (доминировали *Sarocladium kiliense* и *Chaetomium globosum*). Образцы полей «no-till» под подсолнечником (NTSU, NTSUF, NTSU2) и традиционной обработкой под кукурузой, подсолнечником (TRC, TRCF, TRSUF, TRW, TRWF, TRSU2) содержали большое таксономическое разнообразие и значительную численность (10^3-10^4 КОЕ/г почвы) фитопатогенов: в образце поля с нулевой обработкой под подсолнечником с удобрением (NTSUF) доминировали *Phoma herbarum* и *Ph. exhigua*, а без удобрений (NTSU2) – *Alternaria alternata, Al. tenuissima* и *Cladosporium cladosporioides*; в образцах полей с традиционной обработкой под кукурузой и пшеницей (TRC и TRWF) – *Acremonium* sp., *Acr. murorum, Acr. strictum*; в образце полей с традиционной обработкой под кукурузой (TRCF) – *Verticillium tenerum, Verticillium* sp.; в образце полей с традиционной обработкой под подсолнечником (TRSU2) – *Cladosporium cladosporioides* и *Cl. herbarum*. Численность микромицетов условно патогенных для человека (группы BSL-1 и BSL-2) невысока – 10^1-10^2 КОЕ/г почвы. Численность эккрисотрофной группы невысока (10^1-10^2 КОЕ/г почвы) для всех микромицетов, кроме *Fusarium solani*, известного также как фитопатогена и аллергена человека (Kirk et al., 2008)), количество КОЕ которого доходила до 10^3 на грамм почвы в образце поля с нулевой обработкой под пшеницей (NTW2) на среде Гетчинсона. Численность эпифитов была значительна (до 10^3 КОЕ/г почвы) лишь благодаря *Trichotecium roseum* и *Aureobasidium pullulans* в образцах полей с традиционной обработкой под кукурузой (TRC и TRCF). Колонии эккрисотрофов (*Aureobasidium pullulans, Epicoccum nigrum, Monilia geophila, Trichotecium roseum*) были единичны. Чрезвычайно редко с минимально-выявляемой численностью (10^1-10^2 КОЕ/г почвы) встречались энтомопатогенные/нематодопатогенные микромицеты (Driver et al., 2000; Vega et al., 2008) родов *Clonostachys, Metarhizium, Pochonia*.

Важно отметить, что у некоторых микромицетов двойная экологическая роль. Например, представители рода *Fusarium* в одних условиях могут являться эккрисотрофами, а в других – сапротрофами и даже стимулировать рост растений гиббереллинами (Tudzynski, 2005), но при ослаблении растения штаммы *Fusarium* spp. часто проявляют фитопатогенную активность (Kuldau, Yates, 2000; Stewart, Globig, 2016). Также, в зависимости от множества факторов, обычными почвенными сапротрофами или же фитопатогенами являются микромицеты родов *Acremonium, Alternaria, Cadophora, Cladosporium, Cochliobolus, Coniothyrium, Phoma* и *Verticillium*. А представители родов *Clonostachys, Metarhizium, Pochonia* могут быть как сапротрофами, так и энтомопатогенными видами (Driver et al, 2000; Vega et al., 2008). Для определения специфической (фитопатогенной, энтомопатогенной и др.) активности конкретных штаммов необходимо проводить дополнительные лабораторные и полевые эксперименты, которые могут стать темой отдельного исследования.

Высокая численность (10^3-10^4 КОЕ/г почвы) целлюлозолитиков выявлена в данном исследовании, в первую очередь, на селективной среде Гетчинсона в посевах образцов поля с традиционной обработкой под подсолнечником TRSO2 (преобладал *Sarocladium kiliense*), поля с нулевой обработкой под пшеницей NTWF2 (преобладал *Zygorrhynchus molieri*) и кукурузой NTC3 (преобладали *Sarocladium kiliense* и *Chaetomium globosum*) (рис. 2). Показано, что на этой среде численность КОЕ микромицетов вышеперечисленных родов существенно возрастает по сравнению с количеством пропагул, вырастающих на среде Чапека. Значительная целлюлозолитическая активность может быть обусловлена большим запасом растительных остатков на полях.

Сапротрофный блок был представлен типичными почвенными микромицетами – *Aspergillus* spp., *Cuninghamella elongata, Eurotium niveoglaucum, Paecilomyces lilacinus, Penicillium* spp., *Pseudogymnoascus pannorum, Talaromyces flavus, Umbellopsis ramanniana* (рис. 1, 2). Их значительная часть входит в базу данных BSL патогенных для человека видов. Наибольшей численностью (10^3-10^4 КОЕ/г почвы) и видовым разнообразием (8 видов во всех образцах) характеризовался род *Penicillium*. Количество его КОЕ было максимально в образцах полей с традиционной обработкой под соей и подсолнечником TRSO, TRSOF, TRSU. Несколько меньшее количество видов (7 во всех образцах) отмечено в роде *Aspergillus*, почти все представители которого условно патогенны для человека (Domsch et al., 2007). Наибольшая численность видов рода *Aspergillus* (10^2-10^3 КОЕ/г почвы) выявлена в образцах полей с нулевой и традиционной обработкой под пшеницей (NTW2, NTWF2, TRWF2). Среди которых особо отметим образец TRWF2, где значительна численность (10^3-10^4 КОЕ/г почвы) представителей родов *Aspergillus* и *Penicillium*. Сапротрофная группа микроскопических грибов широко специализирована и может одинаково хорошо разлагать разнообразные типы субстрата, как сложные полисахариды (лигнин, целлюлозу и др.), так и легкодоступную органику (сахара, аминокислоты и др.). В результате деятельности сапротрофов разрушается большая часть растительных и животных отмерших остатков, происходит минерализация органики и образование гумуса, увеличивающего плодородие почвы (Irawan et al., 2017).

Образцы полей с нулевой обработкой под подсолнечником NTSU, NTSUF, NTSU2; полей с традиционной обработкой под кукурузой, пшеницей и подсолнечником TRC, TRCF, TRSU2, TRSUF, TRW, TRWF, содержали высокое таксономическое разнообразие и численность (10^3-10^4 КОЕ/г почвы) фитопатогенов. Причем доминантами в каждой из почв были разные роды и виды. Так, в образце поля с нулевой обработкой под подсолнечником NTSUF преобладали *Phoma herbarum* и *Ph. exhigua*; в образце поля с нулевой обработкой под подсолнечником NTSU2 – *Alternaria alternata, Al. tenuissima* и *Cladosporium cladosporioides*; в образцах полей с традиционной обработкой под кукурузой TRC и пшеницей TRWF – *Acremonium murorum, Acremonium strictum*, *Acremonium* sp.; в образце полей с традиционной обработкой под кукурузой TRCF – *Verticillium tenerum* и *Verticillium* sp.; в образце полей с традиционной обработкой под подсолнечником TRSU2 – *Cladosporium cladosporioides* и *Cl. herbarum*.

Численность эккрисотрофной группы для всех микромицетов была невысока (10^1-10^2 КОЕ/г почвы). Однако для представителей рода *Fusarium,* количество КОЕ было велико и доходило до 10^3 КОЕ/г почвы в образце полей с нулевой обработкой под пшеницей NTW2. При некоторых условиях часть представителей данного рода становятся фитопатогенами сельскохозяйственных растений (Kirk et al., 2008; Tadych, White, 2009). Единичные колонии *Geotrichum candidum* выявлены лишь в образце поля с нулевой обработкой под соей NTSO. Данный вид обычно имеет дрожжеподобный рост и имеет широкую экологическую пластичность, хорошо адаптируясь как к относительно засушливым, так и к переувлажненным локусам (Domsch et al., 2007; Deak, 2009). Остальные представители этой экологической группы выявлены практически во всех исследованных образцах. Эккрисотрофы участвуют разложении и преобразовании специфической легкодоступной органики (фотосинтата), выделяемой растениями. Колонии других представители эккрисотрофов (*Aureobasidium pullulans, Epicoccum nigrum, Monilia geophila, Trichotecium roseum*) были единичны.

Эпифитные и эндофитные микромицеты – неотъемлемый компонент филлосферы растений, в том числе и сельскохозяйственных (Tadych, White, 2009). Влияние данной экологической группы грибов на физиологию растений слабо изучено. Однако известно, что если растение здорово, то эпифитные микромицеты защищают его от фитопатогенов и выделяют вещества, способствующие росту растения. С другой стороны, если растение ослаблено, некоторые из этих грибов могут переходить к паразитизму на хозяине или сапротрофизму (Tadych, White, 2009). Численность эпифитов была значительна (до 10^3 КОЕ/г почвы) исключительно за счет *Trichotecium roseum* в образце поля с традиционной обработкой под кукурузой TRC и дрожжеподобного *Aureobasidium pullulans* – в образце поля с традиционной обработкой под кукурузой TRCF. Известно, что *Trichothecium roseum* в некоторых условиях продуцирует значительное количество афлотоксинов, опасных для человека и животных (Žabka et al., 2006). *Aureobasidium pullulans* может переходить к эндофитному образу жизни, по-видимому, помогая растению в реализации некоторых метаболических путей (Domsch et al., 2007; Vega et al., 2008).

На среде Гетчинсона не отмечали микромицетов родов: *Acremonium, Alternaria, Aspergillus, Aureobasidium, Cadophora, Chaetomium, Cladosporium, Cladophialophora, Clonostachys, Cochliobolus, Coniothyrium, Cuninghamella, Doratomyces, Eurotium, Fusarium*, многие из которых, по литературным данным, являются целлюлозолитиками (Domsch et al., 2007). Предварительно можем заключить, что штаммы вышеперечисленных родов, не растущие на среде Гетчинсона, не являются также инициирующими деструкторами целлюлозы.

Таким образом, длительное применение технологии «no-till» сокращает численность сапротрофных и целлюлозолитических, но не влияет на количество фитопатогенных и условно-патогенных для человека микромицетов в агрочерноземах СНИИСХ. Традиционная обработка данных почв увеличивает долю эккрисотрофной группы и количество видов рода *Penicillium*.

БЛАГОДАРНОСТЬ.

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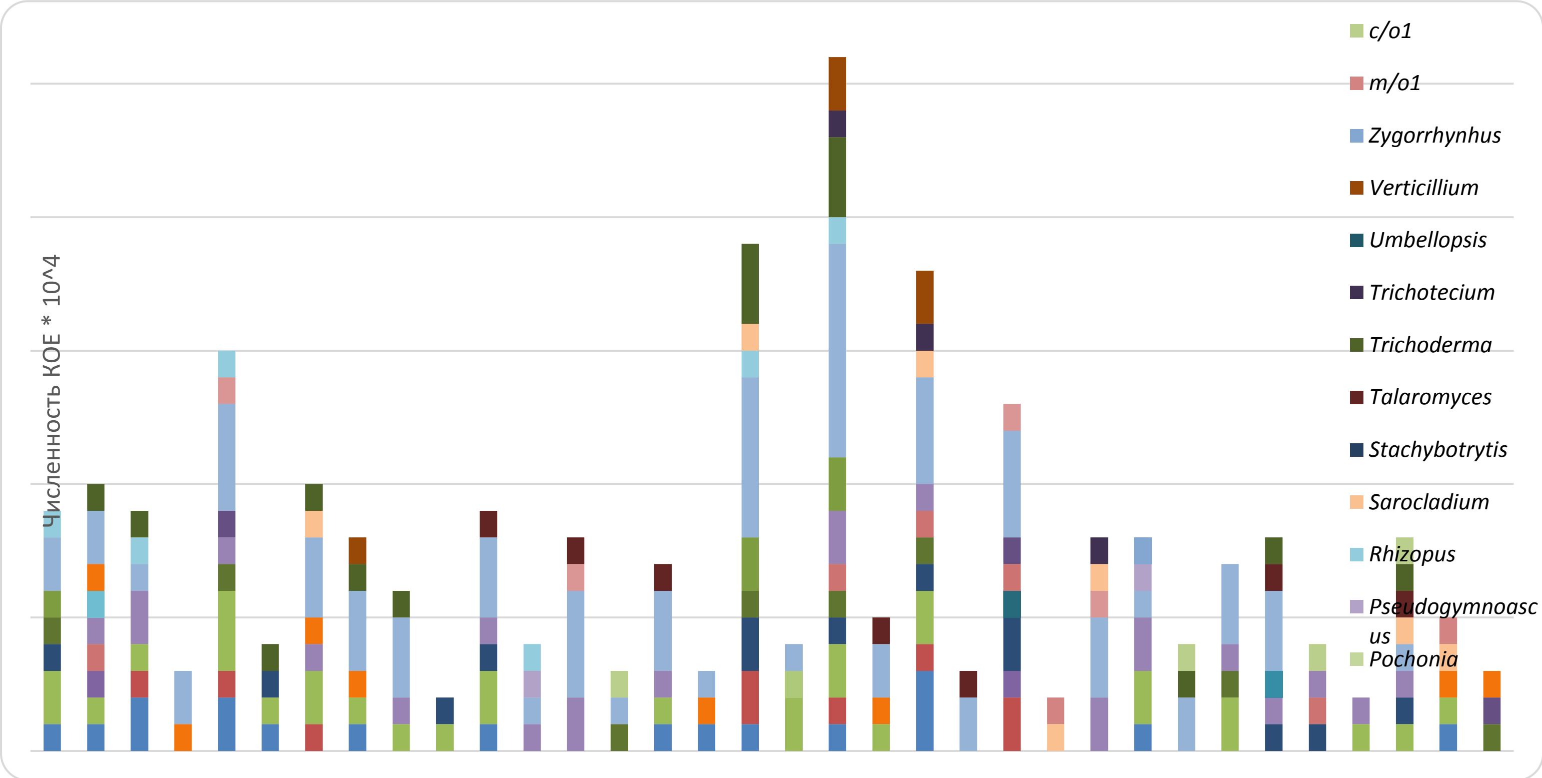

Рис. 1. Численность КОЕ микромицетов на среде Чапека.

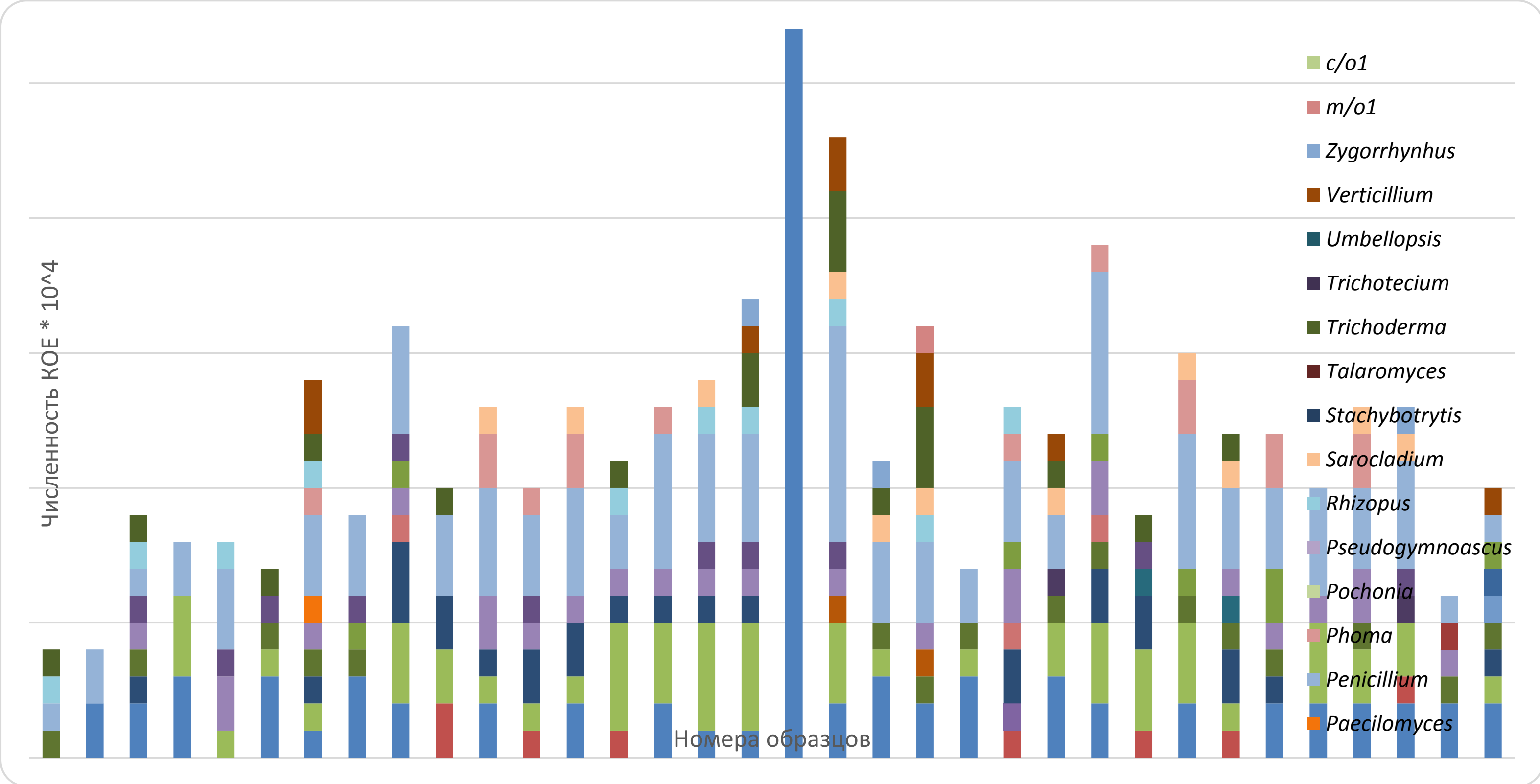

Рис. 2. Численность КОЕ микромицетов на среде Гетчинсона

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Efficacy of indigenous *Pseudomonas chlororaphis* Strains in Promoting Yield and Main Chemical Growth Parameters of Lettuce

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ABSTRACT

Fluorescent Pseudomonas chlororaphis are able to colonize the plants roots and stimulate growth by decreasing the frequency of diseases, using various mechanisms for their action: production of antibiotics, HCN, plant hormones, etc. In this study we examined the effect of two indigenous plant growth promoting rhizobacterial strains of P. chlororaphis (Q4 and Q10) and their mixture (mix Q4+Q10) on the main chemical growth parameters and the yield of dry biomass of lettuce (Lactuca sativa L.). The study was carried out with stagnosol type of soil in pot experiments under semi-controlled conditions in the glasshouse of the Institute of Soil Science, Belgrade, in the period from March to June in 2014. Phosphorus was determined by spectrophotometer, potassium - by flame emission photometry, total nitrogen and carbon - using elemental CNS analyzer Vario EL III, while calcium and magnesium were determined by AAS. The content of proteins in dry biomass was calculated on the basis of nitrogen content. The data on the lettuce dry biomass yield showed that its treatment with both P. chlororaphis strains, as well as with their mixture, had positive effect on this parameter in relation to the control, whereby the strain Q10 was more effective than Q4 and mix Q4+Q10. The obtained results of the studied chemical parameters of lettuce were in accordance with the yield, meaning that their content was the highest in lettuce treated with Q10 strain. Concluding, studied P. chlororaphis strains have high potential in promoting the yield and main chemical growth parameters of lettuce.

***Keywords**: Chemical Growth Parameters, Dry Biomass Yield, Lactuca sativa L., Pseudomonas chlororaphis, Stagnosol.*

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is an annual herbaceous plant from the family Asteraceae, genus *Lactuca*. Nowdays in Serbia it can be found the weed species *Lactuca virosa* L., *Lactuca seriola* L., *Lactuca saligna* L. and other species that interbreed with garden lettuce in low percentage. The true value of lettuce is in the content of minerals and vitamins. Of minerals it mostly contains calcium and iron, and then, significant amounts of magnesium, iodine, manganese, cobalt, sodium, nickel, silicon and copper. Lettuce contains vitamin C, carotene, almost all vitamins of group B, and a significant amount of vitamin E. Of the organic acid it contains malic, citric, uric and oxalic acid. It can be grown in a field and in gardens in an open air and in greenhouses throughout the whole year. As vegetable the lettuce is used fresh (Maksimović and Simović, 1992).

Many vegetable crops, including lettuce, are typically consumed without further processing after harvest and it is important that chemicals are not present in or on any part of the plant (Banchio et al., 2008). Therefore it is essential to avoid chemical fertilizers and pesticides as much as possible by using the non-chemical once for achieving optimal growth and yields of lettuce. Nowadays, the use of biofertilizers in production plays an important role as a supplement to improve the growth and yield of several agricultural, horticultural and medicinal plants (Lugtenberg and Kamilova, 2009). This biological approach is nevertheless considered to be more environment friendly in the long term. One of the emerging research areas for improving the plant growth and yield and for the control of different phytopathogenic agents is the use of plant growth promoting rhizobacteria (PGPR). They are able to improve plant growth by increasing the rate of seed germination and seedling emergence, minimizing the adverse effects of external stress factors, and protecting plants from soil-borne pests and diseases. In this respect, different isolates of fluorescent *Pseudomonas* species take prominent place (Cattelan et al., 1999).

The purpose of this study was to examine the effect of two indigenous *P. chlororaphis* strains (Q4 and Q10) and their mixture (Q4+Q10) on the main chemical growth parameters (content of N, proteins, P, K, Ca and Mg) and yield of dry biomass of lettuce (*Lactuca sativa* L.).

MATERIALS AND METHODS

The bacterial strains *P. chlororaphis* Q4 and Q10 were isolated from different host plants in Serbia. Taxonomic characterization of the isolates was based on tests for Gram staining, cytochrome oxidase, catalase, fermentation or utilization of glucose, lactose and sucrose, utilization of citrate, ability to degrade urea (Jošić et al., 2012; Jošić et al., 2015). The bacteria were grown in liquid King's B medium (KB) for 24 h on orbital shaker at 120 rpm. Concentrations were determined spectrophotometrically (OD_{600}) and all experiments were conducted using 10^6 $CFUml^{-1}$.

The effect of PGP rhizobacteria was studied on lettuce (*Lactuca sativa* L.) cultivar Majska kraljica (eng. May queen), in pot experiments under semi-controlled conditions in the glasshouse of the Institute of Soil Science (Belgrade). The experiment was conducted with stagnosol type of soil (WRB, 2014), during the first week of March until the second week of June in 2014 in plastic pots. Four experimental variants were set up in three replications, as follows: Control (V1); *P. chlororaphis* strain Q4 (V2); *P. chlororaphis* strain Q10 (V3); *P. chlororaphis* strains Q4 and Q10 mixture (mix Q4+Q10 - V4). In every small plastic pot ten lettuce seeds were sown on March 1st. After two weeks five seedlings were transplanted to plastic pots containing 4 kg of homogenized soil. After the emergence on April 24th three plants per pot were left. The inoculation of the soil and lettuce plants in each pot with 10 ml of liquid inoculum of *P. chlororaphis* strains Q4, Q10 and mix Q4+Q10 was performed one day after transplantation in the phenophase of rooting, and another treatment in the period of the rosettes formation. Control (V1) was treated with the same volume of distilled water as V2, V3 and V4 treatment.

The soil samples were air-dried, crushed and passed through a sieve (≤ 2 mm). Before setting up the experiment soil physical and chemical properties were studied.

Soil granulometric composition was analyzed by determination of particle size distribution in mineral soil material (ISO 11277:2009(E), 2009).

Chemical soil properties were determined using the following chemical analyses: soil acidity (pH in H_2O and 1M KCl, v/v - soil:H_2O=1:5, soil:1M KCl=1:5) was analyzed potentiometrically, using glass electrode (SRPS ISO 10390:2007, 2007); humus content was determined using Kotzman method (Jakovljević et al., 1985); available phosphorus (P_2O_5) and potassium (K_2O) were analyzed by Al-method according to Egner-Riehm (Riehm, 1958), where K_2O was determined by flame emission photometry and P_2O_5 by spectrophotometer after color development with ammonium molybdate and stannous chloride. Calcium (Ca) and magnesium (Mg) were extracted by ammonium acetate followed by determination on atomic adsorption analyzer

SensAA Dual - GBC Scientific Equipment Pty Ltd, Victoria, Australia (Wright and Stuczynski, 1996).

Lettuce seedlings were grown according to the standard growing methods until June 13th, when all studied relevant parameters of the plant growth were measured/analyzed. Samples of the plant material were air-dried and the yield was measured. The lettuce plants were dried at 105°C for a period of 2 hours and weighed and for all treatments and replicates the following main chemical growth parameters of the aerial plant parts were analyzed: nitrogen (N) was analyzed on elemental CNS analyzer Vario EL III (Nelson and Sommers, 1996); phosphorus (P) and potassium (K) contents were determined by method named as "wet" combustion, i.e. they were heated to boiling with the mixture of concentrated sulphuric and perchloric acids. In the obtained solution, P was determined by spectrophotometer with molybdate, and K - by flame emission photometry (Jakovljević et al., 1985); calcium (Ca) and magnesium (Mg) were determined by Atomic Absorption Spectrometry (AAS) (Wright and Stuczynski, 1996), where the plant material was converted to a solution by method named as "dry" combustion, i.e., first by heating at 550°C (for several hours) and then by treating the obtained ash with hydrochloric acid (Miller, 1998). The content of proteins in dry biomass was calculated on the basis of N content (Licitra et al., 1996), using the formula: crude proteins (%) = N (%) x 6.25 (factor for conversion of nitrogen content to crude protein).

The obtained data on soil properties represent the arithmetic means of three replicates and standard deviation values. The effects of V1, V2, V3 and V4 treatments on the studied chemical parameters and yield of the plants were evaluated using the analysis of variance (SPSS 20.0, Chicago, USA), followed by Duncan's Multiple Range Test (DMRT). Significant differences between means were tested by the LSD test at P = 0.05.

RESULTS AND DISCUSSIONS

Table 1 displays the results of soil physical and chemical properties. The studied Stagnosol is a clay loam according to the soil textural class determined on the basis of particle size distribution, having very acid reaction and medium humus content, with a very low content of available phosphorus and medium provided with available potassium. It is medium provided with calcium and with high content of magnesium.

Table 1. Physical and chemical properties of Stagnosol

Granulometric composition Fraction (%)	Value (means ± standard deviation)
Bulky sand, 2-0.2 mm	5.0±0.59
Miniature sand, 0.2-0.02 mm	25.9±1.33
Dust, 0.02-0.002 mm	45.1±1.22
Clay, < 0.002 mm	24.0±0.21
Total sand, > 0.02 mm	30.9±1.05
Dust + clay, < 0.02 mm	69.1±1.05
Chemical properties	
pH in 1M KCl	4.45±0.01
pH in H_2O	5.48±0.01
Humus (%)	2.26±0.47
Available P_2O_5 (mg $100g^{-1}$)	3.73±0.28
Available K_2O (mg $100g^{-1}$)	19.8±1.54
Available Ca (mg $100g^{-1}$)	240.0±19
Available Mg (mg $100g^{-1}$)	35.0±3.89

Table 2 displays the data on chemical growth parameters (available macroelements) in lettuce cultivar influenced by the treatment applied.

Table 2. Available macroelements in lettuce and the yield of dry biomass depending on the treatment applied

Variants	Yield (g per pot)	N	Proteins	P_2O_5	K_2O	Ca	Mg
		mg kg^{-1}					
Control	27.39±6.79[a]	4.85±0.13[a]	30.22±0.80[a]	0.73±0.06[a]	2.20±0.01[a]	2.70±0.23[a]	0.70±0.01[b]
Q4	30.29±1.32[a]	4.94±0.32[a]	30.76±1.98[a]	0.85±0.10[a]	2.61±0.30[a]	3.28±0.20[a]	0.76±0.04[a]
B10	36.58±1.06[a]	5.19±0.05[a]	32.35±0.31[a]	0.90±0.08[a]	2.66±0.34[a]	3.62±0.25[a]	0.78±0.01[a]

Q4+Q10	29.93±2.12[a]	4.89±0.47[a]	30.49±2.93[a]	0.77±0.12[a]	2.38±0.38[a]	3.14±0.59[a]	0.73±0.04[ab]
P value	NSD	NSD	NSD	NSD	NSD	NSD	*
LSD (0.05)	6.88	0.54	3.31	0.17	0.55	0.67	0.05

*LSD - least significant differences; NSD - no significant difference at the P=0.05 level of significance, whereas *, ** and *** indicate statistical significant differences at the P<0.05, P<0.01 and P<0.001 levels, respectively; values followed by the same letter in a column are not significantly different.*

The obtained results of N, P, K, Ca, Mg and proteins contents showed that the treatment of lettuce with both *P. chlororaphis* strains, as well as with their mixture, had positive effect on all these parameters in relation to the control, whereby the strain Q10 was more effective than Q4 and mix Q4+Q10. However, this influence was statistically significant only regarding the content of Mg, while for the contents of other macroelements there were no statistical differences between the treatments. This rationale is consistent with the observation that plants inoculated with PGPR take up N, P, K and microelements more efficiently from the soil (Cakmakci et al., 2006). The increase in N, P and K contents in crops inoculated with PGPR *Pseudomonas* and *Azotobacter* strains was previously reported (Vikram et al., 2007; Ordookhani et al. 2011). In addition, as stated by the other authors (Zdravković et al., 2015), and as it was done in this study, it is suggested that bacterial suspension should be applied during the growth phase of plants when the treatment has the greatest impact, i.e. when enzyme activity is the strongest.

Improved mineral nutrition would explain the promotion of root, plant growth and biomass yield. The data on yield of the lettuce dry biomass were in accordance with chemical growth parameters, meaning that the yield was the highest in lettuce treated with Q10 strain (Table 2). According to the studies of Lee et al. (2015), *Pseudomonas chlororaphis* - treated lettuce grew significantly better than nontreated lettuce, as indicated by enhancement of color, mass, length and number of leaves per head. In addition, Cipriano et al. (2016) determined improved lettuce plant biomass yield up to 30% when the strains of P. chlororaphis, with different plant growth promoting traits, were applied on lettuce.

CONCLUSIONS

The data on studied chemical growth parameters and dry biomass yield of the lettuce cultivar showed that its treatment with both *P. chlororaphis* strains had positive effect on all parameters in relation to the control, although this influence was statistically significant only regarding the content of Mg. Concluding, studied *P. chlororaphis* strains (Q4, Q10 and mix Q4+Q10) have high potential in promoting the biomass yield and main chemical growth parameters of lettuce.

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UTILIZATION OF MISCANTHUS WASTE BIOMASS FOR XYLANASE PRODUCTION BY SOIL BACTERIUM *SINORHIZOBIUM MELILOTI*

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ABSTRACT

Miscanthus × giganteus, as a lignocellulosic material, consists of about 30% xylan and represents a good basis for research as a possible raw material in the production of xylanases. Among the soil bacteria, rhizobium is rarely investigated as an enzyme producer. For the first time, Sinorhizobium meliloti strain 207 was used to obtain xylanase during submerged and solid fermentation using miscanthus biomass as a substrate. During submerged fermentation (28°C), the maximum xylanase activity was achieved after 48 h with 10% inoculum and H_2SO_4 *substrate modification. The maximum xylanase activity of 1.215 U/mL was obtained during solid-state fermentation (28°C) by using also* H_2SO_4 *modified miscanthus biomass which was moistened with distillate water. The crude enzyme, produced by strain 207, could be further used in eco-friendly processes of lignocellulose material bioconversion to useful products.*

Keywords: *agroindustrial waste, xylanase activity, solid-state fermentation, Sinorhizobium meliloti.*

INTRODUCTION

The xylanase enzymes (endo-1,4-b-xylanases) cleave the β-1,4-glycosidic linkage between the xylose residues in the backbone of xylans. Thus xylanases form a usable product such as xylose, xylobioza as xylo-oligosaccharides (Gomes et al., 2016). They are a widespread group of enzymes and are one of the most important ones, as their enzymatic activities are required for the depolymerization of the hemicellulosic constituent of the plant cell walls. The xylanase substrate, xylan, is the most abundant hemicellulose and represents approximately one third of the total renewable organic carbon on the earth (Chakdar et al., 2016). Xylan is found in large quantities in hard and soft wood, as well as in many years old plants. It is usually found in the secondary cell wall of plants (Collins et al., 2005). Due to its heterogeneity and complexity, complete hydrolysis of xylan requires a wide variety of enzymes of cooperative action. A complete xylanolytic enzymatic system is quite present among fungi, actinomycetes and bacteria, and some of the most important producers of xylanolytic enzyme include following genera: *Aspergillus, Trichoderma, Streptomyces, Phanerochaetes, Chytridiomycetes, Ruminococcus, Fibrobacteres, Clostridia* and *Bacillus* (Motta et al., 2013).

Xylanases have wide range of industrial and biotechnological applications. For instance, xylanases can be used in paper industry, in the improvement of the quality of animal feed, textile and food processing industries and also in the transformation of lignocellulosic materials in fermentable sugars for the production of second generation ethanol (Gomes et al., 2016; Chakdar et al., 2016).The production of xylanases must be improved by finding more potent fungal or bacterial strains or by inducing mutant strains to excrete greater amounts of the enzymes (Motta et al., 2013). Among bacteria, rhizobial species were rarely investigated as the producer of xylanase.

Agricultural–residues are usually used for enzymatic conversion and for the industrial production of xylanase enzymes, due to its complex structure. They also represent an example of cheap raw material for industrial production of enzymes (Nkohla et al., 2017). Fermentation is the primary technique for the production of various enzymes, and fungi and bacteria play a very important role in the fermentation process. There are two different fermentations, solid-state and submerged fermentation which were used for the production of certain enzymes. Submerged fermentation (SmF) is most often used in the production of bacterial enzymes due to the need for higher water potential. Solid-state fermentation (SSF) is preferred when enzymes have to be extracted from fungi that require a lower aqueous potential. More than 75% of industrial enzymes are produced by SmF, one of the main reasons is that SmF supports the use of genetically modified organisms to a greater extent than SSF (Martins et al., 2011; Buntić et al., 2019). On the other hand, the main advantage of using SSF in the production of enzymes is to use waste lignocellulosic materials as a substrate and thus easily recycle.

Miscanthus × giganteus is a perennial rhizomous grass which is consists of cellulose (33.9%), xylan and araban (32.2%) and lignin (26.3%) (Lee and Kuan, 2015). It grows very rapidly, up to 4 m high, producing a high annual biomass yield of 20-26 t of dry matter/h. Miscanthus is a lignocellulosic plant that is being researched as a possible raw material for obtaining paper, energy, building materials, nursery and greenhouse substrates. Recently, miscanthus biomass attracted interest as a potential raw material for the production of ethanol because it is rich in carbohydrates and grows well even in poor soil (Huyen et al., 2010).

The aim of this study was to examine the hydrolytic potential of rhizobium *Sinorhizobium meliloti* strain 207 through the production of xylanase during SmF and SSF. A modification of the lignocellulosic substrate (Miscanthus × giganteus) was made and the influence of substrate modification on the production of xylanase during SmF and SSF was also tested.

Materials & Methods

Bacterial strain and inoculum preparation

Rhizobium working culture was prepared by using *Sinorhizobium meliloti* strain 207 from the Collection of the Institute of Soil Science (ISS WDCM375-Collection of Bacteria, Institute of Soil Science, Department of Microbiology). This strain was selected according to qualitative test using xylan agar plate (per liter: birch wood xylan 1 g, yeast extract 3 g, K_2HPO_4 3 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g and agar 6 g) (Buntić et al., 2019). A loopfull of purified culture of the strain 207 was transferred from yeast mannitol agar medium on selective agar medium and incubated (3-5 days, 28°C). After incubation, agar plate was flooded with Gram's iodine (2.0 g KI and 1.0 g I_2 in 300 mL distilled water) for 5 min. Clear zones around colonies indicated xylan hydrolysis (Mihajlovski et al., 2015). Microorganism working culture was grown in Erlenmeyer flasks containing yeast mannitol broth (YMB) in a rotary shaker (125 rpm, 48 h, 28°C) (Buntić et al., 2018).

Sample materials and their preparation

The miscanthus (*Miscanthus* × *giganteus*) solid waste material was collected from the local field experiment of Institute of Soil Science, dried and grounded to a particle size up to 3 mm (unmodified substrate). The modification of substrate was performed with 1% solution of following agents: H_2SO_4, NaOH or H_2O_2 in ratio 1:5 (w:v). After 2 h at room temperature (25°C), the solid phase was separated by a vacuum pump and washed with distilled water. The resulting modified substrate was dried for overnight in an oven at 105°C. Obtained miscanthus modified (M) substrate were: M-H_2SO_4, M-NaOH and M-H_2O_2.

Liquid fermentation

Batch experiments were carried out in 100 mL Erlenmeyer flasks which were placed in a rotary shaker at 28°C and under agitation of 125 rpm. Xylanase were produced by growing 1, 5 and 10% inoculum of the *S. meliloti* 207 in media containing waste lignocelluloses material – miscanthus (unmodified or modified). Experiment was conducted in triplicate under following conditions: 2% of predetermined substrate concentration during 24, 48 or 72 h of fermentation process. After incubation, the culture medium was subsequently centrifuged (6000 × g, 15 min) and the cell-free supernatant (enzyme sample) was tested for xylanase activity.

Solid-state fermentation

All experiments related to the production of the xylanase during the SSF were performed in 100 mL Erlenmeyer flasks with 2 g of different substrates (unmodified or modified miscanthus biomass). In order to moisten the substrate, before substrate sterilization at 121°C for 20 min, distillated water (solution 1) or mineral salts solution of K_2HPO_4 (3 g/L), KH_2PO_4 (1 g/L) and $MgSO_4$ 0.5 (g/L) (solution 2) was added in ratio 1:4 (w:v). Overnight bacterial culture in a concentration of 10%, grown in YMB, was inoculated into different sterile solid media and incubated in the thermostat at 28°C. After 2 days of incubation, 10 mL of 0.1 M acetate buffer (pH 4.8) was added for enzyme extraction. All the samples were filtrated and the liquid aliquot was centrifuged and analyzed as enzyme sample, as explained below.

Enzyme assay for xylanase

Xylanase activity was measured by the reduction of 3,5-dinitrosalicylic acid (DNS) in the presence of xylose released by enzymatic hydrolysis of xylan, according to the method of Miller (Miller, 1959). Crude enzyme extract (500 µL) was incubated with 500 µL of 1% solution of xylan (birch wood xylan for xylanase) in acetate buffer (0.1 M, pH 4.8) for 15 min at 50°C. After incubation, 1 mL of DNS reagent was added and the reaction mixture was boiled (90 °C) for 5 min, cooled down and diluted by adding 5 mL of distilled water. The absorbance was recorded on the UV/visible spectrophotometer (UV-160A, Shimadzu Corporation, Japan) at 540 nm against blank (non incubated enzyme). One unit of xylanase activity was defined as the amount of enzyme that released 1 µmol of xylose equivalents per minute. Units were calculated according to the following formulae (Irfan et al., 2016):

$$Xylanase\ activity\ (IU) = \frac{\text{Reductin sugars (mg/mL)} \times 1000}{\text{Incubation time (15 min)} \times 150}$$

Results and Disscusion

Xylanase qualitative test

Sinorhizobium meliloti strain 207 was grown on xylan agar plate for 4 days. The appearance of halo zones around bacterial colony on the plate indicated that strain 207 could hydrolyze xylan and use it as the sole carbon source (**Fig. 1**).

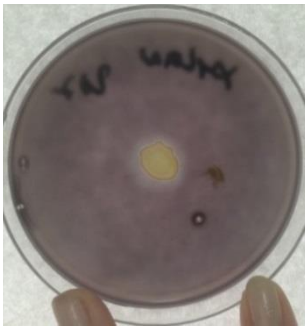

Fig.1. Hydrolysis of xylan agar by *Sinorhizobium meliloti* strain 207

Hydrolysis of xylan by *S. meliloti* has not been reported in the literature yet. Iannetta and authors (1997) reported xylanase activity of 0.41 and 0.76 U/mL in culture supernatant of *Rhizobium leguminosarum* bv. viciae.

This rhizobial species was grown in the presence of bean root and pectin as carbon source in liquid medium (Iannetta et al., (1997). Furthermore, Ivashina and Ksenzenko (2012) described *pssW* gene encoding for glycosyl hydrolase with endo-1,3-β-xylanase and endo-1,4-β-xylanase activities. As it has been determined that *pssW* gene has its counterparts in rhizobial strains such as *R. leguminosarum* and *R. etli*, it is possible that xylanase activity of *S. meliloti* could underline in the presence of similar gene (Ivashina and Ksenzenko, 2012).

Submersed fermentation using miscanthus waste biomass

Among all commercial xylanases which are can be from various origins, around 80–90% is produced in submerged culture. Xylanases are produced in response to xylans from various alterantive substrates: wheat bran, sugarcane bagasse, rice husks and wood pulp (Polizeli et al, 2005; Motta et al., 2013). On the other hand, miscanthus biomass was primarily used in the production of ethanol, but not in the production of enzymes. Considering that the production of enzymes during SmF or SSF occurs at a lower temperature than fermentation during ethanol production, the rest of biomass can still be used in the production of ethanol.

The optimal period for maximal xylanase production in the SmF process varies between different microorganisms. Different experiments were conducted to study this process parameter in enzyme production (Nagar et al., 2010; Anuradha et al. 2007; Breccia et al., 1998). The obtained results of this study indicate that fermentation period of 48 h was optimal for xylanase production by *S. meliloti* strain 207 (**Fig. 2**).

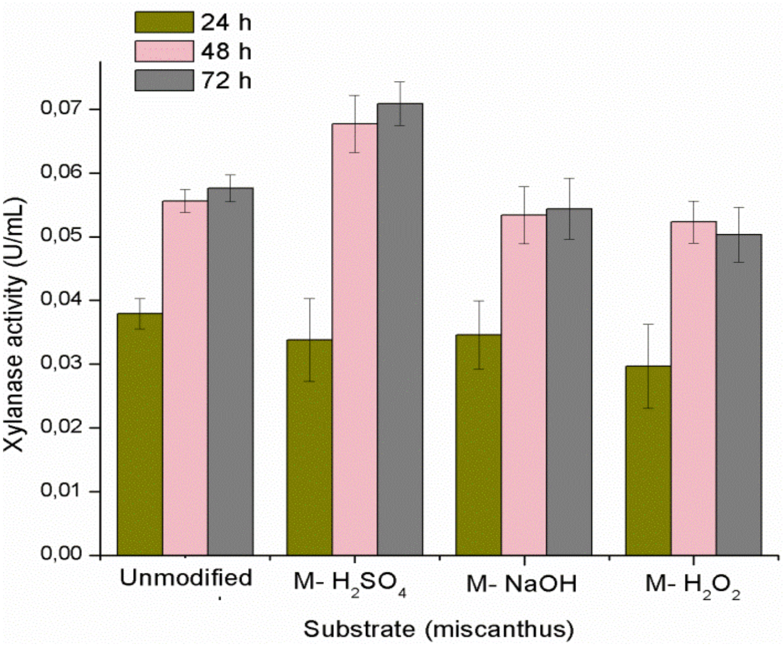

Fig.2. Xylanase production by *Sinorhizobium meliloti* strain 207 during different incubation period of submerged fermentation (5% inoculum, 28 °C, 125 rpm)

A slight increase in activity occurred after 72 h of using unmodified and modified biomass treated with H_2SO_4 and NaOH. That increase was not significant in comparison to the increase of enzyme activity between 24 and 48 h of the incubation time. It was 68%, 49.9%, 64.7% and 56.7% with using of unmodified, M-H_2SO_4, M-NaOH- and M-H_2O_2 modified waste biomass. In the literature, various microorganisms produced maximum xylanase activity after different incubation period. Thus, *Bacillus pumilus* SV-85S and *Streptomyces violaceoruber* produced maximum xylanase activity after 36 h, *Bacillus amyloliquefaciens* and *Bacillus circulans* D1 after 48 h and *Bacillus* SSP-34 showed maximum xylanase activity when grown for 96 h (Nagar et al., 2010; Khurana et al., 2007; Bocchini et al., 2002).

The effect of inoculum size on xylanase production by rhizobium *S. meliloti* 207 was also examined, and the results of obtained xylanase activities were presented in **Fig. 3**.

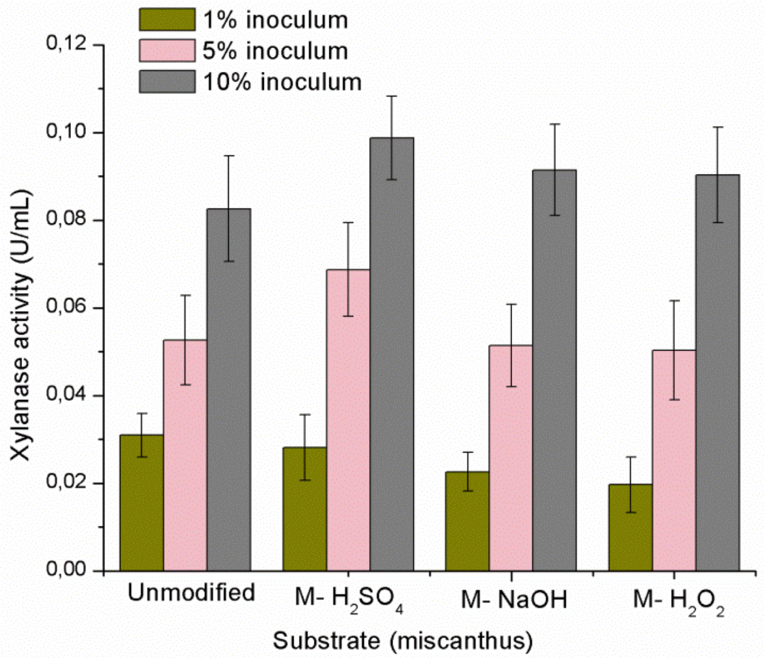

Fig.3. Xylanase production by Sinorhizobium meliloti strain 207 using different inoculum size during submerged fermentation (incubation period of 24 h, 28°C, 125 rpm)

With the increase of the inoculum size from 1 to 10%, xylanase activity was increasing as well. This increase appeared to be linear. Bearing in mind biomass modification, substrate modified with H_2SO_4 gave maximum xylanase activity of 0.0987 U/mL of crude enzyme extract. There were no differences between applying NaOH and H_2O_2 as modification agents on xylanase activity. The activities were similar with increasing of inoculum size. In addition, by comparing these two modified substrates with unmodified miscanthus biomass, there were no significant differences.

The inoculum size must be sufficiently large to colonize all the substrate particles (Shah and Madamwar, 2005). However, high concentration of inoculum is not preferred in industrial fermentation (Nagar et al., 2010). The size of the inoculums varying from 1 to 5% was insufficient to colonize all the substrate particles in the liquid medium. Therefore, they achieved lower xylanase activities.

Several researchers have reported the use of 1.0–5.0% (v/v) inoculum size for hyper production of xylanase (*Bacillus pumilus* ASH and *Trichoderma reesei* SAF3) (Nagar et al., 2010).

Solid-state fermentation using miscanthus waste biomass

Using miscanthus biomass (which on average consists of about 30% of xylan+araban) as a cost-effective substrate, rather than commercial pure xylan is a better economical strategy for the enzyme production (Lee and Kuan, 2015). In this way, problem of the waste biomass disposal could be solved. On the other hand, a huge amount of biomass is used to generate heat by combustion in large ovens. Instead of combustion, it can be used for the production of enzymes and further for the production of ethanol. This method of processing waste biomass can be considered as an acceptable way of protecting the environment.

The results of solid-state fermentation by using unmodified and modified miscanthus biomass for xylanase production are shown in **Fig. 4**.

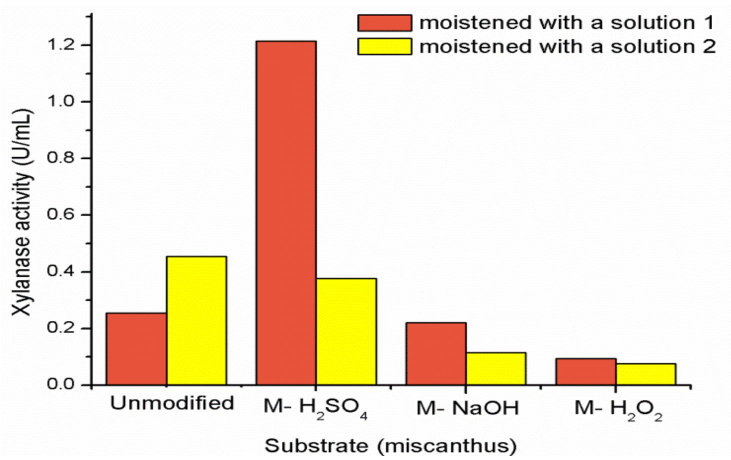

Fig.4. Xylanase production by *Sinorhizobium meliloti* strain 207 during solid-state fermentation (48 h, 28°C)

The maximum xylanase activity of 1.215 U/mL was obtained by using M-H_2SO_4 modified waste biomass by *S. meliloti* 207. Acid hydrolysis of miscanthus by using the dilute sulfuric acid (H_2SO_4) is the most common option, among the chemical pretreatments (acids, alkalis, alcohols, organic acids, pH-controlled liquid hot water, or ionic liquids). During this pretreatment, carbohydrates (mainly hemicelluloses) are depolymerized in the liquid into oligosaccharides and monosaccharides (Lee and Kuan, 2015; Guo et al., 2012). The success of xylanase production was five times lower when the unmodified and M-NaOH modified biomass were used as an alternative enzyme substrate (0.254 and 0.221 U/mL, respectively). It is probably because dilute sulfuric acid more easily renders a yield of xylose than glucose from the biomass, while pretreatment in alkaline solutions like NaOH and ammonia causes solubilization and removal of hemicelluloses (Lee and Kuan, 2015). Furthermore, these values were obtained when the distillate water was used as substrate moistening agent. However, when solution 2 was used as a substrate moistening agent, the obtained xylanase activities were lower. This salt mixture only improved the production of enzyme during the use of an unmodified biomass of miscanthus.

Among xylanases-producing microorganisms, the lower activity was obtained by *Neocallimastix sp*. strain L2 (1.13 U/mL) using Avicel (PH 105) from Serva at 50°C in comparison with this study. However, higher xylanase activity was achieved by using wheat bran, bagasse hydrolysates and oat and urea by *Penicillium clerotiorum* (7.5 U/mL), *Bacillus circulan*s D1 (8.4 U/mL) and *Aspergillus niger* PPI (16 U/mL) respectively (Motta et al., 2013). Therefore, *Sinorhizobium melilot* strain 207 is not as potent in xylanases production, but is a rare case of rhizobial species with this ability.

Conclusion

The cost of producing xylanase has a significant impact on the economics of the waste biomass conversion process. On-site production of xylanase is a potential strategy which could be used in order to reduce costs. The use of the enzymes secreted from microorganisms grown on the same lignocellulosic material that will be further converted to ethanol can become an acceptable way of environmental protection. Therefore, utilization of miscanthus biomass for the production of xylanase was justified. Solid-state fermentation was a better choice for xylanase production by *Sinorhizobium* *meliloti* strain 207. In addition, chemical pretreatment with H_2SO_4 improved xylanase activity in comparison to the unmodified substrate. The crude enzyme, produced by strain 207, could be further used in eco-friendly processes of lignocellulose material bioconversion to useful products, as well as in the manufacturing of animal feed, bread, food and drinks, textiles, cellulose pulp and paper industry.

Acknowledgements

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SANITARY-HYGIENE AND EPIDEMIOLOGICAL STATUS OF LAND

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ABSTRACT

The paper deals with: the status of land as a natural environment for the decontamination of exogenous chemical substances, which fall into it; quantitative and qualitative composition of soil microorganisms; sanitary-hygienic and epidemiological importance of the land; sanitary and hygienic measures for the protection of land from pollution. This is significant from the aspect of organization of environmentally safe and health-safe agricultural production, protection of the environment and population.

INTRODUCTION

The land, as a factor of the surrounding environment, exerts an influence on the health of man. The land consists of mineral and organic matter, organomineral complexes, soil microorganisms, and also from soil moisture and air. One of the most important components of the soil is humus, which determines its fertility (Yemtsev, Djukic, 2000; Djukic et al., 2007).

The character of the soil (stony, sandy, clay, etc.) and its properties (porosity, water sustainability, capillary, water and air penetration) must be taken into account when selecting a plot for the construction of objects for different purposes. Water sustainability, ie. the ability to retain water, determines the state of the level of groundwater. The ability of the soil to pass through the air is important for self-cleaning processes, because the oxygen supply allows rapid oxidation of organic matter (Djukic et al., 2013).

The great hygienic nature of the soil, as an element of biogeospheric, is that it not only accumulates different waste, it is also the natural environment of its detoxification. It is used for the detoxification of solid municipal waste (CCW), solid industrial waste storage (CIO), purification and detoxification of wastewaters in the filtration fields and fields of soaking, etc. (Ayaz and Akc, 2000; Đukić et al., 2012, 2013). Pesticides, mineral fertilizers and a variety of chemical substances are introduced into the land, which are dangerous for human health.

Processes aimed at establishing the natural state of the soil are processes of soil self-purification. Organic matter (proteins, fats, carbohydrates) and the products of their metabolism under the influence of microorganisms are dissolved to inorganic matter (mineralization). At the same time, the processes of creation of humus take place in the soil - complex organic matter of the soil that ensures its fertility (Đukić et al., 2007). Mineralization of the final products of degradation of proteins occurs with the help of nitrification bacteria, with the formation of nitrates. Sampling processes of soil lead to its release from biological pollution, pathogenic microorganisms and helminth eggs.

Land as a natural environment for decontamination of waste

Land is a system that ensures life on earth, that is, a part of the biogeospheric process in which detoxification processes (decontamination, decomposition, transformation into non-toxic compounds) take place. The basic mass of exogenous organic matter that arrives at it. Organic matter (in the form of proteins, fats, carbohydrates and products of their metabolism), after falling into the soil, undergo degradation, until the formation of inorganic matter (the process of mineralization).

Along with this process in soil, the process of synthesis (from organic matter waste) of the new complex organic matter of the soil takes place. This matter has been called humus, and the process of its synthesis is called humification. Both processes (mineralization and humification), aimed at establishing the original state of the land, are called processes of self-purification of the land.

The process of detoxification of exogenous organic matter into the soil with wastewater is very complex and is carried out mainly by microorganisms.

Carbohydrates undergo aerobic conditions with transformations, part is oxidized to CO2 with energy separation, part (monosaccharides) goes on synthesis of glycogen microbial cells. Degradation of fat in aerobic conditions takes place very slowly to the formation of fatty acids with energy separation, and under anaerobic conditions - to the formation of H2, CO2 and others. Proteins break down to amino acids. Part of the amino acid is involved in the life activity of microbial cells. Products of nitrogen exchange are subjected to biochemical oxidation by aerobic bacteria. This process is called nitrification. Simultaneously with the oxidation processes in the soil, the reduction processes take place (Jemcev, Đukić, 2000; Garcia de Lomas et al., 2007; Đukić et al., 2012, 2013).

The degree of reducing effect of bacteria, in addition to their biochemical properties, depends on the composition of the environment, its reaction and other conditions. The denitrification process is followed by the separation of gases.

Quantitative and qualitative composition of soil microorganisms

The land is the basic reservoir of microorganisms in nature. Microorganisms condition the process of important processes in the soil, enable the circulation of all biogenic elements, participate in pedogenesis and soil fertility maintenance (Jemcev, Đukić, 2000, Đukić et al., 2007; Gougoulias et al., 2014).

The number of microorganisms in the soil depends on the type of soil, the content of organic matter and water, the climate conditions, the seasons, the degree of pollution of land with industrial waste and other factors and can reach several billion in grams. Even in the desert sand, in conditions of almost complete absence of moisture, in 1 g can be found up to 105 microbial cells. The basic mass of microorganisms is at a depth of 10-40 cm, in the lower horizons of the soil their number decreases according to the reduction in the amount of nutrients and water (Đukić et al., 1996).

It is known that increasing the amount of organic matter in the soil, as a rule, the number of microorganisms increases. Organic matter is a nutrient medium for most of the soil microorganisms. Total stocks of organic matter of the land reach 400 t / ha, many of which are in the surface layer (up to 30 cm) of land. The main component of the organic matter of the soil is the remains of plant and animal tissue, while the live mass of microorganisms per 1 ha of soil (fertilized) is over 5-6 t (Jemcev, Đukić, 2000; Đukić et al., 2000; Đukić et al., 2007). Microorganisms are the richest chernozem, chestnut soil, serozem and specially treated soils. The number of bacteria in 1 g of such land sometimes reaches several tens of billions. Microorganisms are poor sandy and mountainous terrain, as well as lands uncovered by vegetation (Mandić et al., 2002, 2004).

In qualitative terms, soil microorganisms are presented with bacteria, actinomycetes, mushrooms, algae and viruses.

The number and qualitative composition of soil microorganisms is closely dependent on the degree of soil contamination with faeces, urea, but also from the way it is processed and fertilized (Đukić, Mandić, 1993). Thus, for example, in arable land, there are twice as many microorganisms as in forests. Due to the lack of necessary nutrients, the destructive effect of light, drying, the presence of microbial antagonists and phages, pathogens of asporogenic bacteria are maintained in the soil for several days to several months.

Gram-positive sticks and spongy aerobes of the genus Bacillus (Bac. Mycoides, Bac. Subtilis, Bac. Mesentericus, Bac. Megatherium) are constantly found in the soil. They are thrown widespread everywhere, participating in the decomposition of various organic matter in soil and water, causing the deterioration of nutrients, and some species cause the disease of man, animals and plants.

Bac is most often found in the land. cereus, Bac. megatherium and Bac. subtilis. In the soil, spores of the pathogenic microorganism Bac have been maintained for a long time. anthracis, which is a black challenger challenger. Bac. Cereus belongs to microorganisms that are very widespread in nature. Its presence in the soil depends on the content of organic matter. It develops better in soil with neutral or weak alkaline reaction.

The strains of the genus Clostridium (Cl. Sporogenes, Cl. Putrificum, Cl. Perfringens) are also found in different types of soil, particularly in Cl. perfringens. It is found in land that is constantly contaminated. Cl. perfringens of type A and atoxigenic strains are constantly found in the intestine of animals, as they are often in contact with the soil.

In the soil, spores of tetanus (Cl. Tetanus), gas gangrene (Cl. Perfringens), batulism (Cl. Botulinum) and other soil microorganisms have been maintained in the soil for a long time. Getting into the soil, they transform into spores, which can be maintained in the soil for many years.

Cl. Botulinum is primarily a land saprofit. Its distribution depends not only on the geographical location of the area, but also on the season, the character of the chemical composition of the land, its pollution, etc. Cl. Botulinum is found both in forest (2.5 - 3.3%), and in abandoned soil (3.3 - 10%).

It is believed that under favorable conditions (watering, presence of organic matter) Cl. Botulinum in the soil can not only last but also reproduce (Peretruhina, 2005). It can also be isolated from the animals and fish that reside on land, in the sea and in fresh waters. It also develops at the expense of using dead organic soil substrates. In addition, botulism causes are found in a series of animals without any clinical signs; in animal carcasses Cl. Botulinum begins to reproduce and produces toxin.

In the soil there are also turbid aspheric aerobes and optional anaerobes of the genus Pseudomonas (Ps. Fluorescens), Proteus (Pr. Vulgaris) and others. (Djukic et al., 1994; Mandic et al., 1994)

An important indicator of the sanitary condition of the soil is the finding of bacteria of the intestinal stomach group - coliforms and close grampositive cocks - indicator of faecal contamination Ent. faecalis.

Presence in coliform bacteria and enterococcus soil testifies to its faecal pollution (Landry and Wolfe, 1999).

In addition to these bacteria in the soil, Azotobacter species Azotobacteria, nitrification bacteria of the genus Nitrobacter, Nitrospin, Nitrococcus, Nitrospir; Sumpo-oxidizing bacteria of the genus Achromatium, Beggiatoa, Thioploca, Thiospirillopsis and Thiothirix; gastric bacteria, saprophytic coca genus Micrococcus (M. albus, M. candicans, M. cereus flavus), Sarcina (S. ureae) and from the actinomycetes (genera Actinoplanes, Streptomyces, Kineosporia, etc.)

Pathogens of bacteria, viruses, mushrooms and protozoa can be found in the soil. Land as a factor in the transmission of a number of infectious disease agents is a very complex substrate. The length of maintenance of some pathogenic bacteria is presented in the following table (tab 1.3).

Tab. 1.3. Length of bacteria in soil

Bacteria	**Mid-term, Week**	**Maximum time, month**
Salmonella	2 - 3	12
Shigella	1,5 - 5	9
Vibrio	1 -2	4
Micobacterium	13	7
Bacillus	0,5 - 3	several years
Clostridium	1,5	several years

The land serves as a place of residence for different animals, for example, rodents, in which parasitic agents of plague, tularemia, fever, haemorrhagic fever, encephalitis, leishmaniosis, etc. are parasitized. In the soil, a certain stage of pure protozoa (ameba, for example) takes place. The role of land in the transmission of invasions of the mud (ascari, ankilostoma, etc.) is especially significant. Some mushrooms live in the land. Pouring into the body, they cause alimentary-toxic aloe vera, ergotism, aspergillosis, penicillosis, mucocromycosis, histoplasmosis, chromomycosis, and other fungal diseases.

Sanitary-hygienic character of the land

The great hygienic significance of the soil consists in the fact that it contains certain chemical components that the person enters through food, drinking water and, to some extent, through atmospheric air. Increased or decreased concentration of fluoride, iodine, manganese, selenium and other chemical elements leads to the creation of natural or artificial geochemical zones, which play a key role in the development of endemic diseases like fluorose, drowsiness, etc. (Đukić et al., 2011).

The hygienic assessment of the degree of soil contamination by inorganic compounds is based on the comparison of the quantitative content of the given element in the soil with its MDK: for mercury - 2.1 mg / kg, chromium - 0.05 mg / kg, lead - 20 mg / kg, manganese - 1500 mg / kg, arsenic - 45 mg / kg (Djukic et al., 2015).

Organic matter of the land is presented as organic matter (humic acids...), which were synthesized by microorganisms, as well as allochthonous organic matter, which have come to the soil from outside.

In the form of humus substances, huge reserves of carbon are concentrated. The increase in carbon content of organic compounds shows 2 to 3 times the potential contamination of the soil. The ratio of carbon to humus to carbon of plant origin is called the humification coefficient.

The level of soil contamination is also testified to the content of organic nitrogen and the value of the sanitary number or number of Hlebnikov, as well as the ratio of nitrogen humus to total organic nitrogen. In clean land, the sanitary number is close to 1. The smaller the sanitary number, the land is polluted.

Sanitary-bacteriological soil testing (tab 1.1) consists of determining the total number of microorganisms in 1 g, the number of thermophils in 1 g, the co-titer, the titer-perfringen, and in some cases, also from the presence of staphylococci, proteus and pathogenic microbes (Đukić et al., 2011). A very sensitive indicator of fresh faecal contamination of the soil is the vital helminth eggs (in 1 kg). The basic sanitary and anthropological indicator of soil contamination is per unit area of land (0.25 m2).Hygienic soil diagnostics can be performed on the basis of the chemical composition of the soil air (tab. 1.2) and based on the so-called complex parameters.

The increased content of organic nitrogen and carbon without increasing the amount of ammonium nitrogen, low-titer and a large number of helminth eggs testify to fresh faecal contamination of the soil in the absence of mineralization of organic matter. A similar situation, but with the emergence of ammonium nitrogen, indicates the process of mineralization begun. At the same time, the presence of organic nitrogen and carbon, ammonium nitrate, nitrite, nitrate and chloride indicates longer soil pollution and the presence of intense mineralization of organic products. The presence of nitrate, chloride and low titer perfringens indicates a longer-term soil contamination. High content of humus nitrogen and approaching the number of breadwinner units - a reliable indication of intense humification land.

Tab. 1.1. Hygienic soil assessment based on complex parameters

Characteristics of the land	**Number of larvae and puppets at 0.25 m2**	**Number of helminth eggs per 1 kg of soil**	**Koli-titar**	**Titar-prefringens**	**Sanitary Number of *Hlebnikova***
clean	0	0	1,0 and more	0,1 and more	0,98 - 1,0
little contaminated	1 - 10	Up to 10	1,0 - 0,01	0,1 - 0,001	0,85 - 0,98
contaminated	10 -12	11-100	0,01 - 0,001	0,01 or less	0,70 - 0,85
very contaminated	100 and more	more than 100	0,001 and less	0,0001 and less	0,70 and less

Tab. 1.2. Hygienic soil diagnostics based on the chemical composition of the soil air

Land character	**Content in the land air (at 00C, pressure 760 mmHg) at a depth of 1 m, volume. %**			
	CO_2	**O_2**	**CH_4**	**H_2**
Practically clean	0,38 – 0,80	0,3 – 19,18	-	-
Poorly contaminated	1,2 – 2,8	19,9 – 17,7	-	-
Medium polluted	4,1 – 6,5	16,5 – 14,2	-	-
Very contaminated	14,5 – 18,0	5,5 – 1,7	0,8 – 2,7 and more	0,3 – 3,4 and more

The hygienic significance of soil humidity consists in the fact that all chemical substances, as well as biological soil contaminants (helminth eggs, bacteria, viruses) can be moved in it only with soil moisture. In addition, all chemical and biological processes that take place in the soil, including its self-purification from organic compounds, are carried out in aqueous solutions (Djukic et al., 2012).

The hygienic significance of the soil consists in the fact that it is a huge, natural laboratory, in which the processes of synthesis and degradation of organic matter take place, photochemical processes, the formation of organic and inorganic matter, the death of many bacteria, viruses, protozoa and eggs of helminth. The land is used for purification and detoxification of waste, dirt, waste, exhibits influence on the climate, vegetation development, etc.

So, the land exerts a great influence on the health of the population, it has great hygienic significance and it appears: 1) the main factor in the formation of natural and artificial spaces that play a leading role in the formation and prophylaxis of endemic diseases; 2) the environment that ensures circulation in the system "external environment - the human" of chemical and radioactive materials, as well as exogenous chemical substances that fall into the land with the waste of industrial enterprises, autotransport, wastewater, etc .; 3) one of the sources of chemical and biological pollution of atmospheric air, underground and surface waters, as well as plants that people use for food; 4) the transmission factor of infectious diseases; 5) natural, most suitable environment for disinfection and detoxification of liquid and solid waste (Đukić et al., 2011).

Epidemiological significance of the land

The epidemiological character of the soil consists in the fact that it is a suitable environment for the development of microorganisms and eggs of helminth and serves as a factor in the transmission of many infectious and parasitic diseases.

The largest number of microorganisms are found in soil at a depth of 5 - 10 cm. Permanent landowners are sporadic aerobic and anaerobic bacteria, as well as other bacteria that participate in self-purification processes.

The epidemiological hazard of the soil consists in the fact that pathogenic spore anaerobic bacteria are constantly inhabited there in it - tetanus, gas gangrene, and spores of the black cauliflower and botulinum sticks, which cause severe human disease. Acute intestinal infections can be found in soil contaminated by human faeces. The contaminated land may have the role of transporter of abdominal typhus and paratyphoid, salmonellosis, bacterial and amebic dysentery, cholera, viral hepatitis A, polyomyelitis, tuberculosis, jersiniosis, lambliosis and helminthosis (ascoridosis, trichochecephalitis, etc.). The bacteria of the typhus-paratyphic group survive in the soil for about 2-3 weeks, and in favorable conditions and for several months. Tuberculosis myobacteria and poliomyelitis viruses in the soil can be maintained for more than 3 months (Flores-Tena et al., 2007; Ghodbane et al., 2014).

Egg geohelminate (ascorhide and nematode that parasitize in human blindness, causing trihocephalosus) carry in the soil the maturation stage to the invasiveness state, i.e. the ability to infect a person, for 2-3 weeks to 2 - 3 months. The eggs of these helminates can be kept in the soil for 7-10 years.

The epidemiological importance of the soil consists in the development and fertilization of the flies which are the transmitters of the pathogens of intestinal infections. In the soil, rodents often live in the soil for infectious agents of leptospirosis, jersinrosis, tularemia, etc.

When choosing a plot for the construction of buildings for different economic purposes, it should be kept in mind that the ability of the land for self-purification is limited. Land protection, self-cleaning, sanitation and the fight against insects and rodents is of great hygienic significance.

In order to determine the quality and degree of soil safety in the territory of settlements, hygienic assessment of the land is carried out in the spa zones and other important territories, with the compulsory compilation of the sanitary and epidemiological conclusion on its condition and the benefits for construction, recreation and other purposes (Đukić et al., 2011; Kalwasińska et al., 2012).

Sanitary-epidemiological assessment of soil is done on the basis of sanitary-bacteriological indicators in the presence of the causative agent of intestinal infections, pathogenic bacteria, enteroviruses, sanitary-indicator microorganisms; based on sanitary-parasitological indicators - in the presence of the triggers of intestinal parasitic diseases (geohelmintosis, lambliosis, amebiosis, etc.); based on sanitary and toxic indicators - on the content of chemical pollution; based on sanitary-chemical (sanitary number, content of organic matter) and presence of flies and fly dolls.

For land, maximum permissible concentrations of chemical substances, including heavy metals, pesticides, petroleum products, etc. are determined. Pathogenic bacteria, eggs of helminth, larvae and fly dolls must not be found in clean soil. The index (quantity) of the bacteria of the intestinal tract group (BGCS) and the enterococcus index must not be greater than 10 in grams of soil.

Sanitary-hygienic measures for the protection of land from pollution

The central methodological issue is the determination of MPK (maximum permissible concentration) of exogenous chemical substances in the soil (Figure 1.2). This means that its maximum quantity in the soil (in milligrams per 1 kg of dry soil) which in direct contact with man does not cause a negative effect on his health.

In the first stage, the physical-chemical properties of matter and their stability in the soil are studied.

The second stage is the explanation of the scope of experimental research and the orientation threshold concentrations in relation to each indicator of harmfulness (Semenov and Đukić, 2017; Đukić et al., 1999, 2000).

At the third stage of the study, a laboratory experiment is set up to demonstrate supportive concentrations for six indicators of harmfulness. The organoleptic indicator of harmfulness is characterized by the degree of change in the nutritional value of products of plant origin, as well as the odor of atmospheric air; flavor, color and aroma of water and nutrients.

The common-sense indicator of harmfulness is characterized by the influence of exogenous matter on the self-purifying ability of the soil and its biological activity. The accumulation indicator refers to the standardization of the chemical substance that passes from the soil through the root system to the plant and accumulates therein (Djukic et al., 2009, 2015; Mandic et al., 2009). The migration water indicator is characterized by the process of migration of the studied matter into surface and groundwater. The migration air pollutant characterizes the processes of transferring chemical matter from the soil into atmospheric air by evaporation. The toxicological indicator characterizes the degree of toxicity of exogenous chemical substances for warm-blooded organisms in complex and associated (land dust and chemical matter) the entry of compounds with water, food, etc. in the organism of experimental animals.

The fourth stage determines the size of maximum permitted levels of input (MDNU) and the harmless residual amount (BOD) for chemical substances in the specific soil-climatic conditions.

On the fifth stage, the impact of soil contaminated with exogenous chemical substances is studied on the health of the population in order to correct the hygiene norms for chemical contaminants (MDC, MDNU, BPK).

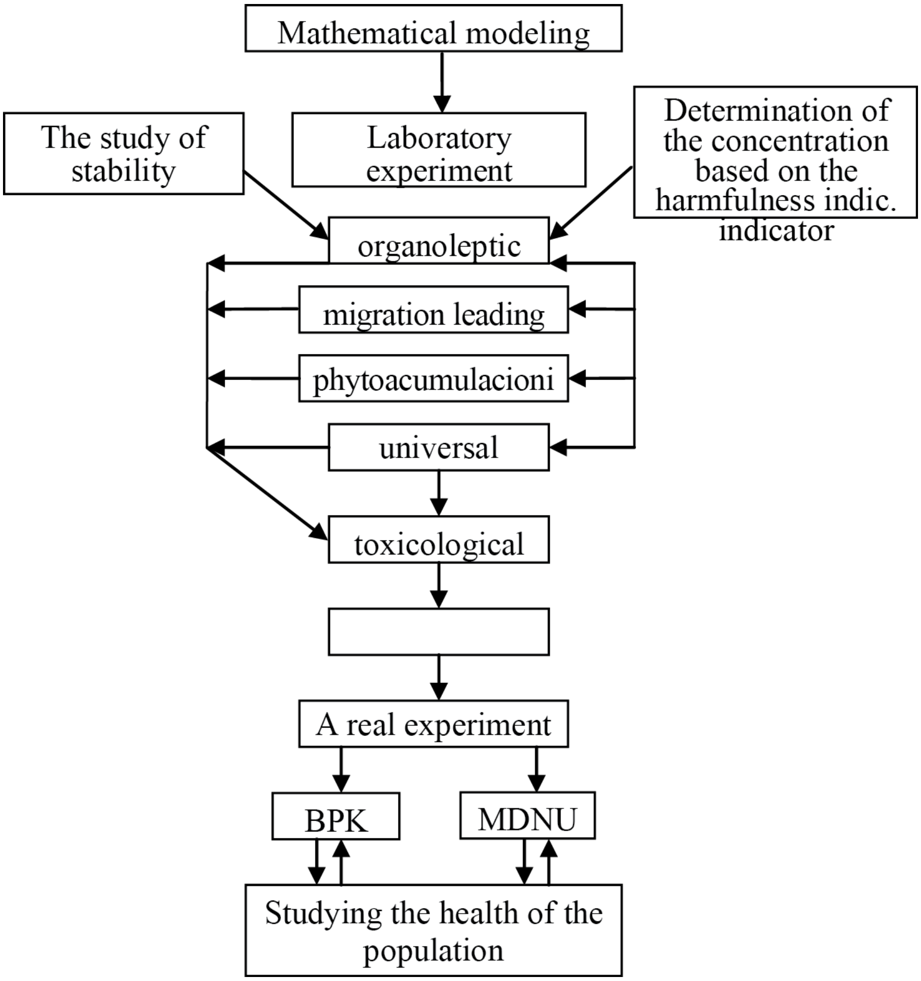

Fig. 1.2. Standardization of exogenous chemical substances in the soil

Sanitary protection of land means a complex of measures aimed at limiting the introduction of polluted land to quantities that do not disturb the processes of self-purification in the soil, do not cause the accumulation of harmful substances in plants in quantities that are hazardous to human health and which do not cause air pollution, surface and groundwater and deterioration of the sanitary condition of settlements (Đukić et al., 2000, 2008).

The objective of sanitary protection of the soil is the prophylaxis of infectious and parasitic diseases, for which the soil has the role of transmitter, and also acute and chronic poisoning with chemical substances.

Measures can be divided into several groups (Djukic et al., 2011):

1. Legislative, organizational and administrative measures, which implies a system of legally established measures aimed at preventing land contamination, ensuring rational utilization of land resources in the interest of preserving and improving the health of the population.

2. Plan measures that include the correct allocation of parcels for the construction of detoxification and waste utilization facilities and respect for sanitary protection zones around them, and others.

3. Technological measures aimed at raising technological production schemes with little or no waste.

4. Sanitary-technical measures for collecting, removing, detoxifying and utilizing waste (sanitary cleaning of settlements).

Sanitary cleaning of populated places means a complex of measures aimed at collecting, removing, detoxifying and destroying solid waste that occurs in populated areas in order to preserve the health of the population and overall editing.

Waste is divided into two groups: liquid and solid. Liquid waste belongs to impurities from the toilet, washed (from preparing food, washing of dishes), waste water (municipal, industrial, atmospheric, from wash of sidewalks, etc.). Solid waste belongs to garbage (household etc.), street garbage, social food waste (food residues), waste of industrial and commercial enterprises, solid manure and animal waste (animal corpses, manure), slag from liquids and industrial waste.

There are three systems of waste disposal:

1. rafting (sewage);

2. export (in non-analyzed populated areas). This way of removing solid waste is called cleaning, and liquid waste - asenization;

3. mixed (in partially sewed points).

Solid municipal waste can be collected by means of garbage pipes (in residential buildings), stationary and mobile containers. More recently, waste water pipelines are used (pneumatic removal of garbage).

The areas on which the containers are placed must be kept away from houses, children's institutions, sports grounds and resorts at a distance of at least 20-25 m.

Waste must be subjected to detoxification to prevent the spread of infection.

Detoxification modes must comply with the following requirements:

- harmlessness of waste, especially medical, in epidemiological terms;
- rate of detoxification of waste;
- preventing the development of fly flies and creating a favorable environment for the development of rodents;
- rapid transformation of organic matter into compounds that do not dissolve and do not pollute the air;
- protection of surface and ground waters against pollution;
- maximum and safe utilization of useful properties of waste.

Solid waste can be sprayed (processing into organic fertilizers, biological fuel, etc.) and removed (burying, landing, burning).

According to technology, detoxification methods are divided into:

1) biotermic (improved dumps, fields of scrubbing, fields of attenuation);

2) thermal (burning in special furnaces at a temperature of 900 - 10000C, pyrolysis with the production of gaseous fuel and naphtholic oils at a temperature of 16400C and a deficit of oxygen);

3) chemical (hydrochloric or sulfuric acid at high temperature for the production of ethyl alcohol);

4) mechanical (pressing into building blocks).

The most common are biochemical and thermal methods. It is a better biotermic way that is often applied in the form of composting. In order to form a compost, a flat surface is charged (blasting) with clay and surrounds it with a clay of 10-15 cm in height and a channel, the width of the space is 1.5 - 2 m, and the length - if desired.

Composting material (peat, soil) is applied to the selected area in a layer of 10 - 15 cm, then a layer of garbage up to 15 cm is applied, sprinkled with a layer of composting material. Then a layer of garbage is applied again, until the height of the compost reaches 1.5 m. Compost is covered by straw ages. Thanks to the activity of thermophilic microorganisms in the compost, biochemical processes take place, and the garbage is heated to 50 - 700C, organic matter is mineralized, and pathogenic microorganisms, eggs of helminth and larva fly are dead. Every 1 - 2 months the compost is shaken or otherwise periodically moistened. The maturation process lasts 3 - 12 months. The matured compost is loose, the bulk of the dark-earthy to the dark-yellow color. The advantages of composting consist in the fact that the surrounding environment is not polluted, pathogenic microorganisms are destroyed and valuable fertilizer is obtained (Đukić et al., 2012).

Conclusion

Theoretical and experimental experiences from this field point us to several basic conclusions:

- in the soil there are processes of humification and dehumification that contribute to the establishment of the original condition in it, which makes its self-purification;

- the quantitative and qualitative composition of the soil microorganisms is in close correlation with the degree and type of pollution, which can serve as reliable indicators of soil contamination;

- sanitary and hygienic assessment of the land is based on the content of soil moisture, air, various chemical elements, inorganic and organic matter, the total number of microorganisms, saprophytic and pathogenic microorganisms, helminth eggs and some insects;

- Sanitary-epidemiological assessment of the land is done on the basis of sanitary-indicator micro-organisms, sanitary-parasitological, sanitary-toxicological and sanitary-hygienic indicators, as well as the presence of flies and fly dolls;

- In order to protect the soil against pollution, measures of limited introduction of various pollutants are undertaken in order to preserve the self-purifying ability of the soil, prevent the accumulation of harmful substances in plants and their emissions into the air, surface and groundwater, thus maintaining the favorable sanitary condition of the settlements, etc..

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ANTAGONISTIC EFFECT OF SOIL-BORNE BACILLUS ISOLATES AGAINST FUSARIUM INFECTION AND INDUCTION OF WHEAT SEED GERMINATION

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ABSTRACT

This research was conducted in order to examine the antifungal potential of soil-borne Bacillus isolates against plant pathogenic fungi Fusarium proliferatum and Fusarium graminearum, as well as their ability to improve the germination of infected wheat seeds. All tested isolates showed good germination potential for uninfected and infected seeds. Values of relative seed germination index increased up to 80% for infected seeds, when Bacillus treatments were applied. All tested isolates significantly improved the growth of shoots and roots, up to 2.33 times (BS2). In general, isolate BS2 had the best activity in the mix with all 3 fungal strains and increased the length of roots up to 78%. The production of indol-acetic acid was observed for all tested isolates in both concentration of tryptophan. F. proliferatum was resistant to all tested bacterial isolates and the tested fractions showed no antifungal activity. Isolate BS2 was the only bacterial isolate with antifungal activity against F. graminearum subspecies 1, with a weak antifungal activity, only up to 4%. PCR analysis indicated the presence of fengycin and subtilin genes for isolates BS1 and BS2 and bacilysin for B1 and B2. The presence of fengycin, subtilin and bacilysin genes can be the basis of the antifungal activity of the tested bacterial isolates.

Keywords: *Bacillus sp., antifungal activity, Fusarium proliferatum, Fusarium graminearum, seed germination, Triticum aestivum.*

INTRODUCTION

Regarded as plant pathogens, fungal species represent one of the most diverse groups of ecologically and economically crop endangering agents. During the last decades, the negative effect of plant pathogenic fungi on agriculturally significant plant species has been studied comprehensively. The use of fungicides was the most successful strategy for protecting the crops from the phytopathogenic fungi, as well as for improving quality and maintaining stabile crop production. This strategy was an inevitable element of contemporary agriculture for more than a century. However, during the time the overuse of pesticides and fungicides has led to a new potential problem for agriculturists. The emergence of resistance to some of the most important classes of fungicides has been observed in many target fungal pathogens. Due to the short life cycles and the production of easily dispersible spores resistant to fungicides, the problem of reducing and losing fungicidal activity against plant pathogenic fungi has increased rapidly (Doehlemann et al., 2017).

Recently, the use of beneficial microorganisms in the control and suppression of plant pathogenic populations has been widely used. This strategy, also referred as biocontrol, mainly relies on the antagonist effects of specific microorganisms on plant pathogenic fungal species. The advantages of microbiological biocontrol, in comparison to the traditional use of fungicides, relate to a wider spectrum of antifungal activities, prolonged activity in the soil, the direct mechanism of plant immunisation, as well as the lower price of its mass-production (Stanojevic et al., 2016).

It has been shown that different species of the genus *Bacillus*, such as *B. megaterium, B.subtilis, B. cereus* etc, have a wide array of antifungal properties, and can be used as effective biocontrol agents. These bacterial strains are also known for their ability to promote plant growth with different mechanisms. Antifungal activity of most *Bacillus* species underlies in their ability to produce substances such as hydrolytic enzymes, antifungal products and antibiotics (Khan et al., 2018). Furthermore, *Bacillus* strains can produce some cyclic lipopeptides (LPs) with previously recognized strong antifungal effects, such as fengycin, surfactin, iturin, subtilin, bacylisin and bacyllomicin (Dimkic et al., 2015). Besides acting as bioprotectants, some of these soil bacteria can act as biostimulants by improving plant growth through direct effects such as Indol Acetic Acid (IAA) production. The production of IAA can stimulate root elongation, as well as the formation of lateral plant roots (Widnyana et al., 2016).

Fusarium proliferatum is a plant pathogenic fungus, which is associated with different crop diseases, mostly in bananas and corn. It has been shown that *F. proliferatum* is the predominant fungal inducer that causes wilting of roots of onion and garlic plants in Serbia, due to toxin production (Stankovic et al, 2007). *F. proliferatum* induces black point symptoms on wheat kernels as well (Conner et al., 1996). Black point symptoms are induced due to the production of fumonisins, a type of polyketide mycotoxins (Desjardins et al., 2007). In addition, this disease causes losses in crop yield, as well as financial losses for farmers, because infected kernels cannot be used for the production of white flour, due to its dark colorization. *Fusarium graminearum* is a type of fungal pathogen, widely recognized as the causal agent of *Fusarium* head blight in wheat (Yang et al., 2013). *Fusarium* head blight is induced by a wide spectrum of fungal mycotoxins which can cause significant losses of crop yield. Moreover, this disease can also be a significant threat for the health of humans and animals, as mycotoxins produced by *F. graminearum* can accumulate in infected plants (Kuhnem et al., 2015).

The aim of this research was to examine the potential antifungal effects of four *Bacillus* isolates against *F. proliferatum* and *F. graminearum in vitro*, as well as to determine the possible presence of genes for the production of antifungal cyclic lipopeptides. The ability of bacterial strains to induce wheat germination (*Triticum aestivum L.*) was tested *in vitro* based on the length of roots and shoots, and the relative germination index of the seed.

Materials & Methods

Bacterial and fungal strains

In this research the antagonistic effects of four bacterial strains were tested against *Fusarium proliferartum* and 2 subspecies of *Fusarium graminearum*. Isolation of bacterial strains was performed on Nutrient agar. Dilutions of soil samples gathered from grassland were heated at 80°C for 15 min in order to gather *Bacillus* spores. After heating, 20 μL of 10^{-5} and 10^{-6} dilutions were transferred to Petri dishes. The plates were incubated at 28°C, during 48h. Based on the cell morphology and Gram staining, four isolates belonging to the genus *Bacillus* were selected for further research. The strains were preliminary characterized as *Bacillus* spp. (B1, B2) and as *Bacillus subtilis* (BS1, BS2). Fungal strains were kindly provided by Faculty of Technology and Metallurgy, Department of Biochemical Engineering and Biotechnology, University of Belgrade, Serbia. Wheat seeds (*Triticum aestivum* L.) were provided by a local farmer.

Seed germination test

The ability of bacterial strains to induce the germination of wheat seed was tested *in vitro* on Petri dishes. A sterile filter paper was placed on a Petri dish, and 1 mL of sterile distilled water was applied to provide humidity for regular seed growth. The control sample consisted of wheat seeds, without any treatment applied. The fungal and bacterial control samples were prepared by adding uniformly 0.5 mL of spore suspension to the seeds and by resuspending seeds in overnight bacterial culture during 2-3 min, respectively. The test samples were prepared by resuspending seeds in overnight bacterial culture, and adding 0.5 mL of fungal spore suspension to the seeds. The experiment was performed in three independent repetitions. The Petri dishes were placed in a transparent sealed box at room temperature (22°C) to provide humidity. The results were gathered after seven days and expressed by the length of shoots and roots and the relative seed germination (%) index (RSGI) (Buntić et al., 2017). The percentage of RSGI is expressed by Eq.1:

$$\mathbf{RSGI}(\%) = \frac{\mathbf{SG_s}}{\mathbf{SG_c}} \times \mathbf{100} \qquad (\mathbf{1})$$

where SG_S is seeds germination in samples and SG_C is seeds germination in control.

Production of indol-acetic acid (IAA)

The ability of bacterial strains to produce IAA was tested in nutrient media enriched with 0.5 mg/mL and 2 mg/mL tryptophan, based on the method previously described by Stajkovic et al. (2011).

In vitro antagonist potential

In vitro antifungal potential was tested for overnight bacterial suspension, cells-free supernatant (CFS) and heat stable fraction of cells-free supernatant (HS-CFS). Bacterial cultures were grown in liquid Nutrient broth on rotary shaker (28°C, 24h, 120 rpm), while the fungal strain was grown on solid PDA medium (28°C, 7 days). CFS was prepared by centrifuging

(13.000 rpm, 5 min) 2 mL of overnight bacterial suspension (approximately 10^6 CFU/mL, and re-centrifuging trough a Millipore centrifuge filters (0.22 μm). Heal stable CFS was obtained by a 25 min heating at 75°C of previously prepared CFS on Biosan CH100 Cooling-Heating Thermostat.

The antagonist effect of each bacterial strain against *F. proliferartum* and *F. graminearum* was tested on PDA medium. Two-mm diameter plug of fungal mycelia was placed in the centre of the Petri dish, and 20 μL drops of bacterial suspension, CFS or HS-CFS were placed approximately 1 cm from the edges of the Petri dish. Petri dishes were then incubated at 28°C, and the results were gathered after 7 days of incubation. The experiment was performed in three independent repetitions. After incubation, the diameter of fungal colony on a Petri dish with bacterial suspensions, CFS or HS-CFS was measured in mm, and compared to the control sample. The antagonistic effect of bacterial strains was calculated based on the following equation (Eq.2) (Ogbebor and Adekunle, 2005):

$$\text{Inhibition (\%)} = \frac{\text{Fungal Control} - \text{Bacterial Treatment}}{\text{Fungal Control}} \times 100 \qquad (2)$$

PCR analysis

PCR amplifications were performed in 50 μL reaction mixture containing 25 μL of PCR MasterMix (Thermo Scientific DreamTaq Green PCR MasterMix 2x), 24 μL MQ sterile water, 0.5 forward primer, 0.5 reverse primer, and 1 μL DNA. The PCR program used for amplification of fengycin, surfactin, iturin C, subtilin and bacylisin genes was as follows: initial denaturation at 95°C for 4 min, 40 cycles of denaturisation at 94°C for 1 min, hybridization at 58°C for 1 min, elongation at 70°C for 1 min, followed by final elongation at 70°C for 5 min, and initial denaturation at 95°C for 4 min, 40 cycles of denaturisation at 94°C for 1min (Mora et al., 2011; Zhang et al., 2015). For the amplification of bacillomycin genes, the following PCR program was used: hybridization at 55°C for 1 min, elongation at 70°C for 1 min, followed by final elongation at 70°C for 5 min (Mora et al., 2011). Sets of used primers for amplification of LPs genes are shown in Table 1. Amplifications were carried out in Eppendorf Mastercycler personal PCR machine. The amplified products were analyzed in 1% agarose gel in TBE buffer, and were run for 60 min at 90 V.

Electrophoresis gels were then stained with ethidium bromide solution, observed under HoeferMacroVue UVis-20, and photographed. Amplificon size comparisons were made with a 1kb DNA ladder (Fermentas Mass Ruller^{TM} Express DNA loading Mix).

Table 1. Oligonucleotide primers used to amplify genes for antibiotic production

Primer	Gene Product	Primer sequence 5'- 3'	Gene	Amplificon length	Reference
FENDF FENDR	Fengycin	GGCCCGTTCTCTAAATCCAT GTCATGCTGACGAGAGCAAA	*fenD*	269 bp	Mora et al., 2011
SRFAF SRFAR	Surfactin	TCGGGACAGGAAGACATCAT CCACTCAAACGGATAATCCTGA	*srfAA*	201 bp	Zhang et al., 2015
ITUCF ITUCR	Iturin	GGCTGCTGCAGATGCTTTAT TCGCAGATAATCGCAGTGAG	*ituC*	423 bp	Mora et al., 2011
SPASF SPASR	Subtilin	GGTTTGTTGGATGGAGCTGT GCAAGGAGTCAGAGCAAGGT	*spaS*	375 bp	Mora et al., 2011
BACF BACF	Bacylisin	CAGCTCATGGGAATGCTTTT CTCGGTCCTGAAGGGACAAG	*bacA*	498 bp	Mora et al., 2011
BMYBF BMYBR	Bacillomycin	GAATCCCGTTGTTCTCCAAA GCGGGTATTGAATGCTTGTT	*bmyB*	370 bp	Mora et al., 2011

Results and Disscusion
Induction of seed germination

The effect of four *Bacillus* isolate on *Triticum aestivum* seed germination and the length of shoot and root are shown on Table 2, Fig.1 and Fig. 2.

Table 2. Germination of *Triticum aestivum* seeds treated with *Bacillus* isolates and fungi

Treatment		RSGI (%)		
		Fungi 1*	Fungi 2**	Fungi 3***
Control	95	40	50	45
B1	100	80#	75#	80#
B2	95	75#	80#	75#
BS1	95	65#	70#	65#
BS2	100	75#	70#	75#

**Fusarium proliferartum*; ***Fusarium graminearum sub.sp1*; ****Fusarium graminearum sub.sp2*; # *fungi +Bacillus isolate*

According to the RSGI, all tested bacterial strains showed good *Triticum aestivum* seed germination. Moreover, isolates B1 and BS2 improved the seed germination process (Table 1) in comparison to control. In the literature, there are studies which also confirmed that various *Bacillus* strains were capable to improve seed germination (incuding wheat seed). Hu et al. (2019) showed that the application of *B. subtilis* on wheat seeds, even under severe salt stress, could improve seed germination. Authors recorded an increase of seed germination potential up to 5.22% in the sample treated with *B. subtilis* (Hu et al., 2019). Another study indicated that inoculation of wheat seeds with *B. lentus* could improve crop yield (Abbasdokht and Gholami, 2010).

On the other hand, seeds treated with these fungi have shown a decrease in seed germination (Table 1). This decrease was two times lower in comparison to control. In combination with *Bacillus* treatment, infected seed had better seed germination and an increased RGSI, up to 80% (Table 1). The least improvement was observed when BS1 strain was used as an antifungal treatment.

In addition, all applied *Bacillus* isolates significantly improved growth of the shoots and roots, recorded over seven days (Fig.1 and Fig.2). In comparison to the control, the growth of shoots was increased by 27.40%, 5.88%, 19.71% and 1.76% with applied B1, B2, BS1 and BS2 isolates, respectively. The strain BS2 slightly increased the length of shoot, but on the other hand it contributed to the greatest improvement of root length (Fig.1). It was 2.33 times higher than the length of root in the control. The increase in root length was 62.41%, 58.62% and 80.69% using B1, B2 and BS1 strain as seed treatment, respectively.

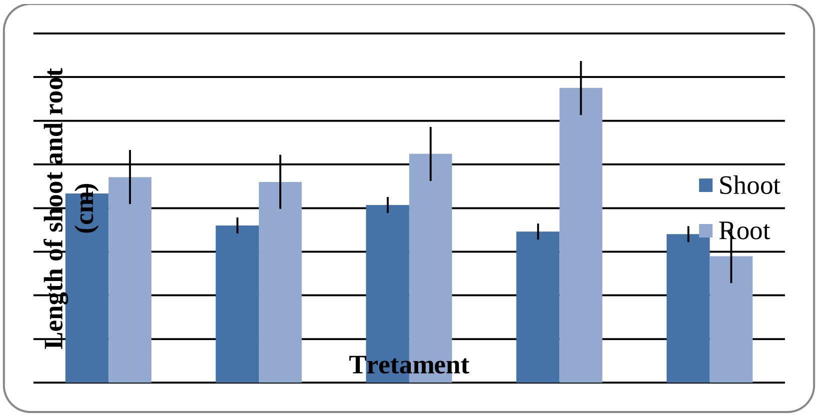

Figure1. The effect of *Bacillus* isolates on the length of shoot and root of *Triticum aestivum* seed

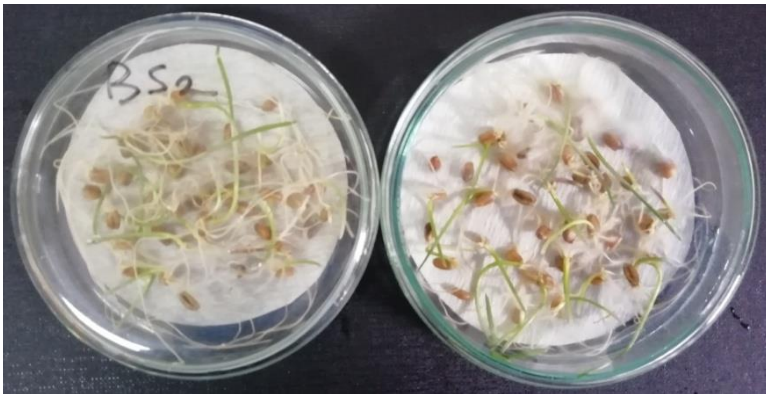

Figure 2. *Triticum aestivum* seeds treated with BS1 isolate (left) and contol (right).

All tested *Bacillus* isolates had lower values for root and shoot length, in comparison to the uninfected control sample (Fig. 3). However, all the isolates induced a slight increase in root length, in comparison to the sample infected with fungi. Although the length of shoot varied between treatments, the highest values were obtained for the uninfected control sample. Isolate BS2 had the overall best activity in the mix with all three fungal pathogens. It increased the length of root by 78%, when applied to the seeds infected with *F. proliferartum.* Moreover, the same isolate increased the shoot length of seeds infected with *F. graminearum sub.sp2* for more than 28%. Isolate B2 was also effective in the mix with *F. graminearum sub.sp1*, and induced an increase of shoot length of 9.09%.

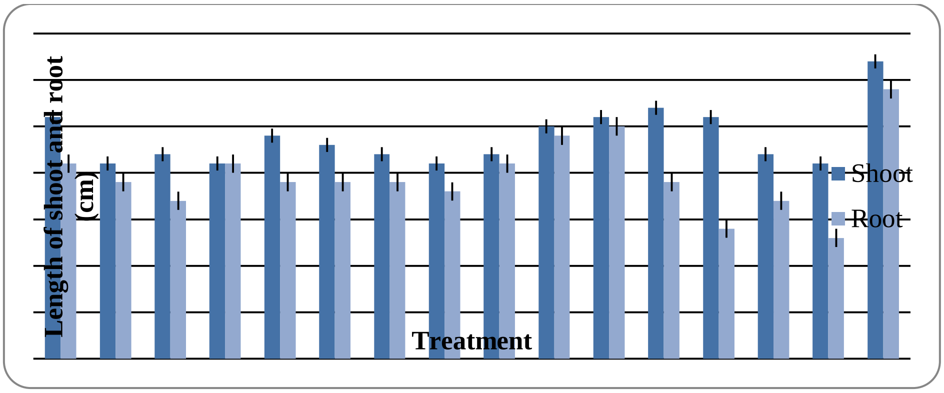

Figure 3. The effect of *Bacillus* isolates on the length of shoot and root of infected *Triticum aestivum* seed (*G1-Fusarium proliferartum*; *G2-Fusarium graminearum sub.sp1*; *G3-Fusarium graminearum sub.sp2*)

Production of indol-acetic acid (IAA)

The production of IAA by *Bacillus* isolates was determined in relation to the standard curve of the synthetic form of auxin. Isolates BS1 and BS2 produced IAA in both tested tryptophan concentrations. Isolates B1 and B2 had a lower IAA production for a concentration of 0.5 mg/mL and 2 mg/mL tryptophan, with a slight change in the colour of solution from yellow to pink. Stajković et al. (2011) also tested the ability of soil isolates to produce IAA. Their research indicated that *B. megatherium* could produce IAA, but *Bacillus* sp. gave a negative reaction. A study conducted by Chagas et al. (2015) demonstrated that soil isolates of *B. thuringiensis* and *B. cereus* can produce IAA. In addition, the same study indicated that a higher IAA production was in direct correlation with longer incubation time, as most isolates showed increased IAA production after 48 h of bacterial growth (Chagas et al., 2015). Since large amounts of phytohormones, such as auxin can have a significant impact on the promotion of plant growth, the positive effect of BS1 and BS2 isolates on the induction of wheat seed germination can be attributed to their ability to produce IAA.

In vitro antagonist potential

The obtained results indicated that *F. proliferatum* was resistant to all tested bacterial isolates (Fig.4). Furthermore, CFS and HS-CFS fractions of all tested isolates showed no antifungal activity against *F. proliferatum* and two *F. graminearum* subspecies.

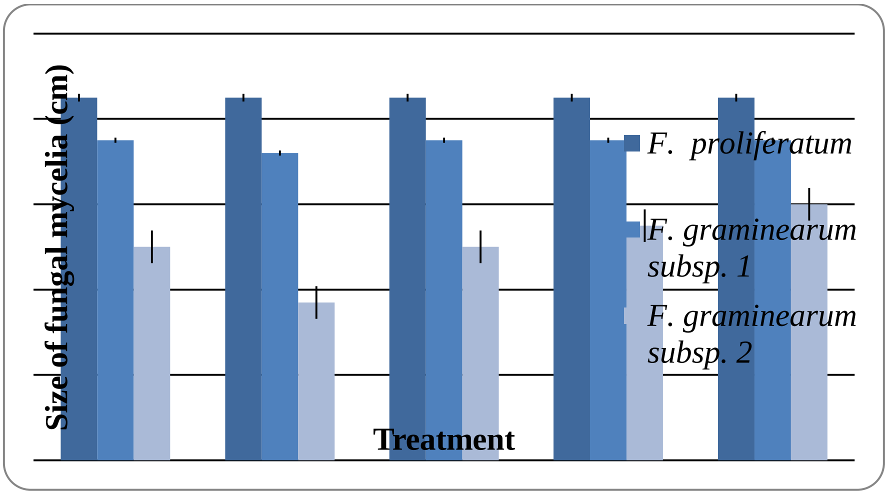

Figure 4. Antagonist effect of *Bacillus* isolates against *Fusarium* species

Isolate BS2 was the only bacterial isolate which showed antifungal activity against *F. graminearum* subsp. 1 However, the inhibition of fungal growth for this isolate was only up to 4%, which can generally be considered as weak (Fig. 4). All tested isolates inhibited the growth of *F.graminearum* subsp. 2. Isolate BS2 inhibited the growth of *F. graminearum* subsp. 2 up to 38.34%, by reducing the size of the mycelia from 6.00 cm to 3.70 cm, in comparison to the fungal control sample (Fig. 4 and 5).

Figure 5. Antifungal effect of BS2 against *F. graminearum* subsp.1 (left) and BS2 (a) and BS1(b) against *F. graminearum* susp.2 (right)

BS1 and B1 had the same antifungal effect against this plant pathogen and inhibited mycelial growth up to 16.67%. Isolate B2 showed the weakest antifungal activity, with the inhibition zone of fungal growth only up to 8.4% (Fig.5). In general, isolate BS2 can be considered as the most potent tested isolate in the suppressing of *F. graminearum* growth.

Bjelic et al. (2018) tested the antagonist activity of soil-borne *Bacillus* strains against the fungal disease inducers in garlic. This study showed that some of the tested *Bacillus* species had a strong antifungal effect against two *F. proliferatum* strains, where *B. subtilis* showed the strongest antifungal effect, with inhibition of up to 62.6% of mycelia growth, as well as the reduction of garlic clove infection up to 58% (Bjelic et al., 2018). In addition, another study showed that *B. megaterium* strain isolated from alfalfa rhizosphere had a strong activity against *F. proliferatum*, with inhibition of mycelial growth up to 41.96% (Bjelic et al., 2017).

PCR analysis

Isolates BS1 and BS2 had amplified fragments corresponding to the fengycin and subtilin genes (269 bp and 375 bp, respectively). Based on their morphological characteristics, these isolates seemed to belong to the *Bacillus subtilis* species. Isolates B1 and B2, which were preliminary characterised as *Bacillus* sp., had specific amplificons at 498bp, which correspond to the length of the amplified bacilysin gene (Table 1 and 3).

Table 3. - Amplification of LPs genes in *Bacillus* isolates

Bacterial strain	**Amplification of LPs genes**					
	Fengycin	Surfactin	Iturin C	Subtilin	Bacilysin	Bacillomycin
BS1	+	-	-	+	-	-
BS2	+	-	-	+	-	-
B1	-	-	-	-	+	-
B2	-	-	-	-	+	-

None of the tested isolates showed the presence of the surfactin, itruin C and bacilomycin genes. However, the presence of fengycin, subtilin and bacilysin genes can be the basis of the antifungal potential of these isolates. The study conducted by Bjelic et al. (2018) indicated that the production of surfactin could be responsible for the strong antifungal activity of *Bacillus* strains against *F. proliferatum*. Ongena and Jacques showed that the presence of genes responsible for the production of LPs is correlated with the stronger antifungal activity of various *Bacillus* species and that these strains can be used as different agents for the biocontrol of plant disease (Ongena and Jacques, 2007).

Conclusion

Isolates B1 and BS2 were the most efficient in improving the germination of wheat seeds in the uninfected sample. All applied *Bacillus* treatments significantly improved the growth of plant shoots and roots and the isolate BS2 had the overall best activity in the mix with all tested fungal pathogens. Moreover, all tested bacterial isolates produced IAA, where BS1 and BS2 are considered to be better producers than B1 and B2. Isolate BS2 was the only bacterial isolate which showed antifungal activity against *F.*

graminearum subsp.1. The same isolate had antifungal activity against *F. graminearum* subsp. 2, probably due to the presence of fengycin and subtilin genes. Based on the results, it can be concluded that the BS2 isolate can be used as a potential agent for inhibiting plant pathogens and improving the germination process in wheat seeds.

Acknowledgments

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LEVEL OF LEAD (PB) IN WATER, SEDIMENT WITH SELECTED FISH SPECIES AT FIVE LOCALITIES, BOTH AT AND NEAR PORT MILENA, ULCINJ, MONTENEGRO

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ABSTRACT

Aquatic ecosystems represent the unity of interacting factors, which are in mutual dependence, within the complex physical and chemical system of water-sediment. Maturation of wastewater containing metals in an aquatic ecosystem can cause numerous physical, chemical and biological changes in it and has a primary role in the deterioration of ecosystem quality.

The aim of this study has been to analyze the anthropogenic impact on the pollution of Port Milena, taking into account the specificity of the pollutants present in the basin and testing the distribution of Pb at selected locations (along the Port Milena stream from the bridge to the Adriatic Sea). The presence of Pb, Cd in water and sediment as well as in the muscle tissue of *Mugil cephalus* has been analyzed.

The highest concentrations of analyzed lead in water has been determined in the autumn period, when the measured concentrations were 8.97 mg / l at the site number 1 (estuary), in the amount of 9.78 mg / l at the site number 4 (waste water from the settlement).

The concentrations of the lead in the sediment varied through the four seasons; the maximum value during the winter period were determined 9.63 mg Pb / kg; during the spring season, maximal concentration was 10.8 mg Pb / kg; during the summer 18.68 mg Pb / kg. while in the autumn period l maximal concentration has amounted 18.5 mg Pb / kg. The analysis of lead in muscle tissue of *Mygil cephalus* has been analyzed on 12 samples. Values ranged from 0.1 to 0.16 mg/kg; Concentration has been compared with the maximum permitted concentration (MPC).

Key words: level of lead (Pb), water, sediment, selected fish species, Port Milena

INTRODUCTION

Ulcinj occupies the southernmost part of the Adriatic coast with its position. It is located in the southeastern part of Montenegro. Due to its distinctive natural and geographical characteristics, Ulcinj and its surroundings belong to the subtropical subtropical zone of the European Mediterranean. The southern part, in the length of 30 kilometers, Ulcinj enters the Adriatic Sea, and in the eastern part is the river Bojana. In the central part of Ulcinj is Lake Saško, and in the bay of Ulcinj it is surrounded by the massive mountain of Rumija. Channel - lagoon - Bay of Port Milena, in Ulcinj, once 4 km long, 80-120 m wide, 4 to 8 m deep, was created by natural forces, which is a catastrophic flood of 1896-1897, when the river Drim in Albania broke out of its gully and formed a new limb that penetrates into Bojana. The devastation of this unique space began more than thirty years ago. Sometimes Port Milen has been the habitat and hatchery of the highest quality species of ichthyofauna, while today, species hardly manage to survive in Port Milena.

Maturing waste water containing metals to an aquatic ecosystem can cause numerous physical, chemical and biological changes in it. These changes are classified in two categories: changes related to the environmental impact on the behavior of metals and changes related to the impact of metals on the state of the environment Veselinovic et al, 1995; Pertsemli, 2007. More than 80% of the chemical elements in their character are metals. The term "heavy metals" refers to a large group of chemical elements that, according to living organisms and the environment, exhibit high toxicity. Phipps,1981.;Siegel,2002. Toxic metals include those non-biogenic metals that act exclusively toxic, such as: lead, cadmium, mercury, arsenic, thallium and uranium, Kastori, 1995; Hoffman 2003.

Depending on the environmental conditions, the density, diversity, structure of the aquatic population may change. The character and degree of these changes depend largely on the level of content and forms of finding metals in water and sediment. The concentration of metals, as biologically non-degradable compounds, depends on the physical and chemical nature of the sediment and water, because they determine the binding force and form of migration, Prica, 2003.

The aim of this paper is to analyze the level of toxic metal lead (Pb) in all four seasons in water, sediment and selected fish species at five localities, at and near Port Millena in Ulcinj.

Material and methods

Water quality and sediment tests, with special emphasis on lead distribution (Pb) in these matrices, were conducted during all four seasons, at selected locations of Port Milena in Ulcinj, as follows: location 1: the entrance to the Adriatic Sea; location 2: before the traffic bridge; location 3: after the traffic bridge; location 4: waste waters from the settlement and location 5: saltworks. The content of heavy metals in water samples (previously acidified to pH <2, was directly determined using the ICP-OES technique (induced coupled plasma-optic emission spectrometer). Determination of total metal content in surface sediment was carried out by wet digestion with imperial water (HCl: HNO3 = 3: 1) (modified method EPA 3050b). Preparation of samples for analysis of the content of metals in flax tissue flathead gray mullet (*Mugil cephalus*, Liunnaeus, 1758) was performed according to the standard method (Analytical Method Perkin Elmer of AAS). Determination of metal content (Pb) has been done using AAS flame technique using the apparatus (Perkin Elmer nalyst 300). PQL for the test metal is given in the following Tab.1.

Tab.1. Working wavelength and practical limits of quantitation

Metal	Working wavelength (nm)	PQL (mg/l)
Pb	283,3	0,005

Results

Analysis of lead (Pb) in water

The concentration of the lead at all five (5) locations along the Port Milen channel,during the winter season were less than 0.005 mg/l. During the spring season, the measured concentrations also did not change (<0.005 mg/l).

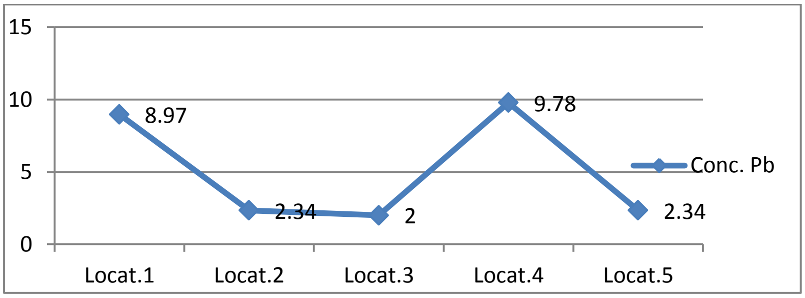

Fig. Analysis of lead (Pb) in water (autumn period) at all five locations

The measured concentrations of lead at all five sites are the same (<0.005 mg/l) during the summer season. An increase in lead concentration (Pb) in relation to the previous 3 seasons were observed in the autumn period, when an increase was found, the concentration of lead (Pb) in the amount of 8.97 mg/l was recorded at locality number 1 (2.34 mg/l at site number 2 (before the traffic bridge), 2.00 mg/l at site number 3 (after the traffic bridge). The highest concentration of lead (Pb) of 9.78 mg/l was determined at the site number 4 (waste water from the settlement), while at the location mo.5 (saltwoeks), the concentration was 2.34 mg/l.

Aanalysis of lead (Pb) in sediment

Sediment is an essential, dynamic component of all aquatic systems which, due to the markedly expressed tendency of binding, are the reservoir of toxic and persistent compounds of anthropogenic origin. The concentrations of the measured lead are different throughout the four seasons, so the measured concentrations in the winter varied in the range of 4.52 mg/kg (Pb) at site number 4; then 4.86 mg/kg at site number 2; 5.50 mg/kg, at site number 5; a concentration of 6.52 mg/kg at site number 3, up to a maximum of 9.63 mg/kg, at location 1-before the traffic bridge.

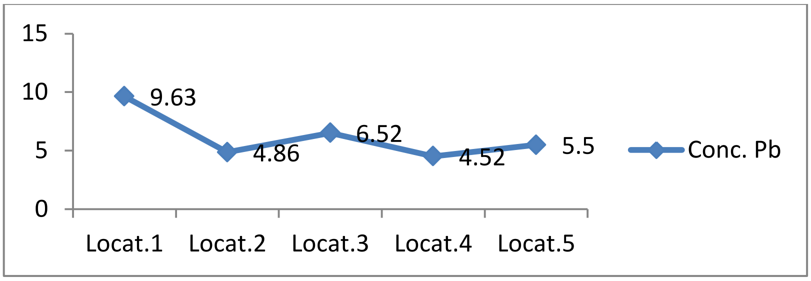

Fig. Analysis of lead (Pb), in sediment during the winter season

During the spring, the concentration of lead was as follows: 10.8 mg/kg at site number 1, which is also the place where the highest concentration of this toxic metal was measured during this season. At site no. 2, the value was 5.7 mg/kg; 8.0 mg/kg was the lead concentration measured at site no.3; while at site no.4, the measured concentrations (6.4 mg/kg) were approximately similar to those obtained in the sample collected and analyzed from site no.5, which is 6.0 mg/kg.

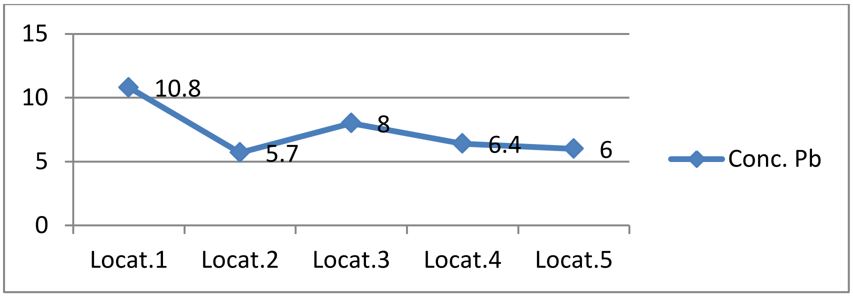

Fig.Analysis of lead (Pb), in sediment, during spring season

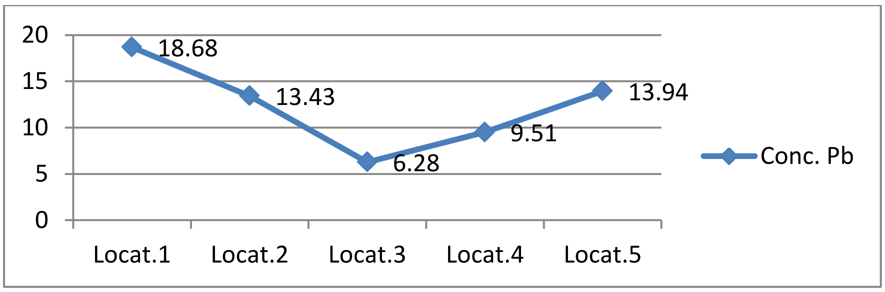

Fig.Analyses of lead (Pb), in sediment, during summer season

The summer season results in a noticeable increase in the concentration of lead in the sediment tested. The measured concentrations had the following values: 18.68 mg/kg (site number 1), 13.43 mg/kg (site number 2), 6.28 mg/kg (site number 3); 9.51 mg/kg), 13.94 mg/kg (site number 5).

During the fall, lead concentrations were also high. 9.56 mg/ kg was measured at site number 1, 14.31 mg/ kg at site number 2, 11.96 mg/kg at site 3, 18.5 mg/kg at site number 4, which is also the highest measured concentration, 13.66 mg//kg at site number 5.

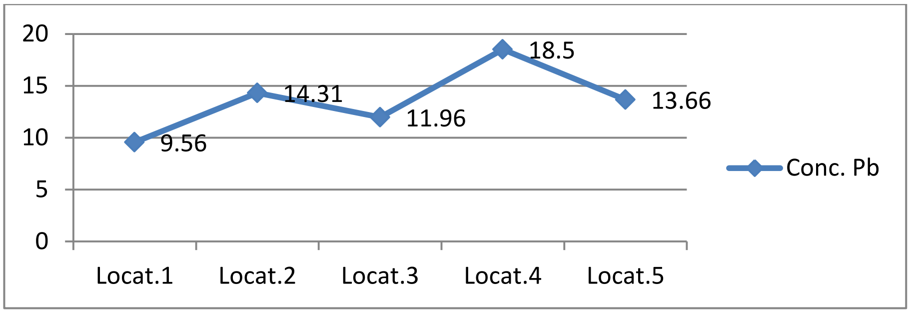

Fig.Analysis of lead (Pb), in sediment, during autumn season

Aanalysis of lead (Pb) in *Mugil cephalus* muscle tissue

The lead in *Mugil cephalus* muscle tissue were analyzed on 12 samples. Values ranged from 0.1 to 0.16 (Tab.2). Lead is toxic to all organisms. Generally, organo-lead compounds are more toxic than inorganic lead components.

Tab.2 Analiza olova (Pb) u mišićnom tkivu *Mygil cephalus*

Number of samples	1	2	3	4	5	6	7	8	9	10	11	12
Conc of Pb	0.1	0.1	0.16	0.1	0.1	0.1	0.1	0.15	0.1	0.1	0.1	0.1

The following histogram shows that the measured lead concentrations (Pb) are less than the MPC values, the law prescribed for this heavy metal.

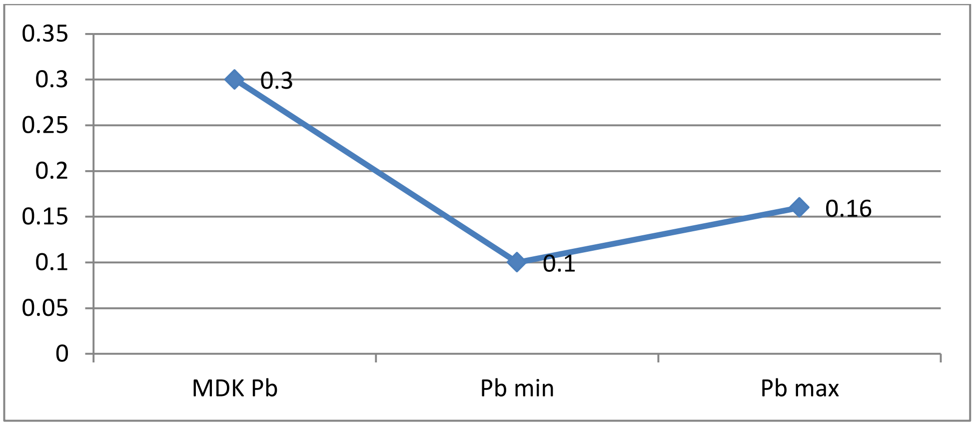

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Fig. Measured lead concentrations (Pb) in relation to maximum permissible concentration (MPC)

Discussion

Port Milena, on the one hand, is a unique creative work of nature and, on the other hand, as an "ecological black point" the example of an irresponsible relationship with nature. Channell Port Milena is exposed to contamination of multiple polluters: urban waste water, traffic, water spill from inhabited areas, agriculture and others, suggesting increased concentrations of lead metal (Pb) and other pollutants in water and sediment Toxic metals include those non-biogenic and exclusively toxic chemicals such as: lead (as well as: cadmium, mercury, arsenic, talum and uranium.The availability of lead is conditioned by concentration in a given medium (water, air, soil) as well as nutrition, growth rate and physiological stress of the exposed organism,Siegel, 2002.

The content of lead in water at selected sites did not change during the winter, spring and fall seasons (<0.005 mg/l). Similar concentration Yilmaz, 2005, states: Lead concentrations vary from station to station (<0.005 mg/l) at Iskenderun Bay (Turkey).

A greater deviation from these values, and also the deviation from MTC (2.0 mg Pb/l) prescribed by the Rule book (Official Journal of Montenegro No. 2/07), has been established for autumn, when values ranged from 2.00 mg

Pb/l to to the site number 3 (after the traffic bridge) to a maximum of 9.78 mg Pb/l, at site number 4 (wastewater from the settlement). It is assumed that increased concentrations of lead during this season, the consequence of traffic pollution or leaking from urban areas, as well as the pollution caused by the consequence of wastewater discharge from the settlement.

In the exhaust gas of the transport means there is tetraethyl lead which is emitted into the atmosphere, and then through precitipation process, also arrives in the aquatic environment, Siegel, 2002. A significant amount of this metal can also be found in urban waste water. The Port of Milena Channel pollutes the waste water of Ulcinj.
In the aquatic environment, lead is primarily bound to sediment, but under low pH conditions, hardness of water and organic matter content (along with a number of other factors) becomes more bioavailable.

The content of total lead (Pb) in sediments of selected sites varied from 4.52 mg Pb/kg dry mass (location 1-winter period) to maximum 18.68 mg Pb/kg dry mass (location number 1-summer period).
Young, immature organisms are more susceptible to lead effects.

The presence of lead (Pb) in the organism of fish is the only consequence of contamination. Lead (Pb) can be bioconcentrated from water, but is not subject to significant biomagnification, even more so the tendency of decreasing the content of lead with the rise of trophic level Eisler, 1988; Brydie & Polya, 2003.

The values of lead in muscle tissue with *Mugil cephalus* ranged from 0.1 to 0.16. Yilmaz, 2005, states: Concretrations of lead in muscle tussue and gonads of gray mullet were observed between 3.59 and 90.97 micog/g.w.wt.respectively. The concentration o lead in muscule tissue of the *Mugil cephalus* from the Maule river were analysed from 0.4 to 11.2 and from the Mataquito river concentration varied between:Pb 0.4-11.1. However the concentrations of Pb in *Mugil cephalus* originating from both estuaries exceeds the levels permitted by current legislation. Tapia et al, 2009,

Krivokapic et al.,2018, states: The level of toxic metals determined by analysis in samples of muscle tissue of the fish reflected the state at the time of sampling; it is necessary to apostropize although some of the approximate boundary, aquatic systems are a dynamic and extremely complex system in which the bioavailability or toxicity of metals is conditioned by chemical speciation, physical as well as numerous biological processes, with ichthyofauna being particularly exposed to the action of toxic pollutants.

Lead is toxic to all organisms. In general, organoleptic compounds are more toxic than inorganic lead components; and young, immature organisms are more susceptible to lead effects. It can be bioconcentrated from water, but is not subject to significant biomagnification, even more the tendency of decreasing lead content with trophic growth (Eisler 1988).

In the aquatic environment, lead is accumulated in algae, macrophytes and benthic organisms, where inorganic forms of lead are not subject to biomagnification. The most harmful effects of lead in the eutrophic aquatic ecosystem are: algae, benthic invertebrates, embryos and young fish, as well as amphibians. Lead detrimental to the reproduction of invertebrates and the growth of algae, and in the amphibians it leads to a decrease in sodium content and developmental problems. At a lead level of 1.0 to 5.1 μg/l in natural waters, disruption of reproduction, death of individuals, and reduction of aquatic organisms growth (Eisler, 1988.; Patrick, 2006.). The effect of lead on fish depends on the type, that is, their ability to secretion this metal over the holster. The fish exposed to high lead concentrations exhibit a wide range of effects including muscular and neurological degeneration and destruction, growth inhibition, mortality, reproductive problems, and etc, Eisler 1988; Horne & Dunson, 1995; Patrick, 2006).

Conclusions

Port Milena, on the one hand, is a unique creative work of nature and, on the other hand, as an "ecological black point" the example of an irresponsible relationship with nature. Channell Port Milena is exposed to contamination of multiple polluters: urban waste water, traffic, water spill from inhabited areas, agriculture and others, suggesting increased concentrations of lead metal (Pb) and other pollutants in water and sediment
Testing of water and sediment quality, with special emphasis on lead distribution (Pb) in these matrices, was carried out during all seasons. In this paper, the concentration of lead (Pb) has been analyzed at five locations, in the water and in the Port Milene sediment and in the muscle tissue of *Mugul cephalus.*

The maximum concentrations of the lead in water has been analyzed in the autumn period 8.97 mg/l at site number 1; 9.78 mg/l at site number 4 (wastewater from the settlement). The lead in *Mugil cephalus* muscle tissue were analyzed on 12 samples. Values are ranged from 0.1 to 0.16. The concentrations of the measured lead in the sediment varied over all four seasons, the maximum value during the winter period 9.63 mg/kg; during the spring season the concentration 10.8 mg/kg; during the summer 18.68 mg//kg, while in the autumn were determined 18.5 mg/kg.

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ESCHERICHIA COLI AND *SALMONELLA SPP.* IN GRUZA RESERVOIR LAKE (SERBIA) PROTECTION ZONE: A DANGER FOR DRINKING WATER SAFETY

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For all living organisms, water is the most vital and important matter for survival. Today, in many under-developed and developing countries, waterborne diseases still pose a major risk in drinking water. Agricultural practices have often proved to be the main source of faecal pollution, with septic systems also identified as sources of serious epidemics of E. coli. Understanding the effects of intensive land use on water resources is essential for the preservation of natural resources and improving environmental quality. In order to determine the presence of certain types of microorganisms in the soil of Gruža Lake protection zone the number E. coli and Salmonella were analysed. Analysing the presence of E. coli and Salmonella in the investigated groups of farmland soils, it has been noted that E.coli is present in 80% of the total plantation area under investigated vegetable gardens, while Salmonella at the same percentage of the area under stubbles. E. coli is present in a significant percentage of land under stubble-fields, meadows and plough fields, in 40 to 58.3% of the samples in the order. Salmonella is present in the soils under arable land and vegetables with 41.7 to 60.0% of the sampled land. The lowest number of both pathogens relates to the land under cereal crops. Management of aquatic ecosystems in the agricultural regions must take into account potential sources of faecal contamination.

Key words: *Gruža Lake, protection zone, E.coli, Salmonella.*

INTRODUCTION

The Gruža reservoir lake was formed in 1984 for the purpose of supplying the city of Kragujevac and the surrounding area (central Serbia) with drinking water. Its accumulation area is 934ha, catchment area 318km² and the surface protection zone with 93 households 1450ha. In the entire basin of the reservoir, there are 20 settlements with 15,000 inhabitants (Stankovic 2000).

The Gruža River with its reservoir is a water source of the first rank with three protection zones (immediate protection zone, narrow protection zone and wider protection zone). Populating inhabitants near the accumulations or uncontrolled use of fertilizers in agricultural production has often had a negative impact on the ecosystem. Understanding the effects of intensive land use on water resources is essential for the preservation of natural resources and improving environmental quality.

Pathogen contamination in some agroecosystems may originate from the point and diffusion sources while improper waste treatment is often the primary source of pathogen. Research on the contamination source has shown that the most common are animal farms (Centers for Disease Control and Prevention, 1998). Management of aquatic ecosystems in the agricultural regions must take into account potential sources of faecal contamination (e.g. faecal on pastures, manure lagoons) such as the time of manure spreading on the fields, as potential sources of faecal contamination. In addition, re-suspension of sediment pathogens may increase the level of pathogens in the case of heavy rainfall. Manure particles and pathogens can be suspended and reach surface waters together with rain (Bradford and Schijven, 2002). We should also bear in mind that pathogens can come with surface and groundwater flows. Studies have confirmed that in addition to the transport of nutrients and sediment in aquatic ecosystems, transport of faecal bacteria from agricultural land occurs as well (Monaghan et al., 2007, Oliver et al., 2005). For almost a century, coliform bacteria, faecal coliforms and E. coli have been used as indicators of drinking water bacterial safety (Leclerc et al., 2001). Namely, the absence of thermal tolerance (faecal) coliform process is a precondition for a water source to qualify as safe drinking water (WHO, 2008). The basis of safe agricultural production is healthy soil, which Doran and Zeiss (2000) define as "the ability of soil to function as a living system in ecosystem boundaries, maintain or improve air and water quality and promote plant and animal health".

In order to determine the presence of certain types of microorganisms in the soil of Gruža Lake protection zone the number of total bacteria, total and faecal coliforms, E. coli and Salmonella were analysed.

MATERIAL AND METHODS

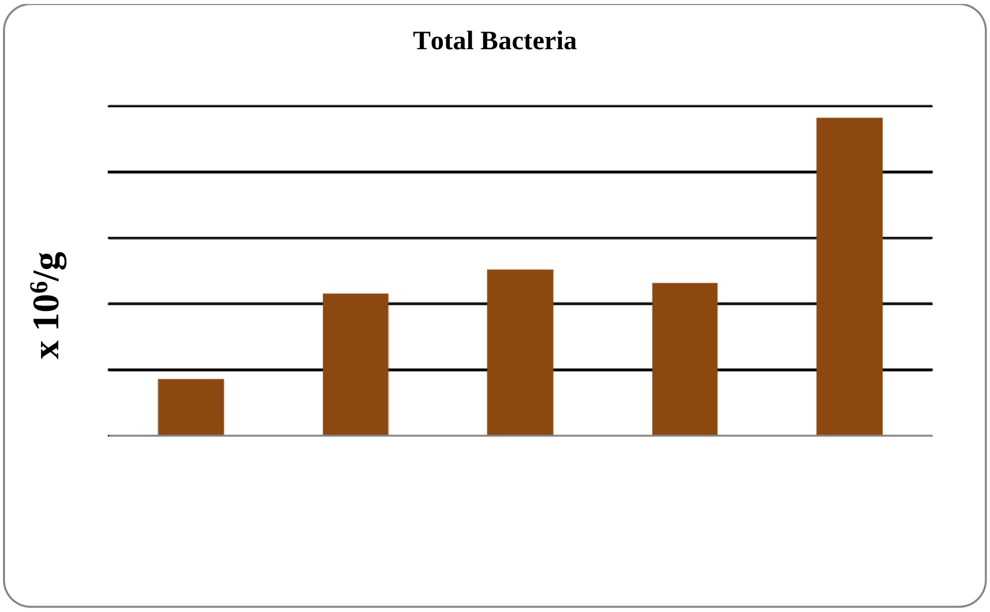
50 samples of soil were analysed under different vegetation and processing: stubble, meadows, fields, vegetable gardens and the sown land under cereal crops in the immediate protection zone of reservoir Lake Gruža (Serbia). Tests were conducted in October 2015 by sampling soil from the depth 0-30 cm with the content of total bacteria, total and faecal coliforms in soil and the total number of E. coli and Salmonella determined.
Total number of bacteria was determined in the 10x diluted Tryptone Soya agar (Torlak) by agar plate method in triplicate at the incubation temperature of 300°C. After a 5-day incubation, the total number of bacteria was expressed per gram of dry soil.

The number of total and faecal colifirms was determined by petrifilm method (3M, USA). Petrifilm is a dehydrated highly selective nutrient base, containing colour indicator, which allows dyeing of the bacterial colonies, so that coliform, and E. coli. bacteria can be clearly distinguished. The incubation for total coliforms and E. coli bacteria was at a temperature of 37 °C, and for faecal coliforms at a temperature of 44°C for a 24h period.
The number of Salmonella spp. was determined by the high-selective homogenous base ChromID™ Salmonella Agar (SM2) (Biomerieux, France). Incubation time was for 18-24 h at a temperature of 35 °C with typical pale pink colonies of Salmonella spp. counted.

For Escherichia coli / Coliform Count-Plate (EC) Petrifilm (3M, USA), inoculation was carried out with 1 ml of the suspension.

RESULTS AND DISCUSSION

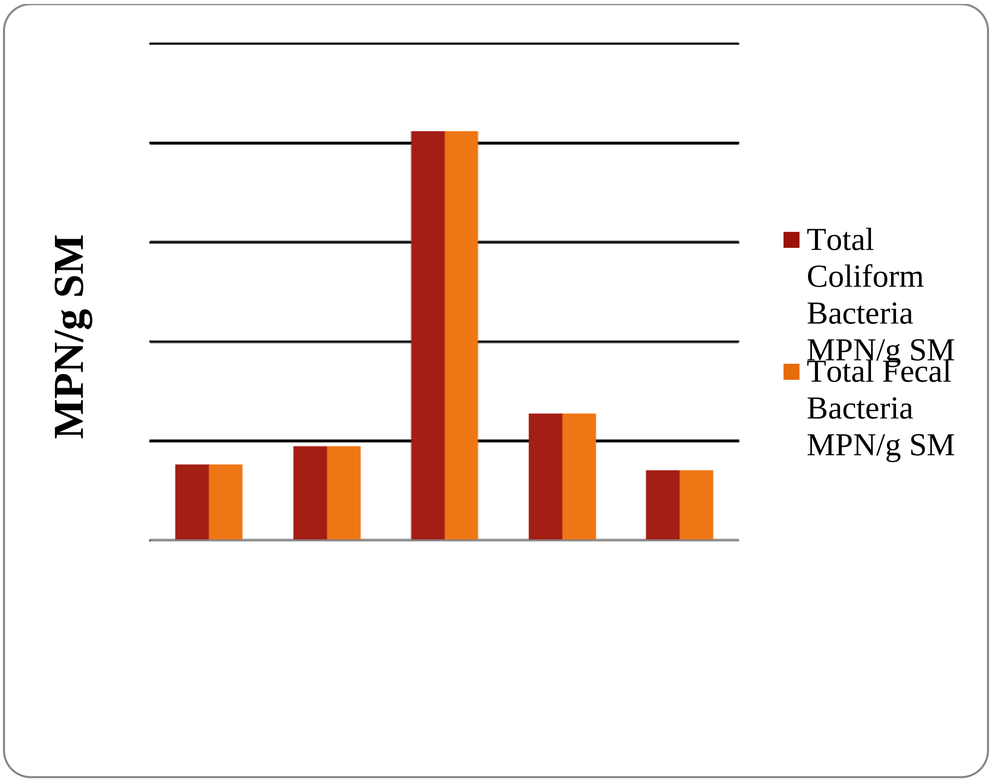
Different practices may modify the soil ecosystem leading thus to the impoverishment of the soil with carbon and biodiversity loss as well contributing to the changed structure of microbial communities (Lauber et al., 2013). Therefore, characterization, genetic and functional diversity of soil bacterial communities as a response to agricultural practices, represents the basis for better understanding of the ecosystem process management Bevivino et al., 2014). Numerous abiotic and biotic factors affect the biodiversity in soil and changes in aboveground ecosystems contribute to changes in underground ecosystems (Hector and Bagchi, 2007). It is known that there is a difference in the structure of microbial communities in uncultivated land in relation to those on which different agricultural practices have been implemented (Jangid et al., 2008), such as liming and fertilizing with mineral fertilisers which can significantly affect the structure of soil microbial communities (Ramirez et al., 2010). During these investigations, the smallest number of bacterial population (Graph.1.) was observed in stubble land, indicating low biological value of the land in relation to arable fields. The restoration of biological processes in stubble land takes longer to activate the processes of organic matter transformation.



Graph. 1. Total number of bacteria in soil ($x10^6$/g)

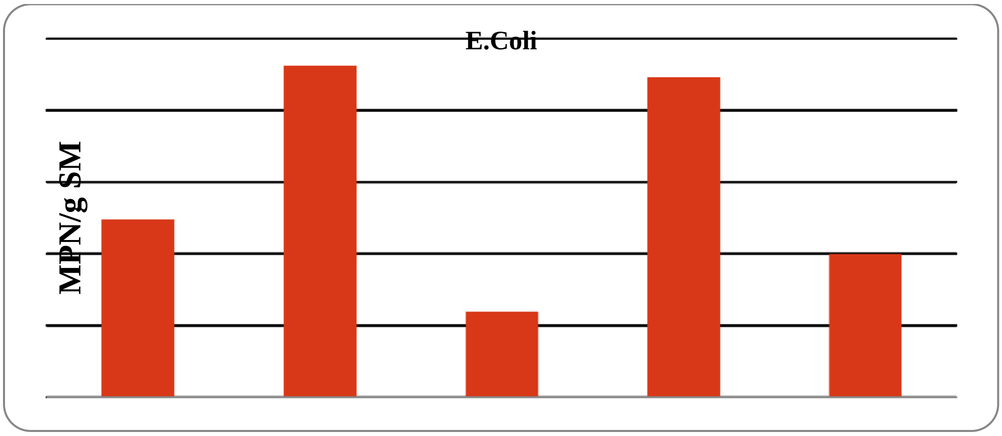
The largest number of bacteria in soils under cereal crops (Graph 1.), with an average of 24.12 $x10^6$/g. Difference in numbers may encourage the applied agricultural measures, as well as chemical and mechanical characteristics of the soil. Soil bacteria perform basic ecosystem functions, such as the circulation of matter, degradation of organic compounds (Nannipieri et al., 2003) and thus contribute to the sustainability of soil ecosystem. Understanding the ecology of microorganisms and their role in the ecosystem is vital for the sustainable ecosystem management and their exploitation.

In these examinations, the largest number of total and fecal coliform bacteria was detected in the plough fields. The high number on individual parcels was measured in the soil under vegetables, with values of 101 to 266 MPN/GSM, provided that the highest individual value is measured on the arable land 1392 MPN/GSM. Possible reason for a large number of these bacteria is the use of low-quality manure in the fall, before sowing corn or wastewater running off, that is, inadequate waste management.



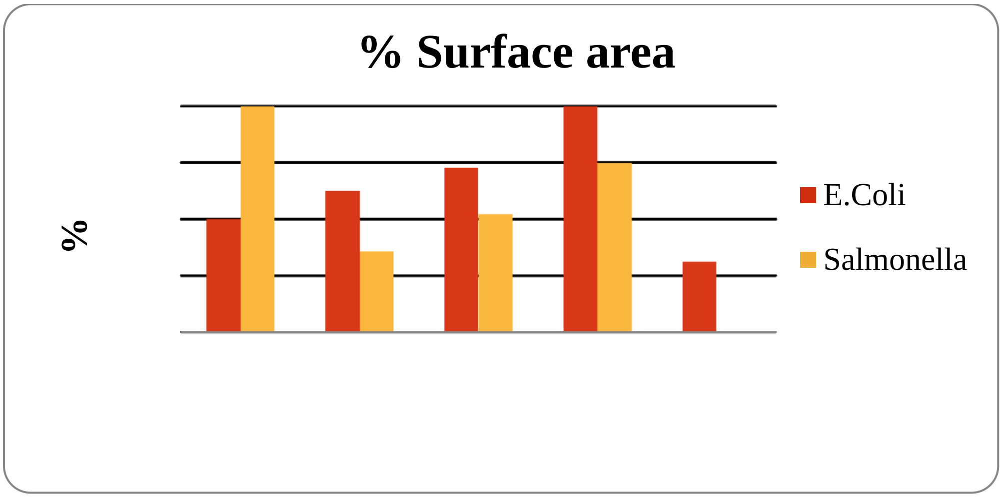
Graph. 2. Total number of coliform and faecal bacteria in soil (MPN/gSM)

Some slight differences were observed in the number of coliform bacteria in the land under stubble, meadows, and cereal field (Graph. 2).
Agricultural practices have often proved to be the main source of faecal pollution (Mosaddeghi et al., 2009), with septic systems also identified as sources of serious epidemics of E. coli. In soils of the investigated locations average number of E. coli ranged from 5,95 MPN g/SM in areas under plough fields to 23.14 in the meadow (Graph. 3).



Graph. 3. Total number of E.coli in soil (MPN/gSM)

However, we should bear in mind that for defining the sanitary-microbiological quality of land each site must be tested separately. Accordingly, in the tested samples, the average number of bacteria in vegetable fields was 22.3 MPN g/SM, and at individual sites, number of E.coli MPN amounted to 62 g/cm. At individual sites of meadow fields, the number of E.coli was121 MPN g/SM.



Graph.4. Percentage of E.coli and Salmonella in soil under different cultures (MPN/gSM)

Analysing the presence of E. coli and Salmonella in the investigated groups of farmland soils, it has been noted that E.coli is present in 80% of the total plantation area under investigated vegetable gardens, while Salmonella at the same percentage of the area under stubbles. E. coli is present in a significant percentage of land under stubble-fields, meadows and plough fields, in 40 to 58.3% of the samples in the order. Salmonella is present in the soils under arable land and vegetables with 41.7 to 60.0% of the sampled land. The lowest number of both pathogens relates to the land under cereal crops (Graph. 4).

CONCLUSION

It is often difficult to control pathogens in agro ecosystem and the absence of sanitary measures in the protection zones significantly endanger water quality, bearing in mind that the hydrological roads connecting agricultural land with water sources might be vectors of disease transmission.

The lowest number of bacterial population was under stubble with the largest numbers of bacteria in soils under cereal crops with mean value 24.12 x106/g. The average highest number of total and faecal coliform bacteria was noted in the fields. However, the presence of pathogenic bacteria should be examined from each individual parcel as the highest individual value measured in reached 1392 MPN/gSM. The presence of E. coli ranged from 5.95 in plough fields to 23,14 MPN g/SM in meadows with the fact that E. coli represented 80% of total planting tested land under vegetables, and the same percentage of Salmonella in the land under stubbles.

Good land management in the protection zones nearby water resources must provide adequate agricultural practices with maintaining or improving the land productivity and reducing the risk of soil and water contamination.

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SPECIFICITY IN THE RELATIONSHIP BETWEEN CELLULOLYTIC BACTERIA AND AZOTOBACTER

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Introduction

The highly diverse soil microbial community includes, inter alia, azotobacter and cellulolytic bacteria which, like other microorganisms, synthesize a wide spectrum of substances and release them into the environment, making them a vehicle for establishing specific interrelationships.

Relationships between azotobacter and cellulolytic bacteria have been studied by Jensen (1940, 1941) and Fedorov (1940), who defined them as being metabiotic, whereas Štucer (1945), Voznjakovskaja (1954) and Rubenčik (1960) determined the existence of symbiotic relationships between these bacteria, as later confirmed by the research results of numerous authors (Holsall and Gibson, 1985, 1986$_a$; Holsall and Goodchild, 1986$_b$; Chunzhi et al., 2002; Haiying; Xiuhong, 2012; Ahmad et al.,2013;).

The objective of this study was to conduct further research into the relationship between cellulolytic bacteria and azotobacter.

Material and methods

Two methods were used in this study. In the disk method, two layers of Fedorov's medium containing 1.5% and 0.8% agar, respectively, were poured into Petri dishes. The upper layer of the medium was inoculated with a thick suspension of azotobacter spread over the surface of the Petri dish. Agar disks impregnated with cellulolytic bacterial cultures were placed on the surface of the inoculated medium. Thereafter, the Petri dishes were incubated for 48 hours at a temperature of 27^0C.

The effect of cellulolytic bacteria on azotobacter was estimated based on heavier growth or absence of growth in the zone around the disk, and the intensity of the effect of some cellulolytic bacterial cultures was determined by the size of the zone around the disk.

The second method involved placement of Fedorov's medium inoculated with an azotobacter suspension on the surface of 15-day-old cultures of cellulolytic bacteria. Upon incubation for 48 hours at 27^0C, the growth of azotobacter was monitored.

Results and discussion

The soil hosts many cellulolytic bacteria which are involved in the decomposition of harvest residues containing more than 50% of cellulose. In addition, the azotobacter present in the soil plays an important role in maintaining its nitrogen regime. Azotobacter utilizes cellulose decomposition products, while cellulolytic bacteria uptake azotobacter-producing nitrogen compounds. Therefore, importance was given to this research on the relationship between these highly beneficial microorganisms (Tabs. 1, 2).

Tab. 1. Effect of some cellulolytic bacteria on *Azotobacter chroococcum*

Cellulolytic bacterial cultures	Strain designation	Azotobacter stimulators	Azotobacter inhibitors
Sporocytophaga myxococcoides	1	+	-
,,	4	+	-
,,	17	+	-
,,	23	+	-
,,	115	+	-
,,	118	-	+
,,	120	+	-
,,	122	-	+
,,	128	-	+

Sporocyophaga ellipsospora	21	-	+
’’	127	-	+
’’	716	-	+
’’	771	+	-
’’	780	-	*
Cytophaga sp.	176	+	-
’’	176*a*	+	-
Cellfalcicula sp.	101	-	+
Sorangium sp.	796	-	+

Tab. 2. Cellulolytic bacterial cultures as stimulators and inhibitors of *Azotobacter chroococcum*

Cellulolytic bacterial cultures	Total number of strains	Stimulators	Inhibitors
Sporocytophaga myxococcoides	9	6	3
Sporocyophaga ellipsospora	5	1	4
Cytophaga sp.	2	2	-
Cellfalcicula sp.	1	-	1
Sorangium sp.	1	-	1

The tables show that 18 cellulolytic bacterial cultures, including 9 belonging to the species *Sporocytophaga myxococcoides*, 5 to *Sporocyophaga ellipsospora,* 2 to *Cytophaga sp.*, 1 to *Cellfalcicula sp.* and 1 to *Sorangium sp.*, and a culture of *Azotobacter chroococcum* were used in the study. The results revealed that some strains of cellulolytic bacteria stimulated azotobacter growth, as azotobacter utilized cellulose decomposition products for its source of carbon and energy. Similar findings were reported by Voznjakovskaja (1954), Rubenčik (1960), Ocampo et al. (1985), Holsall and Gibson (1985, 1986_a, 1986_b), Chunzhi et al. (2002), Alyl et al. (2012), Haiyin and Xiuhong (2012) and Ahmad et al. (2013), who found physiological synergism between cellulolytic bacteria and free-living nitrogen-fixing bacteria.

Moreover, some strains of the tested cellulolytic bacteria were inhibitory to azotobacter growth. Of the 9 tested strains of *Sporocytophaga myxococcoides,* the number of strains (6) stimulating azotobacter growth was twice as high as that of inhibitory strains of this species. Almost all strains of *Sporocyophaga ellipsospora* had an inhibitory effect (4 out of 5 strains) on azotobacter growth. Both strains of *Cytophaga sp.* were found to be stimulators, whereas *Cellfalcicula sp.* and *Sorangium sp.* strains acted as inhibitors.

The intensity of the effect of cellulolytic bacteria on azotobacter varied across strains. The strongest stimulators were strains of *Sporocytophaga myxococcoides* designated by the numbers 4, 17, 23 and 120. The *Cytophaga sp.* strains designated as 176 and 176*a* exhibited a marked stimulatory effect on azotobacter. As for the inhibitory strains, the size of the inhibition zone ranged from 3.3 to 4.1 cm in *Sporocytophaga myxococcoides,* and from 3.7 to 4.2 cm in *Sporocyophaga ellipsospora*.

In most antagonistic cultures, next to the inhibition zone, there was a stimulation zone, with antibiotic substances released by the tested strains stimulating azotobacter growth (Jemcev, Đukić, 2000). The morphology of azotobacter was changed by almost all tested cultures of cellulolytic bacteria. For example, *Azotobacter chroococcum* was of doughy dry consistency and formed wrinkled colonies, but when affected by antagonistic strains or stimulators its consistency became slimy or even drier with colony surface increasingly wrinkling. Microscopic examination showed that azotobacter cells were somewhat smaller or even deformed within the inhibition zone range. In cases when the growth of the test cultures was stimulated, azotobacter cells were found to be larger in control Petri dishes.

Conclusion

The following conclusions can be drawn from this study:

- cellulolytic bacteria include both stimulators and inhibitors of azotobacter growth;
- representatives of *Cytophaga sp.* have a positive effect on azotobacter growth;
- a number of representatives of *Sporocytophaga myxococcoides* species act as stimulators of azotobacter growth, whereas *Sporocyophaga*

ellipsospora is dominated by strains which are inhibitory to azotobacter growth;

- the tested strains of *Cellfalcicula sp.* and *Sorangium sp.* have an adverse effect on azotobacter.

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THE SENSITIVITY OF *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS* AGAINST BACTERIOPHAGES ISOLATED FROM SOIL

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ABSTRACT

Bacteriophages present significant biological agents in the modern concept in control of phytopathogenic bacteria. Owing to their high specificity and environmental harmless, they are gaining importance. The study examined the sensitivity of 10 isolates of Xanthomonas campestris pv. campestris (Xcc) against 7 bacteriophages originating from the soil. The examined bacteriophages showed lipolytic activity for all Xcc isolates as well as reference strain NCPPB 1144. In control strains Pseudomonas syringae pv. syringae Tk21 and X. a. pv. phaseoli KBNS 204 tested bacteriophages did not show lipolytic activity.

Key words: sensitivity, bacteriophages, Xanthomonas campestris pv. campestris

INTRODUCTION

Among many biological agents, bacteriophages have the potential to control zoopathogenic and phytopathogenic bacteria (Balogh et al., 2003; Djukić et al., 2011). They represent a special group of viruses which do not have their own enzyme system but depend directly on the host cell. Bacteriophages parasitize bacterial cells and thus reduce the bacterial population in the environment. They can be found at sites where bacteria are located and are highly specific by eliminating only targeted bacteria, without the influence on other organisms (Gašić et al., 2007). They only multiply when the host bacteria is present and quickly decompose when the host is absent (Kutter and Sulakvelidze, 2005; Obradović, 2009). So far, several types of bacteriophages have been studied which differ in shape, size and composition. All contain nucleic acid, most often DNA wrapped with a protein capsule or capsid (Obradović et al., 2008).

Xanthomonas campestris pv. *campestris* (Xcc) causes significant losses on brassicas in moderate climates (Williams, 1980), at temperatures of 20-30 °C (Mariano et al., 2001). If infection occurs immediately after transplanting plants in the field, yield losses can be up to 90% (Dey et al., 2015). Characteristic symptoms of black rot caused by Xcc are V-shaped chlorotic to necrotic lesions extending from the leaf margins and blackening of vascular tissues. Pathogen is present in Serbia, and depending on environmental factors, it infects with different intensity (Balaž, 1988; Obradović and Arsenijević, 1999; Vlajić et al., 2017). With the aim of introducing integral and organic concept in the brassicas production, this study examined the sensitivity of the Xcc isolates originating from the various hosts of the Brassicaceae family to the bacteriophages isolated from the soil.

MATERIAL AND METHODS

Bacteriophage isolation. Bacteriophages were isolated from the soil by the substrate enrichment method. This method allows selective multiplication of bacteriophages specific to the host being added to the substrate (Crosse and Garrett, 1963). The soil samples were collected from three localities where cabbage and cauliflower were grown (Rimski Šančevi, Futog, Kovilj, AP Vojvodina). Isolate Xcc (KS-17) was used in the enrichment process. For isolation, 10 g of soil, 50 ml of nitrogen yeast glycerol broth (NYGB) was taken and 5 ml of KS-17 isolates were added. The prepared suspension was placed on a rotary mixer in a thermostat at 26 °C for a period of 24 h. Phages were extracted by centrifuging 1 ml of the enriched suspension over 5 min to 16000 g (Hermile Z 300 K). The supernatant was then transferred to a tube and treated with chloroform (10:1 v/v) for 20 min, with occasional stirring. After precipitation of the chloroform, the suspension was decanted and transferred to new tubes, wrapped with aluminum foil and stored in the refrigerator at 4 °C until used.

Testing the sensitivity of Xcc isolates to isolated bacteriophages. Sensitivity of obtained bacteriophages was tested according to Spot Test (Double-Layer Agar), (Adams, 1959). In the Petri dish 90 mm in diameter, 30 ml of the medium (Flat Agar) was placed. After drying, flat agar is poured with 10 ml of Top Lader Agar, a temperature of 45 °C, in which the tested Xcc bacterial isolates (0.1 ml of suspension of bacteria cultivated in LTP medium) was added. After cooling 1 µl of bacteriophage suspensions was applied. After 48 h, plaque appearance was observed at the places of the applied suspension. Ten isolates of Xcc and three control strains (Xcc - NCPPB 1144, *X. axonopodis* pv. *phaseoli* - KBNS 204, *Pseudomonas syringae* pv. *syringae* - Tk21) were used for sensitivity testing (Table 1).

Table 1: Isolates and strains used in sensitivity testing

<table>
<tr><th>Isolate</th><th>Host</th><th>Locality</th></tr>
<tr><td>KS-1</td><td>cabbage</td><td>Rumenka</td></tr>
<tr><td>KL-1</td><td>cauliflower</td><td>Kovilj</td></tr>
<tr><td>BL-1</td><td>broccoli</td><td>Jasenovo</td></tr>
<tr><td>KS-9</td><td>cabbage</td><td>Rimski Šančevi</td></tr>
<tr><td>KL-4</td><td>cauliflower</td><td>Futog</td></tr>
<tr><td>KS-20</td><td>cabbage</td><td>Mrčajevci</td></tr>
<tr><td>KS-24</td><td>cabbage</td><td>Golubinci</td></tr>
<tr><td>UR-1</td><td>oilseed rape</td><td>Rimski Šančevi</td></tr>
<tr><td>BL-2</td><td>broccoli</td><td>Zvečka</td></tr>
<tr><td>KS-44</td><td>cabbage</td><td>Vršani</td></tr>
<tr><td colspan="3">Reference strain</td></tr>
<tr><td>Tk21</td><td>oil pumpkin</td><td rowspan="2">Collection of Phytopathogenic Bacteria, Faculty of Agriculture Novi Sad</td></tr>
<tr><td>KBNS 204</td><td>bean</td></tr>
<tr><td>NCPPB 1144</td><td colspan="2">National Collection of Plant Pathogenic Bacteria</td></tr>
</table>

RESULTS AND DISCUSSION

The appearance of resistant strains in the population of phytopathogenic bacteria to copper compounds and antibiotics imposes the application of biological agents, such as bacteriophages (Minsavage et al., 1990; Gašić et al., 2012). Bacteriophages were first found in association with plant pathogenic bacteria in 1924 when Mallman and Hemstreet (1924) isolated *X. c.* pv. *campestris* from diseased cabbage tissue and observed that filtrate of liquid collected from decomposed cabbage inhibited growth of the pathogen *in vitro*. The bacteriophage application has proved effective in controlling various diseases caused by bacteria of the genus *Xanthomonas* (Flaherty et al., 2001; Obradović et al., 2005; Lang et al., 2007). Since Xcc is a tracheobacteriosis, and so far there have not been sufficiently effective control preparations, bacteriophages could be used as biological agents due to their lipolytic activity.

Seven bacteriophages were obtained in this study from 10 collected soil samples. The presence of phages in the samples was determined by the appearance of plaque after 48 hours of incubation. Bacteriophages were coded as: FXcc1/1, FXcc1/3, FXcc1/4, FXcc2/4 origin from R. Šančevi, FXcc3/, FXcc3/3 isolated from Futog, while FXcc3/6 was obtained from Kovilj locality. Isolates of the bacteriophage used in the study exhibited lipolytic activity for all Xcc isolates and reference strain NCPPB 1144. Formation of plaque due to lipolytic activity indicated the sensitivity of the tested bacterial isolate. The diameter of the plaque was 11-17 mm (Figure 1).

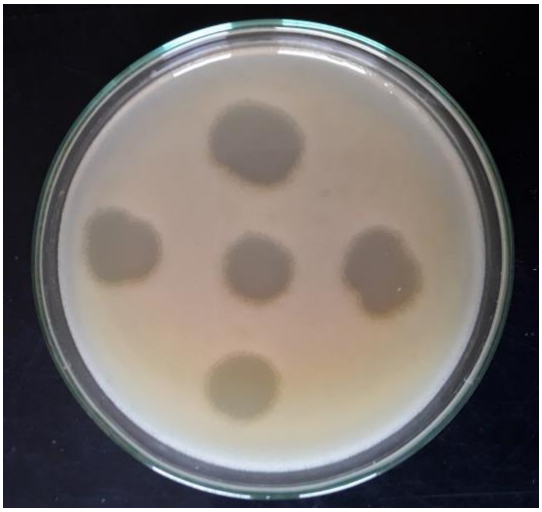

Figure 1: Plaque formation (bacteriophage 2/4)

None of the examined bacteriophages showed activity in the control strains of *P. s.* pv. *syringae* Tk21 and *X. a.* pv. *phaseoli* KBNS 204 (Table 2).

Table 2: The sensitivity of the tested bacterial isolates

Isolate	**Bacteriophage**						
	1/1	**1/3**	**1/4**	**2/4**	**3/1**	**3/3**	**3/6**
KS-1	+	+	+	+	+	+	+
KL-1	+	+	+	+	+	+	+
BL-1	+	+	+	+	+	+	+

KS-9	+	+	+	+	+	+	+
KL-4	+	+	+	+	+	+	+
KS-20	+	+	+	+	+	+	+
KS-24	+	+	+	+	+	+	+
UR-1	+	+	+	+	+	+	+
BL-2	+	+	+	+	+	+	+
KS-44	+	+	+	+	+	+	+
KBNS 204	-	-	-	-	-	-	-
Tk21	-	-	-	-	-	-	-
NCPPB 1144	+	+	+	+	+	+	+

Legend: + forming a translucent plaque; - absence of plaque formation

Our results indicate that isolated phages may be used as biological agents in Xcc control. There are many studies based on bacteriophage application in the control of bacterial pathogens (Kotila and Coons, 1925; Moore, 1926; Schnabel et al., 1999; Petrušić, 2017). Moore proposed that phages may be utilized in disease control (Moore, 1926), and soon thereafter phages were successfully used for the prevention of potato tuber rot caused by *Erwinia carotovora* subsp. *atroseptica* (Kotila and Coons, 1925). According to Schnabel et al. (1999) there was significantly less fire blight on inoculated blossom clusters treated with phage. High population of phage was dependent on the presence of *E. amylovora* and the strategies have to be found for maintaining phage populations during the blossom period.

The limiting factor of bacteriophages application in the agricultural practice is their sensitivity to environmental influences. The main limiting factors for the survival of bacteriophages on leaves are ultraviolet radiation and draining (Gill and Abedon, 2003). According to Balogh et al. (2003), a mixture of bacteriophage and skim milk with sucrose has shown good efficacy in control of bacterial spots on tomato in Florida. According to some authors, due to high specificity of the bacteriophage, they can be used as additional methods for the detection, identification and differentiation of phytopathogenic bacteria (Stolp and Starr, 1964; Gašić et al., 2012; Zlatković, 2018).

The results of this study indicated the presence of bacteriophages in the vicinity of the diseased cabbage plants, which have the ability to lipolyse the bacterial cells. Based on these data, there is a possibility of bacteriophage application in the control of cabbage and other brassicas against bacterial diseases.

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ANTIBACTERIAL ACTIVITIES OF ESSENTIAL OILS OF WILD OREGANO, CLOVE BUD, ROSEMARY, PEPPERMINT, BASIL AND LEMONGRASS AGAINST GROWTH OF SOFT ROT BACTERIA

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ABSTRACT

Essential oils (EOs) from wild oregano, clove bud, rosemary, peppermint, basil and lemongrass were tested for their antibacterial properties against soft rot causing bacteria. The disc-diffusion method was used for the determination of theit inhibitory effects conducted in vitro. The results revealed that the tested EOs exhibited moderate to high antibacterial activity against all three tested bacteria. EOs from wild oregano and clove bud were found to have the strongest inhibitory effect against tested phytopathogenic bacteria. The antibacterial activity of EOs on plant pathogenic bacteria remains an important task for future research.

Key words: essential oils, soft rot, disease, *Pectobacterium*, *Dickeya*

INTRODUCTION

Storage is a major step in the handling of fruit and vegetables up to several months, depending on the commodity and storage conditions with the purpose of extending the marketing season, delaying marketing until prices rise, providing a reserve for more uniform retail distribution or reducing the frequency of purchase by the consumer or supermarkets (Antunes & Cavaco 2010). Although most of the pathogenic microorganisms have optimal growth temperatures above 20 °C, and therefore are controlled from growing, some of them, known as psychotrophs, such as soft rot causing pathogens, grow well at refrigeration temperatures, leading to spoilage, which makes them among the most important decay agents in fruit and vegetables (Antunes & Cavaco 2010). Soft rot disease is caused by various bacterial species within genera *Bacillus*, *Pseudomonas* and *Erwinia* (Agrios 2006). However pathogenic *Enterobacteriaceae* belonging to the genera *Pectobacterium* and *Dickeya* are considered to be the most common. Thay cause disease on plant species from wide range of plant families, including a wide variety of economically important crops, but commonly occur on fleshy vegetables such as potato, tomato, onion, pepper, carrot, eggplant, squash and cabbage (Ma et al. 2007; Shurtleff et al. 2018). Untill now, described bacterial species responsible for soft rot are *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium carotovorum* subsp. *brasiliensis*, *Pectobacterium atrosepticum*, *Pectobacterium wasabiae*, *Dickeya dianthicola* and *Dickeya solani* (Czajkowski et al. 2011). Some isolates of *P. c.* subsp. *carotovorum* were recently reclassified into the new species, named *Pectobacterium polaris* (Dees et al. 2017) and isolates of *P. atrosepticum* into the new species *Pectobacterium zantedeschiae* (Waleron et al. 2018).

Soft rot pathogens can cause substantial yield and quality losses. They possess secrete enzymes capable to decomposing cell wall structures, thereby destroying the texture of plant tissue and which becomes macerated, soft and watery (Shurtleff et al. 2018). Besides symptoms, the recognizing sign, characteristic for disease presence is specific odour i.e. disagreeable smell of tissues affected with bacterial soft rot that accompanies the breakdown of plant tissue. Disease has the same appearance on each plant host.

The soft rot disease can be found on crops in the field, transit and storage or during marketing (Bhat et al. 2010). Soft rot pathogens cause a total loss in production greater than any other bacterial pathogens (Agrios 2006). The greatest economic losses were recorded especially in post-harvest, when estimated losses varied from 15-30% of the harvested crop (Agrios 2006).

Control of soft rot pathogens faces many challenges, since effective ways for chemical control of any of them does not exist. Prevention is still the only solution in their controlling. Antibiotics are essential for control of plants bacterial diseases, but their usage is forbidden in many countries because of their effects on human health or persistent impacts on the environment. These facts have led to prompted research into the identification of new substances with antibacterial activity as an alternative strategy to prevent the spread of diseases. Plant extracts or their derivatives, such as essential oils (EOs), have been recently tested for their usage in plant protection. EOs are substances with strong odor of aromatic plants, usually obtained by extraction, fermentation, or expression, but most commonly using steam distillation method (Moreira et al. 2016). They are liquid, volatile, limpid, coloured and soluble in lipids and organic solvents with lower density than water; present in all plant organs, including buds, flowers, leave, seeds, twigs, stems, flowers, fruits, roots, wood or bark, but are generally stored in plant in secretory cells, cavities, canals, glandular trichomes or epidermic cells (Nazzaro et al. 2013). EOs are very complex natural mixtures which contain about 20–60 components in quite different concentrations (Moreira et al. 2016). This products could be a source of new pesticides or serve as templates for new, more effective compounds (Elkovich, 1988). The replacement of synthetic compounds by EOs with proven inhibitory properties may contribute to the sustainable agriculture production.

Antimicrobial studies of the effects of different EOs are in progress. Thus, different EOs have been observed to be effective against *Pectobacterium* spp. (Deans & Ritchie 1987; El-Zemity et al. 2008; Jeong et al. 2009; Alamshahi et al. 2010; Nezhad et al. 2012; Guerra et al. 2014; Mehrorosh et al. 2014; Rojas et al. 2014; Umunna & Anselem 2014; Alamshahi & Nezhad 2015).

The present study shows antibacterial activity of EOs of wild oregano, clove bud, rosemary, peppermint, basil and lemongrass against soft rot causing bacteria and their reduced concentrations required to achieve a particular antibacterial effect. Their activity potentials were assessed by the presence or absence of inhibition zones, zone diameters and minimum inhibitory concentration (MIC) values.

Materials and Methods

The strains of *P. c.* subsp. *carotovorum* (strain coded as KFB85, obtained from culture collection of the Faculty of Agriculture, Belgrade, Serbia), *P. c.* subsp. *brasiliensis* (strain coded as Pcb62, obtained from T. Popović, culture collection of the Institute for Plant Protection and Environment, Belgrade, Serbia) and *D. dianthicola* (strain coded as Dd31 obtained from T. Popović, culture collection of the Institute for Plant Protection and Environment, Belgrade, Serbia) were used in experiment. Strains were grown before experiment onto Nutrient Agar for 48 h at 26 °C.

Antibacterial activity was tested with six EOs, presented in Table 1. Their inhibitory effect on the strains growth was evaluated by Agar-diffusion assay using holes in medium (Gojgić-Cvijović & Vrvić 2003). Bacterial suspension of each the tested strains was added to Nutrient Agar to obtain the final concentration of 10^8 cells/mL and poured in sterilized Petri plates (ø 9 cm). As soon as the medium had solidified, the holes (ø 0.6 cm) were made on agar in Petri plates where per 100µL of the tested oil was added. Petri plates were then placed for incubation at temperature 26 °C for bacteria development. Three replicates were made for each of the tested strains and for each treatment. Plates with or without bacterial culture in medium treated with sterile distilled water served as positive or negative control treatments, respectively.

To obtain the minimum inhibitory concentration (MIC) values of tested EOs, serial dilutions were made to a concentration ranging from 5 to 50% (5%, 10%, 25%, 50%). The MIC value was defined as the lowest concentration of the EOs required for inhibition of the bacterial growth. All experiments were performed twice.

Table 1. Essential oils tested

Latin name	Common name	Manufacturer
Origanum minutiflorum	Wild oregano	Probotanic
Aetheroleum romarini	Rosemary	Kirka
Syzygium aromaticum	Clove bud	Probotanic
Ocimum basilicum	Basil	Balev
Mentha piperita	Peppermint	Elina
Cymbopogon citratus	Lemongrass	Profissimo

The interaction between the bacteria and the EOs is expressed by the formation of the inhibition zone with diameters (expressed in cm) determined two days after incubation. Data were analyzed by Minitab 16.1.0 Statistical Software. The results were analyzed by using analysis of variance (ANOVA). For testing the difference between the mean treatment, Tukey's Multiple Range test was performed.

Results

According to the results given in Table 2, all six tested EOs (wild oregano, clove bud, rosemary, peppermint, basil, lemongrass) had great potential of antibacterial activitiy against three tested bacteria causing soft rot disease. Wild oregano and clove bud achieved the maximal inhibition zones ≥ 2.0 cm in diameter when applied undiluted and in most of the tested reduced concentrations (Table 2), exhibiting high antibacterial activity. Significant statistical differences were evident in rosemary, peppermint, basil, and lemongrass which exhibited moderate antibacterial activity against soft rot bacteria. Inhibition zones ranged from 1.12 cm to 1.85 cm when applied undiluted.

According to the obtained results it can be noticed that efficacy of EOs was not in direct correlation with reduction of their concentration i.e. oils expressed almost similar efficacy when used in reduced concentration and in undiluted form (Figure 1). Thus, wild oregano caused inhibition haloes ranging from 1.67 – 3.13 cm for 5% concentration and undiluted, respectively; for clove bud values were between 1.43 – 2.28 cm; for basil from to 0.97 – 1.47 cm and for lemongrass among 0.80 – 1.28 cm. EOs of rosemary and peppermint did not inhibit bacterial growth when they were used in concentration of 5%. Rosemary caused inhibition haloes ranging from 0.90 – 1.95 cm for 10% concentration and undiluted, respectively; for peppermint haloes were among 0.78 – 1.57 cm (Table 2).

MIC values for bacterial strains which were sensitive to the tested EOs, were in the range of < 5% for wild oregano, clove bud, basil and lemongrass and > 5% for rosemary and peppermint.

The obtained results also showed that all isolates (*P. c.* subsp. *carotovorum*, *P. c.* subsp. *brasiliensis* and *D. dianthicola*) were similarly susceptible to the tested EOs.

Table 2. *In vitro* growth inhibition (cm) of soft rot pathogens subjected to six different EOs

Tested oil	**Concentration (%)**	***P. c.* subsp. *carotovorum* (strain KFB85)** **Inhibition zone (cm)**	**Tested oil**	**Concentration (%)**	***P. c.* subsp. *brasiliensis* (strain Pcb62)** **Inhibition zone (cm)**	**Tested oil**	**Concentration (%)**	***Dickeya dianthicola* (strain Dd31)** **Inhibition zone (cm)**
Clove bud	100	2.10 a	Wild oregano	100	2.43 a	Wild oregano	100	3.13 a
Wild oregano	100	2.03 a	Wild oregano	50	2.27ab	Wild oregano	50	3.00 a
Wild oregano	50	1.98 ab	Clove bud	100	2.27 ab	Wild oregano	25	3.00 a
Wild oregano	25	1.95 abc	Clove bud	50	2.07abc	Wild oregano	10	3.00 a
Wild oregano	10	1.93 abcd	Rosemary	100	1.95bcd	Clove bud	100	2.28b
Clove bud	50	1.85 abcde	Wild oregano	25	1.90 bcd	Wild oregano	5	2.25b
Wild oregano	5	1.67 bcdefg	Wild oregano	10	1.90 bcd	Clove bud	50	2.13bc
Rosemary	100	1.65 cdefg	Wild oregano	5	1.87 bcde	Rosemary	100	1.85cd
Clove bud	25	1.62 defg	Clove bud	25	1.77cdef	Clove bud	25	1.85 cd
Clove bud	10	1.62 defg	Rosemary	50	1.63defg	Clove bud	10	1.82d
Rosemary	50	1.58 efgh	Clove bud	10	1.60defgh	Clove bud	5	1.80d
Clove bud	5	1.48 fghi	Peppermint	100	1.57defghi	Rosemary	50	1.67de
Peppermint	100	1.33 ghij	Basil	100	1.47efghi	Peppermint	100	1.55def
Basil	100	1.27 hijk	Clove bud	5	1.43fghij	Basil	100	1.43efg
Basil	50	1.18 ijkl	Basil	50	1.33ghijk	Basil	50	1.42efg
Rosemary	25	1.18 ijkl	Basil	25	1.28ghijk	Basil	25	1.40efg
Basil	25	1.13 jklm	Lemongrass	100	1.22ghijk	Basil	10	1.28fgh
Basil	10	1.12 jklmn	Lemongrass	50	1.20hijkl	Lemongrass	100	1.28fgh
Lemongrass	100	1.12 jklmn	Rosemary	25	1.18hijkl	Lemongrass	50	1.27fgh
Lemongrass	50	1.05 jklmno	Lemongrass	25	1.18hijkl	Rosemary	25	1.15ghi
Peppermint	50	1.02 jklmno	Basil	10	1.15 ijkl	Lemongrass	25	1.03 hij
Lemongrass	25	1.02 jklmno	Rosemary	10	1.15ijkl	Lemongrass	10	1.03hij
Basil	5	0.97 klmno	Lemongrass	10	1.15ijkl	Peppermint	50	1.02hij
Rosemary	10	0.90 lmno	Peppermint	50	1.03jkl	Basil	5	1.02hij
Lemongrass	10	0.88 lmno	Lemongrass	5	1.00 kl	Lemongrass	5	1.00hij
Peppermint	25	0.83 mno	Basil	5	0.98 kl	Rosemary	10	0.95hij
Lemongrass	5	0.80 no	Peppermint	25	0.92 kl	Peppermint	25	0.92 ij
Peppermint	10	0.78 no	Peppermint	10	0.78 l	Peppermint	10	0.80j
Peppermint	5	0 p	Rosemary	5	0 m	Peppermint	5	0 k
Rosemary	5	0 p	Peppermint	5	0 m	Rosemary	5	0 k

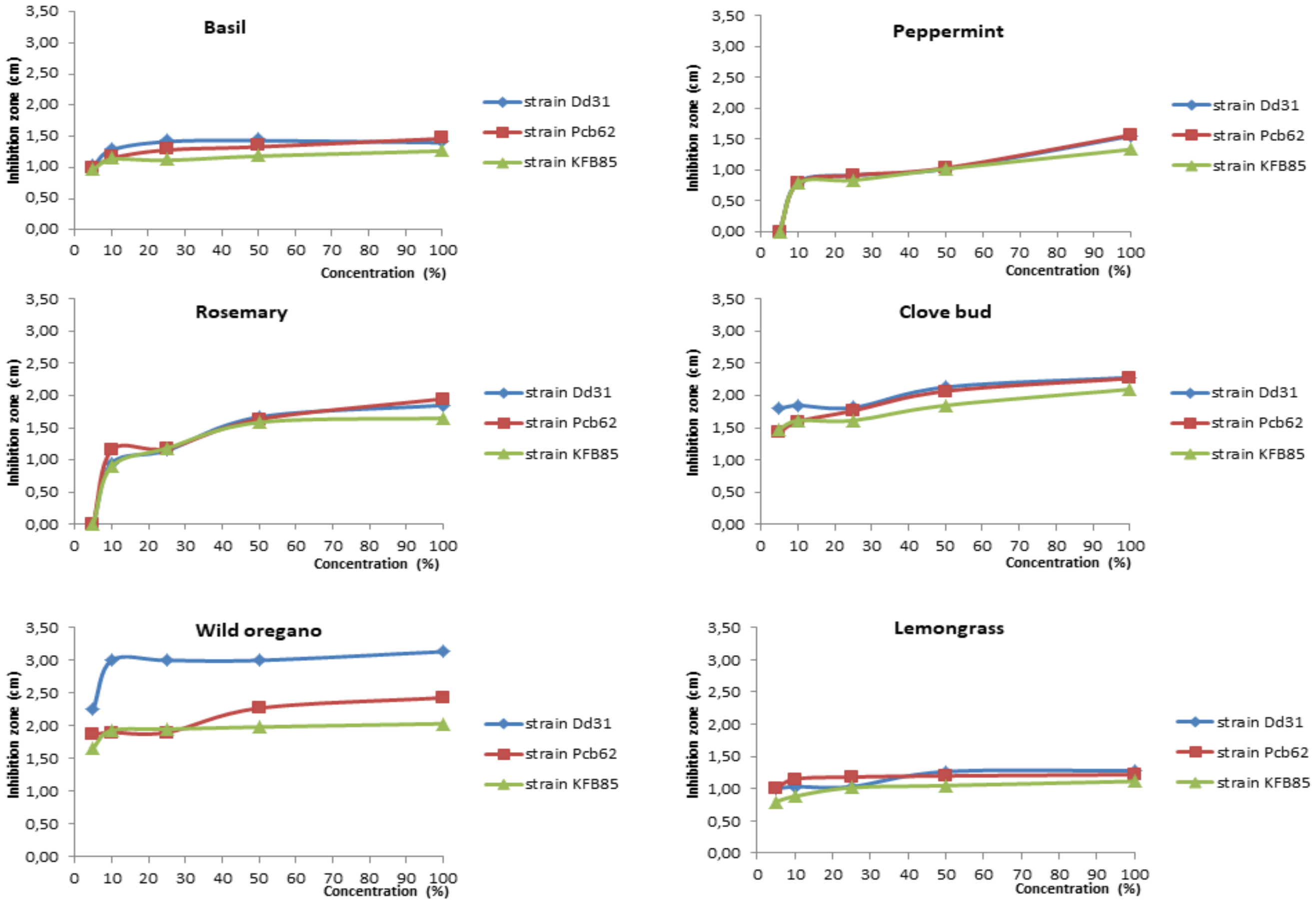

Figure 1. Antibacterial activity of essential oils of wild oregano, clove bud, rosemary, peppermint, basil and lemon grass used in different concentrations against growth of soft rot bacteria

Figure 2. Antibacterial activity of EOs against *P.c.* subsp. *brasiliensis* strain Pcb62; wild oregano in different concentrations (left), clove bud undiluted (right)

Discussion

Nowadays EOs undoubtedly present the most promising group of natural compounds for development as alternative to the usage of synthetic pesticides in plant protection. They have been tested against bacteria and fungi, including both human and plant pathogenic strains and positively documented from a wide number of *in vitro* experiments, including those with the efficacy against postharvest pathogens (Antunes & Cavaco 2010). The necessary concentrations of EOs for inhibition of microbial growth are usually higher in food than in culture media, which might be the result of interactions between phenolic compounds and the food matrix, which is why all of these factors should be taken into account in commercial applications (Tzortzakis & Economakis 2007). Their effects, as well as the minimum concentrations gain effectiveness without affecting fresh produce quality and storage, therefore deserving further research (Tzortzakis & Economakis 2007).

EOs such as rosemary (*Rosmarinus officinalis*), clove (*Eugenia caryophyllus*), thyme (*Thymus vulgaris*), lemon grass (*Cymbopogon flexuosus*), eulcalyptus (*Eulcalyptus globules*), peppermint (*Mentha piperita*) and basil (*Ocimum basilicum*) are well known for their pest control properties against various microorganisms and insects (Mohan et al. 2011). This study therefore proved antimicrobial properties of wild oregano, clove bud, rosemary, peppermint, basil and lemongrass against bacterial soft rot pathogens. Wild oregano and clove bud provided the highest degree of inhibition growth of soft rot pathogens, *P. c.* subsp. *carotovorum*, *P. c.* subsp. *brasiliensis* and *D. dianthicola* because at the lowest concentration, it was a more potent inhibitor of growth than the other oils studied.

Potential antibacterial properties of EOs of clove and rosemary against bacterium *P. carotovorum* in *in vitro* trials were previously described by El-Zemity *et al.* (2008). Jeong *et al.* (2009) showed that EO from *Cymbopogon citrates* effectively inhibited *P. carotovorum* even in reduced concentration of 0.5% when the growth of bacterium was completely inhibited. Popović et al. (2016) found 7 EOs (*Thymus vulgaris*, *Cinnamomum cassia*, *Cassia angustifolia*, *Origanum vulgare*, *Boswellia serrata*, *Eucalyptus globulus*, *Satureja montana*), among 51 tested to be the most efficient against *P. c.* subsp. *carotovorum*; however many other tested oils were also effective, but with lower power for bacteria inibition (*Melaleuca alternifolia, Rosmarinus officinalis, Anethum graveolens, Syzygium aromaticum, Laurus nobilis, Ravensara aromatica*, *Pimpinella anisum, Cinnamomum verum, Gaultheria procumbens, Cymbopogon martinii, Lavandula angustifolia, Cedrus atlantica, Citrus bergamia, Ocimum basilicum, Pinus sylvestris, Mentha x piperita, Lippia citriodora*).

Some of the EOs such as oils of copaiba, sweet orange, spearmint, *Eucalyptus citriodora* and bergamot, used in concentration 0.5%, were reported as stable in reduction of the severity of soft rot causal agent *P. c.* subsp. *carotovorum* in Chinese cabbage (Guerra et al. 2014). According to Awadalla et al. (2008) lemongrass, spearmint, peppermint, geranium, fennel, bisilicum, marjoram and carnation markedly inhibited the growth of *E.*

carotovora in vitro, emphasizing on lemongrass to have the lowest MIC value. Authors stated that treatments of stored potato tubers with the tested EOs were successful in controlling soft rot diseases during storage periods.

The EOs of the *Lavandula angustifolia*, *Rosmarinus officinalis* (rosemary), *Salvia fruticosa* (sage), as well as those of *Coridothymus capitatus* (Spanish oregano), *Origanum dictamnus* (Cretan dittany) and *Satureja thymbra* were found to possess potent antimicrobial activity against *Erwinia carotovora* strains (currently name *P. c.* subsp. *carotovorum*) (Vokou et al. 1993). Neem leaf and seed aqueous extracts significantly reduced the incidence and severity of soft rot pathogen *E. carotovora*, while ironweed and Siamese cassia aqueous leaf extracts gave moderate control of the disease (Bdliya & Dahiru 2006). The antibacterial activity of the chitosan nanoparticles added with thyme essential oil presented a significant inhibitory effect on the growth of *P. carotovorum* (Sotelo-Boyás et al. 2015).

With the aim to determine the minimum inhibitory concentration (MICs) Alamshahi et al. (2010) tested EOs of *Thymus vulgaris*, *Rosmarinus officinalis*, *Coriandrum sativum*, *Cuminum syminum* and *Eucalyptus camuldulensis* at concentrations of 0.01% , 0.05% , 0.1% , 0.5% , 1% , 5% , 10% , 25% , 50%, 75% , 100% (v/v) against the bacterium *P. c.* subsp. *carotovorum*. The largest inhibition zone (16.5 mm) and lowest MIC (5 µl/ml) were shown by *T. vulgaris*, followed by *C. sativum*, *C. syminum* and *R. officinalis*. MIC values of EOs were from 5-250 µl/ml.

Two important variables appear when EOs are applied either *in vivo* or *in vitro*; one is the contact time for oils to exert their effect and the other is the concentration required to achieve the same degree of inhibition and these two variables would be interacting (Moreira et al. 2016). Despite all apparent advantages, the detailed examination of the biological activity and dispersion of EOs and their compounds in plant tissues remains to be done with the aim to develop a formulation that maintains fungicidal or bactericidal activity without causing undesirable effects to the product to human health and the environment (Antunes & Cavaco 2010).

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ИННОВАЦИОННЫЕ ИНФОРМАЦИОННЫЕ ТЕХНОЛОГИИ В СЕЛЬСКОМ ХОЗЯЙСТВЕ.

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АННОТАЦИЯ

Современное сельское хозяйство ожидает от разработчиков отраслевых технологий эффективных инструментов для производства высококачественной продукции, при том условии, что новые технологии будут отвечать требованиям сохранности окружающей среды, просты в эксплуатации и будут приносить ощутимую прибыль. В статье излагаются результаты применения инновационной технологии, основанной на принципиально новом подходе – информационном управлении физиологией живого организма. Рассказывается о новом классе технических устройств, о репринтерах, и способах их использования для производства разнообразной сельскохозяйственной продукции.

ABSTRACT

Modern agriculture expects from the developers of industry technologies effective tools for the production of high-quality products, provided that the new technologies will meet the requirements of environmental protection, easy to operate and will bring tangible profits. The article presents the results of the innovative technology application based on a fundamentally new approach – information control of the physiology of a living organism. The article describes a new class of technical devices - reprinters, and methods of their use for the production of various agricultural products.

Ключевые слова Инновационные информационные технологии, информационный перенос, гомеопатия, репринтер, матрицы биологически активных веществ, растениеводство, животноводство.

Keywords Innovative information technology, information transfer, homeopathy, reprinter, matrix of the biologically active substances, plant growing, animal husbandry.

Современное сельское хозяйство может по праву считаться стратегической отраслью экономики любого государства. Высокий уровень развития отрасли, ее способность обеспечивать собственное население продуктами питания, создает условия для устойчивого развития нации и гарантирует ее продовольственную безопасность.

В современном мире характерным признаком развитого сельского хозяйства является высокая концентрация передовых технологий в отрасли, которые и обеспечивают ее эффективность. Разработка инновационных технологий для сельского хозяйства является крайне сложной и наукоемкой задачей. Причина в том, что эти технологии должны быть не только высокопродуктивными, но и экологически дружественными, просты для включения в сложившийся хозяйственный уклад и иметь приемлемые эксплуатационные характеристики.

Инновационная технология, отвечающая всем выше перечисленным требованиям, была создана группой российских ученых, аффилированных с секцией «Ноосферные знания и технологии» Российской Академии Естественных Наук, в первой декаде текущего столетия.

В основе этой технологии лежит универсальное явление природы - способность всех объектов к информационному обмену. Явление это известно давно, хотя его существование не признается представителями ортодоксальной российской и мировой науки. Однако, как бы это не звучало неожиданно, явление информационного переноса успешно используется на протяжении двухсот лет последователями немецкого врача Самуэля Ганемана (Hahnemann), известного как основоположника современной гомеопатии. В своем фундаментальном труде «Органон врачебного искусства», опубликованном в 1810 году, Ганеман описал простую практическую процедуру переноса свойств лекарственных препаратов на обыкновенную воду. Процедура эта получила название потенцирование. В процессе потенцирования гомеопатических препаратов происходит одновременный перенос свойств биологически активных веществ (БАВ) на воду и понижение концентрации самого вещества в растворе за счет его пошагового разведения. В практике подготовки гомеопатических препаратов используются как десятичные, так и сотенные разведения. Легко видеть, что если суммарная степень разведения молярной массы БАВ больше степени числа Авогадро, то в таком растворе вероятность присутствия даже одной молекулы БАВ практически равна нулю. А современная гомеопатия с успехом использует препараты, имеющие тридцатую, шестидесятую и более высокие степени разведения. При этом оказывается, что влияние гомеопатических препаратов с высокими потенциями на состояние физиологии человека особенно эффективно. Вода, прошедшая процедуру потенцирования становится информационным носителем свойств БАВ. Ее принято называть активированной и, как правило, она доставляется в организм пациента в контейнере, известном под названием «сахарная крупка». Каждая гранула гомеопатического препарата несет в себе несколько микро капель активированной свойствами БАВ воды, что оказывается вполне достаточным для требуемого воздействия на больного и запуска процессов корректировки его физиологического состояния.

Предложенная Ганеманом процедура потенцирования представляет собой ручную и достаточно трудоемкую технологию переноса информации о свойствах БАВ на воду. По этой причине небольшого объема активированной воды, который производится гомеопатами, достаточно лишь для обеспечения потребностей собственной медицинской практики. Из-за принципиальных ограничений по объему подготовки активированной воды, за все двести лет существования гомеопатии, как самостоятельного направления в медицине, вопрос о возможности применения информационного подхода к управлению физиологическими процессами других живых организмов (например, сельскохозяйственных растений и животных) даже не поднимался. И только в последнее время с созданием технических устройств, получивших название репринтеры, стало возможным использовать методологию информационного переноса для управления физиологией любого без исключения живого организма.

Разработанное российскими специалистами и запатентованное на территории РФ устройство «Акватор» (патент № 2297392) является высокопродуктивным репринтером, способным производить до 10 тонн активированной воды в час. В процессе активации воды непосредственно используется само устройство (рисунок 1), а так же матрица БАВ (рисунок 2). Матрица представляет собой контейнер с нейтральным веществом, на которое предварительно «записаны» свойства БАВ. Процесс активации воды происходит бесконтактным способом (рисунок 3), а полученная таким образом вода становится носителем свойств БАВ и в дальнейшем используется для полива растений или выпаивания животных. Результаты, полученные при применении технологии «Акватор», убедительно доказывают, что информационное воздействие такой водой на объекты сельскохозяйственного производства приводит к существенным изменениям продуктивности и качества продукции [1-5].

Рис. 1 Промышленное устройство «Акватор»

Рис. 2 Твердофазные матрицы препаратов

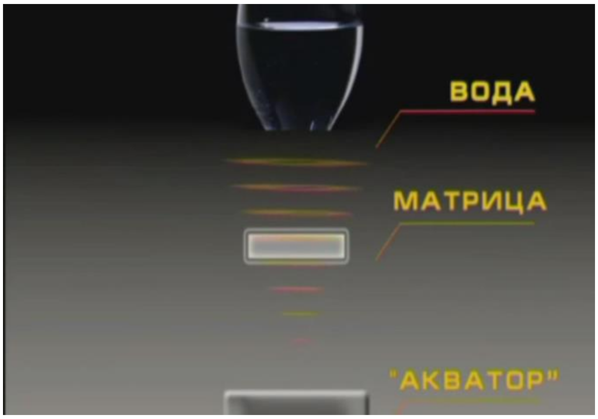

Рис. 3 Схема бесконтактной обработки воды устройством «Акватор»

Устройство «Акватор» может применяться как самостоятельно, так и в составе модуля. Модуль включает в себя проточно-накопительную емкость, само устройство и матрицу биологически активного вещества. На рисунках 4-5 представлены варианты исполнения модуля, предназначенные для решения различных сельскохозяйственных задач.

Рис. 4 Модуль «Акватор», используемый на свиноферме компании ALZU Квагафонтеин, Миделбург, провинция Мпумаланга, ЮАР

в основании кейс с устройством «Акватор» и матрицей,

сверху, проточно-накопительная емкость, встроенная в систему подачи воды

Рис. 5 Модуль «Акватор»,

используемый на птицеферме в области Зала в Венгрии

В различных областях сельского хозяйства, как растениеводства, так и животноводства, технология «Акватор» используется уже более десяти лет.

ПРИМЕНЕНИЕ ТЕХНОЛОГИИ В РАСТЕНИЕВОДСТВЕ

В Кубанском государственном аграрном университете КубГАУ (Россия, г. Краснодар) в учебно-опытном хозяйстве «Кубань» на винограднике технического сорта Бианка изучалось влияние активированной воды со свойствами биологически активных веществ Росток и Кремний. Применение активированной воды осуществлялось четыре раза за сезон в виде некорневой обработки кустов по фазам вегетации: в фазу роста побегов и соцветий, накануне цветения винограда, роста ягод, в начале созревания ягод винограда. Сравнительные данные показателей урожая при использовании растворов препаратов и активированной воды по годам приведены в таблицах 1-3.

Таблица 1 – Влияние некорневой обработки кустов биологически активными веществами и активированной водой на урожай и качество винограда сорта Бианка 2007г.

Препарат	Урожай		Средняя масса грозди, г	Масса 100 ягод, г	Сахаристость сока ягод, г/100см^3
	ц/га	Процент к контролю			
0.1% раствор Росток	108,0	151,6	104,1	135,9	24,2
вода, активированная матрицей Росток	122,2	171,6	128,4	148,2	25,6
0.1% раствор Кремний	116,6	163,8	88,7	136,4	25,5
вода, активированная матрицей Кремний	120,9	169,8	101,0	144,8	25,6
контроль - простая вода	71,2	100,0	89,5	144,3	24,8

Таблица 2 – Влияние некорневой обработки кустов биологически активными веществами и активированной водой на урожай и качество винограда сорта Бианка 2008г.

Препарат	Урожай		Средняя масса грозди, г	Масса 100 ягод, г	Сахаристость сока ягод, г/100см^3
	ц/га	Процент к контролю			
0.1% раствор Росток	96,2	102,3	100,7	158,3	22,8
вода, активированная матрицей Росток	112,0	119,1	106,4	168,1	23,6
0.1% раствор Кремний	94,7	100,7	73,0	122,0	24,7
вода, активированная матрицей Кремний	131,7	140,1	75,1	148,9	23,4
контроль - простая вода	94,0	100,0	76,3	141,7	23,8

Таблица 3 – Влияние некорневой обработки кустов биологически активными веществами и активированной водой на урожай и качество винограда сорта Бианка 2009г.

Препарат	Урожай		Средняя масса грозди, г	Масса 100 ягод, г	Сахаристость сока ягод, г/100см^3
	ц/га	Процент к контролю			
0.1% раствор Росток	147,0	151,9	103	156	18,6
вода, активированная матрицей Росток	176,1	181,9	122	95	21,2
0.1% раствор Кремний	158,9	164,2	98	154	18,6
вода, активированная матрицей Кремний	188,4	194,6	109	162	18,6
контроль - простая вода	96,8	100,0	99	130	21,8

Там же в КубГАУ проводились испытания по применению технологии «Акватор» при выращивании озимого короткодневного сорта лука селекции КубГАУ Эллан. Данные по урожаю, полученному с использованием активированной воды на матрицах БАВ Радикс и Радифарм, приведены в таблице 4.

Таблица 4 - Урожайность лука-репки озимого сорта Эллан при обработке матрицами стимуляторов роста Радикс и Радифарм

Вариант	Урожайность луковиц					
	общая		стандартных		товарных	
	т/га	Процент к контролю	т/га	Процент к общей	т/га	Процент к общей
контроль – простая вода	19,8	100,0	16,0	80,8	17,3	87,4
вода, активированная матрицей Радикс	27,1	136,9	24,6	90,9	25,1	92,7
вода, активированная матрицей Радифарм	26,5	133,8	22,0	83,0	22,6	85,3

ПРИМЕНЕНИЕ ТЕХНОЛОГИИ В ЖИВОТНОВОДСТВЕ

В 2015 году на одной из птицеферм, расположенных в области Зала в Венгрии, проводился откорм бройлеров с применением технологии «Акватор». Предприятие включало восемь птичников, каждый из которых имел площадь порядка 1100 кв. м и вмещал около 20 тысяч кур. Для испытания технологии были привлечены два отделения — тестовый и контрольный. Помещения заселялись бройлерами породы Ross-308 с племенным индексом 241069. Птица содержалась напольным способом. Ее откорм начинался с первого дня поступления цыплят из инкубатора и продолжался примерно 40 дней. Условия нахождения, состав и качество корма, санитарно-гигиенические мероприятия в обоих птичниках были идентичными. Различие состояло лишь в том, что в опытный цех подавалась вода, прошедшая обработку на репринтере. Биотехнология использовалась в промышленном варианте с применением комбинации матриц. Одна из них обладала ярко выраженными иммуно-протекторными свойствами, а две другие были носителями характеристик стимуляторов роста птицы. Испытания показали, что бройлеры опытной группы превзошли кур из контрольного птичника по всем показателям, входящим в европейский бройлерный индекс (среднесуточный привес, коэффициент выживаемости и конверсия корма). В результате в тестовой группе Европейский Бройлерный Индекс составил 346 единиц, а в контрольной 300 – величина близкая к среднестатистической по предприятию [4].

В 2015 году на свиноферме (Квагафонтеин, Миделбург, провинция Мпумаланга, ЮАР) компании ALZU проводился откорм свиней с использованием технологии «Акватор». Применение активированной воды, полученной с матрицы соматотропного гормона (гормона роста), осуществлялось в период с 56 по 112 день жизни животных. Обработка воды проводилась на модуле, встроенном в систему подачи воды в свинарник (Рисунок 4). В результате к окончанию откорма средняя масса тестовых животных составила 59.4 кг, в то время как средний показатель по ферме в различные сезоны года колебался в районе 50 кг.

Весной 2018 года на базе одного из фермерских хозяйств (Россия, Краснодарский край, Северский район) был проведен экспериментальный откорм кроликов. Для этих целей были отобраны две группы животных в возрасте 2 месяцев. Каждая группа состояла из 8 особей породы «Белый великан» и 8 особей породы «Советская шиншилла» по четыре самца и четыре самки в каждой (всего 16 особей). Обе группы были привиты вакцинами от миксоматоза и геморрагической лихорадки. Одна группа считалась контрольной, другая — экспериментальной. Период откорма составил два месяца — с 3 февраля по 3 апреля.

Кормление производилось сеном люцерны и комбикормом для кроликов компании «ПРОВИМИ», а также зернопродуктами, травяной мукой, жомом свекловичным, шротом зерновым, витаминно-минеральным премиксом. Еще использовался известняк, масло растительное, соль, фосфаты, аминокислоты, пробиотик, антиоксидант.

Поение животных осуществлялось водой из скважины, забор с нижнего горизонта — 50 метров. Вода имеет санитарный сертификат.

В этот период экспериментальная группа животных получала воду, обработанную устройством «Акватор» через матрицу соматотропного гормона с добавлением в микродозах пептидного препарата гидролизата цельной крови компании «Биоэрагрупп».

Убой животных производился в возрасте четырех месяцев. Контрольное взвешивание тушек показало, что в экспериментальной группе имеется устойчивое превышение веса над показателями контрольной группы. Средний вес тушки контрольной группы составил 2,08 кг, тогда как средний вес тушки в экспериментальной группе достиг 2,3 кг, (более чем на 10% выше контроля), что говорит об устойчивости эффекта воздействия специально подготовленной воды на организм животного.

Применение воды, полученной с матрицы эритропоэтина, привело к еще одному эффекту. При поении крольчихи в период беременности активированной водой у нее отмечалось сокращение срока вынашивания плода, в некоторых случаях до трех дней [5].

ВЫВОДЫ

Технология «Акватор» является универсальной и может применяться практически в любой отрасли сельского хозяйства. Ее внедрение не требует дополнительных затрат и не нарушает сложившихся методов хозяйствования. К тому же технология является абсолютно экологически чистой и высокоэффективной, приносящей производителям сельскохозяйственной продукции ощутимую дополнительную прибыль.

Возможности технологии и результаты ее применения были отмечены дипломом ежегодного конкурса «EcoWorld», проводимого Российской Академией Естественных Наук [6]

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MICROPLASTICS AS ENVIRONMENTAL THREAT

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ABSTRACT

The reason for initiating this topic is the fact that the presence of microplastic in surface waters has become an alarming global problem. Except in water, microplastics can be found in sediment and soil, but also in groundwaters, bottled waters, sea salt, food from the sea, other foods (for example, honey) and drinks (for example, beer), air, etc. Although both national and European regulations do not prescribe determination of microplastics in wastewaters discharged into the environment, as well as in drinking water, food and beverages, it is necessary to develop methods for their sampling and analysis. Considering the prevalence of the problem it can be expect this item to be entered into the relevant regulations. In addition, the European Marine Strategy Directive requires EU Member States to ensure that by 2020 "the characteristics and quantities of waste in the sea do not harm the coastal and marine environment". Preventive action and shared responsibility in combating damage would result in the preservation of biological balance. Regardless the regulations, it is desirable to know the extent of load of sea water and individual surface waters by microplastic.

Key words: *Microplastic, Microbeads, Natural waters*

INTRODUCTION

The production of plastic in the world has increased about 20 times, in the last few decades. Although the most of the used plastic is trying to recycle, a significant part gets to the environment. World production of plastic surpassed the 320 million tons mark in the 2016. Also, it is estimated that between 5 and 13 million tons of plastic waste leaks into the World´s oceans every year as a result of inappropriately dumped and mismanaged [1]. Plastic products are produced to be long lasting and therefore pose a long lasting environmental hazard, which has been growing over the years. When they get into the water, due to a different effects (wind, rain, UV light, mechanical action), the plastic objects are braking to the micro sizes of so-called microplastics (secondary microplastics). In addition, plastic origins from personal hygiene products (toothpastes, washing agents, roll-on deodorants, exfoliating body washes, facial scrubs etc.) gets into the wastewaters as micro-size balls of plastic so-called microbeads (primary microplastics).

Microbeads are tiny polyethylene spheres which, contrary to microplastics, do not come from the degradation of large pieces of plastic. Instead, they are manufactured on purpose to be put into consumer products. These tiny plastic microbeads are usually less than 1 millimetre at their largest [2]. Due to their small size as well as small density, they can pass through the wastewater treatment plant and end up in the water in nature. Therefore, their use in the United States, Canada and the United Kingdom is already prohibited, and a ban is also expected in the European Union. However, the risk of microplastic originating from larger plastic objects is and will remain a big problem for a long time.

There are many different types of plastics that can commonly be found in everyday objects (e.g plastic bottles and plastic bags, CDs, umbrellas, coats etc.). These include polyethylene (PE), polystyrene (PS), polypropylene (PP), nylon, polyvinyle chloride (PVC) etc.

SOURCES OF MICROPLASTICS

Sources of microplastics are widespread. It originates from diverse sources and can be broadly classified into land- and sea-based sources. They include: agricultural runoff, urban runoff, stormwater, aquaculture, textiles, tyres, paints, cruise ships, ocean dumping, etc.

The land-based sources (Fig. 1) are considered to be the dominant input of microplastics into oceans, which can be of both primary and secondary origins [3]. A common source of microplastics in the oceans is clothing made from synthetic fibres. Tiny fibres of polyester and acrylic (both a type of plastic) are shed when clothing is washed [4]. When we wear or wash any clothing made from synthetic material, small cloth fibres are rubbed and come apart from the larger piece of clothing. These small pieces of synthetic fibres are known as microfibres. When microfibres rub off your clothing during normal use, they can end up in the air we breathe. And when washed, synthetic clothing will shed small plastic particles, which end up in your home wastewater system and eventually make it to the ocean [5]. These are worse than the relatively spherical microplastics because they have a larger surface area.

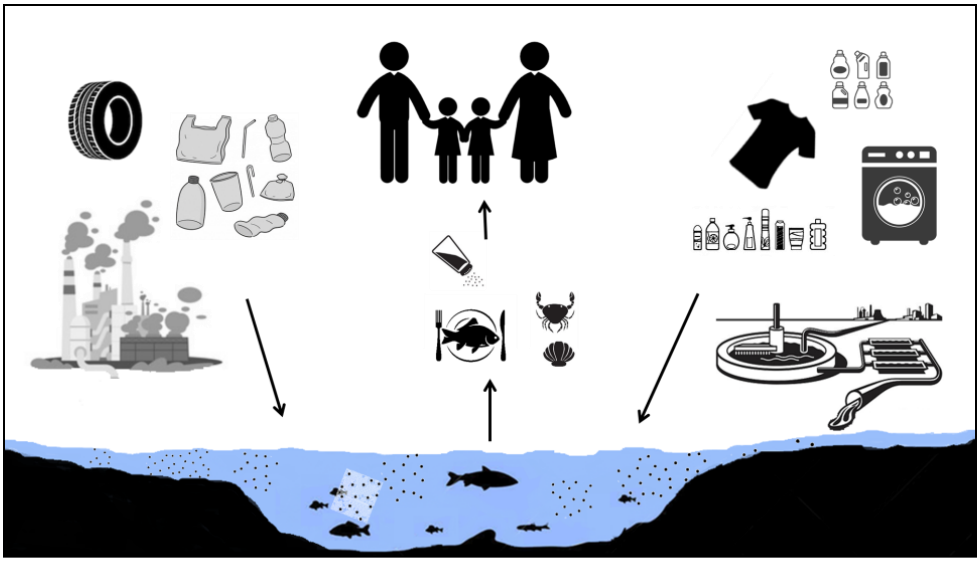

Figure 1. Land-based sources of microplastics

Terrestrial emissions are the dominant source of microplastics. Microplastics originate predominantly from automobile tire wear, household and laundry dust, industrial processes (e.g., blasting and deflashing of plastics), and through deterioration of surfaces made of or coated with plastic, for example, artificial turf and polymeric paint, etc. Most of these emissions occur in urban and residential areas [6]. About 30 vol% of the microplastic particles that pollute rivers, lakes and oceans consist of tire wear [7; 8; 9; 10]. Also, microplastics can come from road-surface marking paints [11].

Sea-based sources of microplastics are attributed to the fishing, shipping, and offshore industry sectors. It is estimated that there were about 4.7 million fishing vessels globally in 2012. Lost, abandoned or worn-out of fishing and aquaculture gears and other equipment during use can easily result in the introduction of microplastic particles in the marine environment, which continue to entangle or kill marine life and damages habitats, ultimately impacting fish stocks and quality. Besides, operational and accidental spills from large shipping vessels and cargo are also considered as significant source of resin pellets. Furthermore, microplastics of various polymers including PE, polypropylene, and PS in the atmospheric fallout have been first detected due to inadequate attention to sources from dust emission and deposition [3].

Among mentioned sources, wastewater treatment plants are one of the dominant sources of microplastics. In his work Li et al. [12] lists the key results from the microplastics studies on several wastewater treatment plants. Althou the results showed that removal efficiency of microplastics is high (90 – 95%), there is substantial amount of microplastic being discharged into natural waters through wastewater treatment plants.

EFFECTS OF MICROPLASTICS ON ENVIRONMENT AND HUMAN HEALTH

Variety of animals living in the water adopt microplastic as food, which due to stigmatization in the digestive tract, can lead to their death. In addition, recent researches suggests that plastics can accumulate organic pollutants such as carcinogenic polychlorinated biphenyls, polycyclic aromatic hydrocarbons (PAHs) and organochloric pesticides (DDD – dichloro-diphenyl-dichloroethane and DDT – dichloro-diphenyl-trichloroethane). Based on the literature data, it is believed that the remains of plastics can attract and concentrate up to one million times more organic pollutants compared to their amount in seawater, and when marine animals enter them into the body they endanger not only them but also human health.

Ingestion of microplastics by marine organisms in most cases is accidental because the particle is often mistaken for food [13]. Microplastics ingested by marine organisms can cause chemical and physical harm (hindering mobility and clogging of the digestive tract, inflammation, hepatic stress, decreased growth). The consuption of microplastics is common to a wide range of marine organisms, such as invertebrates, mussels, barnacle, sea cucumbers, zooplankton and fishes. Also, turtles, fish-eating birds and mammals can consum microplastics, which can interfere with the food chain as microplastics ingested by organisms in the lower trophic level could pass up the food chain when lower trophic organisms are fed upon by organisms in the higher trophic level [14].

Based on information found in the website [15], the results of the new research have shown that microplastics can be transferred from contaminated water through flying insects and pollute the environment. Also, this newly discovered phenomenon poses a risk to insect-feeding animals. Scientists have fed mosquito larvae with microplastics and have found that plastics remain in their organisms even when they transform into adult individuals. A new study, published in the Biology Letters, suggests that many larvae of mosquitoes in their diet include microplastics. They can not distinguish plastic from food pieces because the algae they use in diet are the same size as microplastic particles. Scientists are currently focusing on the damage that these plastic particles make to mosquitoes. Therefore, it can be assumed that other flying insects that start their life in the larval stage in water also consume microplastics.

According to the same source [15] it can be concluded that birds, bats and spiders are just some of the animals that eat enormous amounts of insects every day, which means they eat large amounts of microplastics. Plastics have been discovered inside the stomachs of many seabirds, but land birds are also endangered and we still do not know what the consequences will be.

The presence of microplastics in the sea could lead to contamination of sea products that humans use as food. One of such product is sea salt. Study conducted by Yang et al. [16] showed that 7 – 240 particles/kg, 550 – 681 particles/kg and 43 – 364 particles/kg has been found in 15 brand of rock/well salts, sea salt and lake salt, respectively.

These particles have also been reported in drinking water. Eerkes-Medrano et al. [17] reviewed studies about findings of microplastics in drinking water (bottled water and drinking water treatment plants). This work presented studies that showed that in the bottled water PET was among the most dominant polymer detected. Also, studies about investigating the presence of microplastics in water samples from drinking water treatment plants (DWTP) were reported. According to the results obtained from different DWTPs it can be found that particles of plyester, polyvinyl chloride, polyethylene, polyamide, epoxy resin, as well as polypropylene polymers appeared in water samples.

Presence of microplastics in food for human consumption and in air samples has been reported. Thus, microplastics exposure via diet or inhalation could occur, and cause the effects on human health. In addition to seafood, potential microplastics have been reported in other foods. The presence of synthetic microfibers and fragments was reported in honey and sugar. Also, the contamination of beer by potential microplastics has been reported [18]. In the same work, potential human health risks of microplasstics were reviewed. For example, studies among nylon flock workers and workers in textil industry were conducted. Interstitial lung disease, a work-related condition that induces coughing, dyspnea (breathlessness), and reduced lung capacity, has been identified in 4% of workers from nylon flock plants. Histopathological analysis of lung biopsies from workers in the textile (nylon, polyester, polyolefin, and acrylic) industry showed interstitial fibrosis and foreign-body-containing granulomatous lesions, postulated to be acrylic, polyester, and/or nylon dust.

According to the presented results Wright and Kelly [18] it can be concluded that, if inhaled or ingested, microplastics may accumulate and exert localized particle toxicity by inducing or enhancing an immune response. Chemical toxicity could occur due to the localized leaching of component monomers, endogenous additives, and adsorbed environmental pollutants. Chronic exposure is anticipated to be of greater concern due to the accumulative effect that could occur.

Therefore, it is clear that it is necessary to take all necessary measures to minimize damaging impact of microplastics on the environment, human and animal health.

WHAT ARE THE SOLUTIONS?

- Education and raising awareness about harmfull effects of microplastics on the environment
- Good management of plastic waste (e.g. recycling)
- Reduce the use of plastic packaging
- Improve wastewater treatment processes (for example usage of microorganisms that are able to degrade microplastics)
- Developing and improving of methods for microplastics detection and analysis

These are things to think about when it comes to the use of plastic and its impact on the environment. First of all, this refers to education, primarily children and young people, in order to raise awareness about the importance of this problem.

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PLANT PRODUCTION

THE REGRESSION AND CORRELATION AMONG PH AND READILY AVAILABLE POTASSIUM IN THE SOIL

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ABSTRACT: Potassium (K) belongs to the macro-regulatory elements in the soil and is not a part of plant-derived organic compounds. Potassium affects the water regime of plants, stimulates the growth of young tissues, and activates enzymes, which increases the resistance of plants to diseases and lodging. Too much potassium inhibits the assimilation of calcium, magnesium and nitrogen. The solubility of many compounds that plants need depends on the soil solution reaction (pH). Using regression and correlation, we analyze the connection (dependency, association, ratio) of two or more variables. The research was held in the Republic of Serbia, in the municipality of Aleksinac, for two consecutive years (2015, 2016). One thousand parcels were monitored, out of which the first 500 were under wheat and the other 500 under corn. The pH was determined as specified by the method of potentiometric determination in a suspension of 1N KCl, while the phosphorus content was calculated using the AL method. A statistically significant positive correlation between these parameters was found in both years (in 2015, $R = 0.310$, sig < 0.001; in 2016, $R=0.238$, sig < 0.001). A regression analysis of the data on both experimental years showed that a model with a statistically significant predictive power was obtained (sig $<$ 0.001).

Keywords: potassium, pH, correlation, regression, soil

INTRODUCTION

Potassium increases the osmotic pressure and regulates water in plants. It affects the assimilation of water from the soil, assimilating it to longer distances in plants via xylem and phloem. Potassium is a mobile element; its deficiency causes necrosis, which appears at the edges of lower leaves. There is more potassium in the vegetative parts of plants than in the reproductive parts. Potassium deficiency occurs usually during the rapid growth of fruits. However, potassium should not be added in excessive amounts, because it is an antagonist to Mg, Ca and N. High potassium reduces yields and the profitability of production. Nitrogen deficiency leads to high potassium. Too much sodium leads to potassium deficiency, and large amounts of potassium can alleviate the toxic effects of ammonium (KASTORI *et al*., 2013; EL OMARI, 2018). Humus is also a very important soil matter, due to its ability to bind large amounts of water and nutrients, which are easily available for plants (GRČAK *et al.*, 2017).

Correcting the soil pH is essential because it ensures optimal plant growth and higher yields, since it allows plants to use nutrients from the soil. Knowing the soil pH helps to make the right choice of cultures to be planted (sown). By determining the pH, we obtain information on what and how much of it should be added to the soil (BARROW, 2016). Macroelements (N, P, K, S, Mg and Ca) are best assimilated at a pH range from 6-7, while microelements prefer a more acidic environment with pH 4.5-5.5, with the exception of molybdenum, for which a neutral acid is more suitable (GRČAK *et al*., 2018).

If there is not enough time and capacity to carry out a pH analysis in the laboratory, it is possible to perform a soil analysis using two glasses or plastic cups, vinegar and sodium bicarbonate. The soil sample is uniformly distributed in two glasses or cups. Vinegar is added to one glass or cup, while sodium bicarbonate is added to the other one. If there is rustling and foaming in the glass or cup with vinegar, it means that the soil is alkaline. If the soil is acidic, there will be a reaction with sodium bicarbonate, which is a clear indication that the soil is acidic. If there is a very weak reaction or no reaction whatsoever, it means that the soil has a neutral reaction (RADULOVIĆ, 2017).

The distribution of potassium ("surface fertilization without ridging," "surface fertilization with ridging" and "meliorative fertilization") is much less dependent on the manner of fertilizer application, in contrast to phosphorus, where the satisfactory distribution of K is up to a depth of 30 cm (RADANOVIĆ, 2016)

Correlation is the interrelationship between various phenomena that are represented by values in two or more random variables. In addition, the connection means that, based on the knowledge of the value of one variable, with a certain probability, it is possible to predict the value of the other variable, since these values occur in a particular ratio (PALLANT, 2007).

Soil reaction (pH) is by definition a negative decimal logarithm of the concentration of hydrogen ions (H^+) and tells us about the degree of soil acidity. Soil reaction, i.e. soil pH, influences plants: directly (influence on the cell sap pH) and indirectly (influence on the accessibility of nutrients for plants, the activity and composition of microorganisms in the soil) (MARINKOVIĆ, 2016).

Changes in soil reaction (pH) may be conditioned by natural (alternation of phytocoenoses) and anthropogenic factors (HUE, 1992). The pH varies in the same soil depending on the time of year, thus, it is lower during summer when microbiological processes are greater, and higher during winter when microbiological and chemical processes are minimal. Potassium deficiency usually occurs in sandy, lime and clay soils (VARGA, 2015).

Potassium deficiency affects the structure of the tree trunk and roots. The first symptoms of K deficiency appear on the oldest leaves. The tree trunk is shorter and thinner, and the roots are shorter, poorly branched and have a smaller number of root hairs. The symptoms that can be observed are in the form of necrosis at the top of leaves and along the edges, the number of necrotic spots that are yellow-dark or dark in color is gradually increasing, and eventually necrosis affects a larger surface of leaves, which in extreme cases can lead to premature leaf falling (VARGA, 2015).

In a high concentration of hydrogen ions (pH 3 to 3.5), the release of potassium ions is two times higher than in soil reaction ranging from 4.5 to 9.0 (KASTORI *et al.*, 2013). Readily available (available to plants) potassium (K_2O) is its water-soluble and changeably sorbed form (ĐURĐEVIĆ, 2014).

A report on the soil status in the Republic of Serbia (Izveštaj o stanju zemljišta u Republici Srbiji, 2009) published data on the basic parameters of soil fertility, and some of them are: pH in N KCl soil, the amount of humus, the abundance of readily available forms of potassium and phosphorus, CaCO3. In Serbia, acid soils are dominant; of the total number of samples tested, there are 18% of soils with an extreme acid reaction (pH<4.0), 17% of soils with a strong acid reaction (pH 4.0-4.5), 30% of soils with a moderate acid reaction (pH 4.5-5.5), 22% of soils with a weak acid reaction (pH 5.5-6.5), and 18% of soils with neutral and alkaline reactions. Acid soils represent more than 60% of Republic Serbia, out of which about 30% is extremely acidic (JELIĆ *et al.*, 2015).

At pH 7.00 (neutral reaction), the utilization of nitrogen is 70%, of phosphorous 30%, and of potassium 60%. A decline in pH to 6.00 changes the utilization of nitrogen to 63% and of phosphorus to 15%, while the utilization of potassium remains the same as at neutral pH – 7.0, i.e. it is 60%. However, at pH 5.0 (acidic soil), the usability of all nutrients is much lower, about 40% for nitrogen, 10% for phosphorous, and 30% for potassium (BOROJEVIĆ, 2016).

There is approximately 0.2-3% of the potassium in the soil. Clay soils have a higher K content, but the organic reserves of potassium are very small. Of the total amount of potassium in the soil, only a small part is available for plant nutrition. Only mobile (movable) potassium is available to plants, while fixed potassium is unavailable to plants (McLEAN and WATSON, 1985). After liming the pseudogley soil, the content of readily available phosphorus was increased by 4.3 mg 100-1 g of soil in relation to the control variate, while the content of potassium did not change significantly (JERINIĆ *et al.*, 2015). Liming the pseudogley soil for a period of three years resulted in a decrease in soil acidity and an increased content of readily available phosphorus compared to the control variate, while the potassium content did not change significantly (JERINIĆ *et al.*, 2015).

High alkalinity is not recommended, since it blocks a large number of trace elements, and accelerates the mineralization of organic matter (STEFANOVIĆ, 2019).

Acid soils contain high levels of H, Al, Fe and Mn ions, while Ca, Mg, P are either missing or are in an insufficiently accessible form in these soils. For example, fruit species are more successfully grown in soils that have a weak acid reaction or a slightly alkaline reaction. A low pH may be a natural feature of the soil or a consequence of a continuous application of acidic nitrogen fertilizers. Based on the data from literature and practical experience, the optimal level of readily available phosphorus in fruit growing is about 15 mg P_2O_5 per 100 mg of soil, or 25 mg K_2O/100 g of soil (GUDŽIĆ, 2015). The physiological role of potassium is the neutralization of organic acids formed in the process of metabolism; it participates in the synthesis, degradation and transfer of carbohydrates from leaves to roots (sugar beet) or fruits (fruit), has an effect on the osmotic pressure by reducing transpiration, which is important in drought periods. It would be good to reduce the potassium content to an extent where this element becomes limiting (restrictive) for achieving high yields (RÖMHELD and KIRKBY, 2010). The influence of potassium fertilizers (KCl and K_2SO_4) leads to physiologically acidic fertilizers, and plants assimilate potassium more intensely than the accompanying anion, which, with a hydrogen ion, creates acid, resulting in an increased acidity of the soil. Potassium chloride (KCl) is the most commonly used mineral potassium fertilizer in Serbia for economic reasons (KASTORI *et al*., 2013). FINCK (1982) states that the influence of the application of potassium fertilizers on soil reaction is of little significance, and that a reduction in soil pH upon application of these fertilizers is usually not observed, except for a slight increase in the loss of calcium. The soils in Serbia are more abundant with potassium than with other elements necessary for plant nutrition. Therefore, it is recommended that the future research pays more attention to studying the mobilization capacity of the soil regarding potassium, that is, the use of its reserves in the soil for meeting the needs of cultivated plants for this element (JAKOVLJEVIĆ *et al.,* 1983). There is a correlation between an increase in pH and activities of microorganisms, as well as an increase in the humus content and activities of microorganisms. With an increase in micro-organisms, the mineralization of nitrogen is higher, and thus, the total amount of N that is returned to the soil is lower (KOMLJENOVIĆ and KONDIĆ, 2011).

MATERIAL AND METHODS

The objective of the research and paper was to establish a correlation between soil pH and readily available potassium in the soil. The research lasted from 2015 to 2016 (two years). We chose 1000 plots to examine in the municipality of Aleksinac, in the Republic of Serbia. The territory of the municipality of Aleksinac is located in the southeastern part of the Republic of Serbia, between 43°27' and 43°44' of the north latitude and 21°29' and 21°56' of the east longitude. The largest number of areas in this district are under corn and wheat. Therefore, we chose plots under these cultures for our research. Of the total number of plots tested, 500 plots under wheat and 500 plots under corn were analyzed. The plots used for the research were generally those where these two cultures alternated year after year. The samples were collected from the plots on the basis of the guidelines for soil sampling prescribed by the Institute of Field and Vegetable Crops in Novi Sad (Institut za ratarstvo i povrtarstvo, 2006) and on the basis of the Agrochemistry Practicum (GUDŽIĆ, 2011). The depth from which samples were taken was up to 30 cm. A chemical analysis of the soil was performed in a laboratory of the Agricultural Extension and Advisory Service in Nis.

After the analyses accomplished, statistical processing of the obtained data was done in IBM SPSS Statistics 20 (trial version).

Soil pH is a measure of acidity and alkalinity in the soil. The optimum pH range for most plants is between 5.5 and 7.0. The pH level controls many chemical processes taking place in the soil, such as, the availability of nutrients (McCAULEY *et al*., 2009).

Table 1. Soil classification as regards the readily available potassium supply (Jelić, 2012)

Soil classes	**Limit values of mg K_2O $100g^{-1}$ of the soil**
Abundant (rich) soil	> 20
Moderately abundant soil	10 – 20
Poor soil	< 10

The principle of determining readily available potassium in the soil by applying the AL-method (Egner-Riehm) is based on the extraction of readily available potassium using the AL solution (the AL solution consists of 0.1 N ammonium lactate and 0.4 N glacial acetic acid), is expressed in mg K_2O 100 g^{-1} (K_2O/100 g) in the air-dry soil. The potassium from the extract is determined by flame photometry (GRČAK *et al*., 2018; HENDERSHOT *et al*., 1993). In order to determine pH, it is necessary to sieve the soil through a sieve whose pore diameter is 2 mm for available forms of phosphorus and 1 mm for available forms of potassium (PREDIĆ, 2011).

The total content of potassium in the soil varies within wide limits and typically ranges from 1% to 3.5%. Potassium that is readily available for plants is in the form of K^+ cations that is adsorbed on adsorption complex of the soil or is in the soil solution in the form of highly soluble salts. The concentration of available forms of potassium in the soil is expressed in mg K_2O/100 g of the soil (PREDIĆ, 2011).

RESULTS AND DISCUSSION

As part of the correlation analysis, the Pearson correlation coefficient between the pH determined in KCl and K_2O was calculated. The analysis of 2015 data (Table 2) found that there was a statistically significant positive correlation between these parameters ($R=0.310$, $sig < 0.001$).

Table 2. Pearson correlation performed for the samples from 2015

Correlations

		pH in KCl	K_2O in 100g (a.d.s.)
pH in KCl	Pearson Correlation	1	,310**
	Sig. (2-tailed)		,000
	N	1000	1000
K_2O in 100g a.d.s.	Pearson Correlation	,310**	1
	Sig. (2-tailed)	,000	
	N	1000	1000

** Correlation is significant at the 0.01 level (2-tailed)
air dry soil – a.d.s.

Using the same method, we performed a statistical analysis of 2016 samples (Table 3). The correlation analysis showed that there was a statistically significant positive correlation between these parameters (R=0.238, sig < 0.001).

Table 3. Pearson correlation performed for the 2016 samples

Correlations

		pH in KCl	K_2O in 100g a.d.s.
pH in KCl	Pearson Correlation	1	,238**
	Sig. (2-tailed)		,000
	N	1000	1000
K_2O in 100g a.d.s..	Pearson Correlation	,238**	1
	Sig. (2-tailed)	,000	
	N	1000	1000

**. Correlation is significant at the 0.01 level (2-tailed).

After the Pearson correlation results showed a statistically significant correlation, we decided to perform a prediction model. In order to predict K_2O based on the pH, a regression analysis of data was done.

By the regression analysis of 2015 data, we obtained a predictive model with statistically significant predictive power ($F = 106.192$; sig < 0.001), which explained 9.6% (R square $= 0.096$) of the dependent variable, in this case the amount of readily accessible phosphorus (Table 4).
The predictive model is (Table 5): $K_2O = 5.867\, pH - 10.233$

Table 4. Evaluation of the model for the 2015 data

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	,310[a]	,096	,095	9,03711

a. Predictors: (Constant), pH in KCl
b. Dependent Variable: K_2O in 100g a.d.s.

Table 5. Coefficients for data from 2015.

Coefficients[a]

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95,0% Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
(Constant)	-10,233	3,567		-2,869	,004	-17,232	-3,234
pH in KCl	5,867	,569	,310	10,305	,000	4,749	6,984

a. Dependent Variable: K_2O in 100g a.d.s.

By the regression analysis of 2016 data, we obtained a predictive model with statistically significant predictive power (F = 106.192; sig < 0.001), which explained 5.70% (R square = 0.057) of the dependent variable (Table 6).
The predictive model is (Table 7): $K_2O = 5.171\ pH - 5.815$

Table 6. Evaluation of the model for the 2016 data

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	,238[a]	,057	,056	9,60989

a. Predictors: (Constant), pH in KCl
b. Dependent Variable: K_2O in 100g a.d.s.

Table 7. Coefficients for data from 2016

Coefficients[a]

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95,0% Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
(Constant)	-5,815	4,496		-1,293	,196	-14,638	3,008
pH in KCl	5,171	,667	,238	7,748	,000	3,862	6,481

a. Dependent Variable: K_2O in 100g a.d.s.

CONCLUSION

Potassium is not included in the organic composition of plant compounds, but it does affect a number of vital processes in plants. Chemical properties of the soil are important factors of soil fertility, especially pH, on which the assimilation of nutrients by plants depends. For examined years of research, the regression models i.e. the predictive models, had statistically significant prognosticative power, but the 2016 model pointed out almost two times less variance of the dependent variable with respect to the 2015 model. The conclusion is that sustaining the optimal pH value is essential to the optimum level of potassium in the soil.

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SELECTIVITY AND STABILITY OF HERBICIDES AND HERBICIDE COMBINATIONS BASED ON GRAIN YIELD FROM SORGHUM (*SORGHUM BICOLOR* MOENCH.)

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ABSTRACT

In the period 2012 – 2014 a field experiment was carried out with sorghum hybrid Arcansiel (Sorghum bicolor Moench.). It were tested 4 soil-applied herbicides: Tender EC (S-metholachlor), Silba SC (S-metholachlor + terbuthylazine), Sharpen 33 EC (pendimethalin) and Wing P (dimethenamid + pendimethalin) and 4 foliar-applied herbicides: Casper 55 WG (prosulfuron + dicamba), Cambio SL (bentazone + dicamba), Camix 560 SE (mesotrione + S-metholachlor) and Maton 600 EK (2,4-D ethylhexyl ester). In all variants seeds were treated with the herbicide antidote Concep III (fluxofenin) to protect sorghum from the phytotoxic effect of anti-cereal herbicides. Herbicide combinations Silba + Casper and Wing + Cambio result in obtaining high grain yields of sorghum. High grain yields are also obtained in the herbicide combinations Silba + Cambio, Sharpen + Cambio, Tender + Casper and Wing + Casper. Combining of Camix and Maton with soil herbicides results in lower grain yields. The most unstable are the herbicide Maton and its combinations with the herbicides Tender, Silba, Sharpen and Wing. Technologically the most valuable are the herbicide combinations Wing + Cambio and Silba + Casper. They combine high grain yield with high stability in the years. The single use of the herbicides Camix and Maton gets lower ratings due to their weaker efficacy against weeds and sunflower self-sown plants.

Keywords: *grain sorghum, herbicides, grain yield, selectivity, stability*

INTRODUCTION

Under conditions of limited moisture and insufficient and expensive water for irrigation, growing drought-resistant crops such as sorghum under technologies that help maximize the use of scarce water resources becomes more and more important (Dongiovanni et al., 2000).

The main species weeding crops of sorghum belong to the group of late spring weeds. Of the perennial weeds problematic in sorghum are *Sorgum helepense* Pers., *Cirsium arvense* Scop., *Convolvulus arvensis* L., etc. In the conventional technology for sorghum growing chemical control of cereal weeds is impossible, which limited the dissemination of the crop due to the need for costly vegetation earthling - mechanized and manual (Archangelo et al., 2002; Bibard, 2004; García, 2005; Tsuru et al ., 2005; Vajs, et al. 2007).

The issues in controlling cereal weeds necessitated the introduction of the new technology Concept in sorghum. The Concept technology is based on the use of the herbicide antidote Concep III. It is used for seed treatment to protect sorghum from the phytotoxic effects of the active substance S-metolachlor. This makes it possible control of annual cereal weeds to be conducted by applying the herbicide Dual gold 960 EC in the post-sowing period prior to emergence of the sorghum (Joaquin, 1998; Roy et al., 2002; Mubarak et al., 2006; Abdel-Gadir et al., 2009; Delchev, 2018).

The aim of this study is to examine the selectivity and stability of the manifestations of a group of soil and foliar herbicides and their combinations on sorghum grain grown under the Concep technology under various weather conditions.

MATERIAL AND METHODS

In the period 2012 – 2014 a field experiment was carried out with sorghum hybrid Arcansiel (*Sorghum bicolor* Moench.). The experiment was plotted by the block method, in 4 replications, size of the crop plot 20 m^2, on leached vertisol soil type, after predecessor durum wheat. A total of 25 variants were tested. Active substances and doses of the studied soil and vegetation herbicides are given in Table 1.

In all variants seeds are treated with the herbicidal antidote Concep III to protect sorghum from the phytotoxic effect of anti-cereal herbicides.

Seeds are treated pre-sowing with the growth stimulator Lactisem sorghum at a dose of 3 l/100 kg of seed to overcome the biologically set delay of initial growth. All variants are treated in stage 8 leaf with Lactofol sorghum at a dose 10 l/ha to accelerate the development and bringing flowering before the onset of drought in summer. Preparations are applied at working solution consumption 200 l/ha.

Statistical evaluation to rate the representativeness and reliable effect of the studied parameters has been applied through dispersion analysis and Fischer’s parametric criterion F (Shanin, 1977; Barov, 1982). In the variance analysis the ANOVA123 software is used for calculation (Lidanski, 1988).

The selectivity of herbicides was established through their effect on grain yield and the following variances have been calculated:

Shukla, (1972) stability variance (σ_i^2)

$$Sh-\sigma_i^2 = [1/(e-1)\,(t-1)(t-2)] \times \left[t(t-1)\sum_{j=1}^{s}\left(u_{ij}-\bar{u}_i\right)^2 - \sum_{i=1}^{t}\sum_{j=1}^{s}\left(u_{ij}-\bar{u}_i\right)^2\right],$$

where $u_{ij} = X_{ij} - \bar{X}_{.j}$, $X_{ij} =$ observed trait value of i^{th} cultivar in j^{th} environment, $\bar{X}_{.j}$ = mean of all cultivars in j^{th} environment, $\bar{u}_{i.} = \sum_{j=1}^{m} u_{ij}/e$, e = number of environments, and t = number of cultivars.

In this study, calculation of adjusted stability variance (Sh-Si^2) was necessary, because the heterogeneity term was significant ($P < 0.01$). The stability statistic Sh-Si^2 calculated following removal of heterogeneity due to environmental index ($Z_j = \bar{X}_{.j} - \bar{X}_{..}$) as a covariate from GE interaction variance, where $\bar{X}_{.j}$ = mean of all cultivars in j^{th} environment and $\bar{X}_{..}$ = mean of all cultivars across all environments, using the following equation (Shukla 1972):

$$Sh - S_i^2 = [t/(t-2)(e-2)] \times \left[s_i - \sum_{i=1}^{t} s_i/t(t-1)\right],$$

where $s_i = \sum_{j=1}^{s}\left(u_{ij} - \bar{u}_{i.} - b_i Z_j\right)^2$, and $b_i = \sum_{j=1}^{s}\left[\left(u_{ij} - u_{i.}\right)Z_j\right]/\sum_{j=1}^{s} Z_j^2$.

Cultivar stability across multiple years and locations also was evaluated using the ecovalence (W_i) (Wricke 1962):

$$W_i^2 = \sum_{i=1}^{t}\left(X_{ij} - \bar{X}_{i.} - \bar{X}_{.j} + \bar{X}_{..}\right)^2$$

Greatest stability is when $W = W_i^2 = 0$.

For grain yields stability parameters have been calculated. Stability variances (σi^2 and Si^2) by Shukla (1972) and ecovalence W_i by Wricke (1962) show what portion of variation related to interaction of the preparations and years are accounted by the specific variant.

Through the stability criterion (YS_i) of Kang (1993) the value of each variant has been shown by simultaneous taking into account the parameter value and the stability of the variant. The value of that criterion is that by using non-parametric methods and statistical reliability of differences we obtain a combined valuation ranking variants in a descending order according to their economic value.

To calculate these parameters the STABLE software of Louisiana State University Agricultural Center, Baton Rouge, USA (1993) was used.

The following model was applied to assess the stability of various variants in their interaction with years:

$$X_{ij}=m+N_i+Y_j+NY_{ij}+L_{ij}\ ; \qquad \text{where:}$$

X_{ij} – grain parameter (yield, mass) of the i-th variant with j-th environment (year),
m – general mean,
N_i – effect of the i-th variant,
Y_j – effect of the j-th environment (year),
NY_{ij} – effect of interaction of the i-th variant with the j-th environment (year),
L_{ij} – error relating to the i-th variant in the j-th environment (year).

RESULTS AND DISCUSSION

Grain yield is a result of the additive effect of the efficiency and selectivity of the studied herbicides and herbicide combinations (Table 2). When used alone soil herbicides Tender, Silba, Sharpen and Wing and vegetation herbicides Casper, Cambio, Camix, Maton, grain yield increases, because part of the weeds present are destroyed. The increase in yield is less compared to the combined use, because when herbicides are applied alone part of the prevailing weeds in the experiment cannot be destroyed.

The highest grain yields are achieved by treating with herbicide combinations Silba + Casper, and Wing + Cambio – 27.9 % over control. Very high and close to these are yields through herbicide combinations Silba + Cambio, Sharpen + Cambio, Tender + Casper, and Wing + Casper.

Grain yields are lower when gained under the influence of combinations involving herbicides Camix and Maton. In herbicide Camix decrease in grain yield compared to other herbicides is due to the greater phytotoxicity. This herbicide should be used primarily in secondary weeding with annual weeds. So far Camix is the only solution to combat them in sorghum grain. In herbicide Sanafen the decrease in grain yield compared to the other herbicides is due to the lower efficiency of the herbicide against perennial broadleaf weeds.

By analysis of variance with respect to grain yield (Table 3) it was found that the effect of the tested variants is 96.8 % of the total variance of the data shown in differences d≤0,1 %. The years have the strongest impact on grain yield - 57.4 % of the variants. It is conditioned by unequal variant response to changes in environmental conditions. The reason is the large differences in weather conditions over the three years of the study. The power of influence of soil herbicides is 11.7 % and that of vegetation herbicides - 11.4 %. The effect of years, soil and vegetation herbicides are very well shown in d≤0.1 %. There is an interaction of soil herbicides with the year conditions (A x B) - 6.4%, of the vegetation herbicides with the terms of years (A x C) - 6.3 % and the three experiment factors (A x B x C) - 2.0 %. They are proven for differences d≤5 %. The interaction between soil and vegetation herbicides (B x C) is unproven.

Based on the proven interaction year x soil herbicide and year x vegetation herbicide the stability of manifestations of each variant was evaluated in terms of grain yield of sorghum (Table 4). Stability variances σ_i^2 and S_i^2 have been calculated by Shukla, W_i ecovalence by Wricke and stability criterion YS_i by Kang.

Stability variances (σ_i^2 and S_i^2) by Shukla accounting for the linear and non-linear interactions, respectively, unidirectional assess the stability of variances. Those variants that show lower values are assessed as more stable because they interact less with the environmental conditions. The negative values of parameters σ_i^2 and S_i^2 are considered to be 0. In reliably high values of any of the two parameters - σ_i^2 and S_i^2, the variants are considered unstable. In the ecovalence W_i by Wricke, the higher the values of the parameter, the more unstable the respective variant.

On this basis, using the first three stability parameters, it has been established that the most unstable is the untreated control, herbicide Maton and its combinations. In these three variants the values of the stability variance σ_i^2 by Shukla and the ecovalence W_i by Wricke are the highest and mathematically proven. Instability is of linear type - proven value of σ_i^2only. The reason for this instability is the wide variation in grain yields as a result of the greater number of non-destroyed weeds in Maton due to its weaker abilities to control secondary weeding with perennial weeds. The other soil and vegetation herbicides included in the experiment show high stability during the three years of the study.

In order to fully assess the effectiveness of each variant one should take into consideration both its impact on grain yield of sorghum and its stability - the reaction of the crop to that variant throughout the various years. Very valuable information on the technological value of the variants is provided by the parameter YS_i by Kang for simultaneous assessment of production and stability, based on the reliability of differences in yield and the variance of interaction with the environment. The overall criterion for stability YS_i by Kang, taking into account both the stability and value of yield gives a negative assessment solely of the weeded, untreated control, characterizing it as the most unstable and low yielding.

According to that criterion technologically the most valuable are herbicide combinations Wing + Cambio и Silba + Casper. These herbicide combinations combine high grain yields and high stability of that parameter over the various years.

In terms of technology for growing grain sorghum, combinations Wing + Casper, Tender + Casper, Silba + Cambio, Tender + Cambio, Sharpen + Casper and Sharpen + Cambio are also rated high. In these herbicide combinations of the Concept technology high grain yields are combined with good stability throughout the years of the study. The combinations of Camix and Maton with the four soil herbicides also receive good ratings.

The single use of the herbicides Camix and Maton receives lower ratings due to their weaker efficacy against weeds and sunflower self-sown plants, although they also show high selectivity to the grain sorghum hybrid Arcansiel.

CONCLUSIONS

Herbicide combinations Silba + Casper and Wing + Cambio lead to highest yields of grain from sorghum.

High grain yields are also obtained in herbicide combinations Silba + Cambio, Sharpen + Cambio, Tender + Casper and Wing + Casper.

Combining Camix and Sanafen with soil herbicides leads to lower grain yields.

The most unstable are herbicide Maton and its combinations with herbicides Tender, Silba, Sharpen and Wing.

Technologically the most valuable are the herbicide combinations Wing + Cambio and Silba + Casper. They combine high grain yield with high stability over the years.

The single use of herbicides Camix, Maton receives lower marks because of their lower efficacy against weeds and sunflower self-sown plants.

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SELECTIVITY AND STABILITY OF HERBICIDES AND HERBICIDE COMBINATIONS BASED ON GRAIN YIELD FROM WINTER OILSEED CANOLA (*BRASSICA NAPUS* L.)

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ABSTRACT

In the period 2012 – 2014 a field experiment was carried out with the conventional Maximus hybrid PX113 (Brassica napus L). A total of 16 variants were tested. The efficacy of the herbicide Salsa 75 WG (ethametsulfuron-methyl) in single use is higher when applied with the adjuvant Trend 90 compared to the adjuvants Codacide and Silwet L-77. In tank mixtures of Salsa 75 WG and Butisan max (metazachlor + quinmerac + dimethenamid) and Butisan duo (metazachlor + dimethenamid), the herbicide efficacy and aftereffect against graminaceous weeds are higher when applied with the adjuvant Codacide. In concomitant use of Salsa 75 WG and Select super 120 EC (clethodim) with the adjuvant Codacide synergetic effect is reported. In the tank mixture of Salsa 75 WG + Targa super 5 EC (quizalofop-P-ethyl), higher efficacy is reported in its combined use with the adjuvant Trend 90. Technologically the most valuable are the tank mixtures of the herbicides Salsa 75 WG, Butisan max and Butisan duo with the participation of the adjuvant Codacide, followed by those with the participation of the adjuvant Trend 90. From the point of view of the technology for growing winter oilseed canola, tank mixtures of Salsa 75 WG with the herbicides Select super 120 EC and Targa super 5 EC are also highly rated, followed by that of Salsa 75 WG with Galera super (clopyralid + picloram + aminopyralid). The combinations of Salsa 75 WG with the adjuvants Trend 90, Codacide and Silwet L-77, but without the participation of a partner herbicide and the herbicide combination Modawn 4 F (bifenox) + Aramo 50 (tepraloxydim) are rated low.
Keywords: *winter oilseed canola, herbicides, seed yield, selectivity, stability*

INTRODUCTION

A number of authors in their research establish the efficacy of different herbicides and herbicide combinations in weed control in canola crops and their positive impact on the yield of seed (Oliveira Junior, 2001; Pérez Fernández et al., 2001; Bulavin et al., 2007; Luzhinskiy et al., 2011; Leonov

et al., 2011). The concomitant use of herbicides with adjuvants greatly increases their efficacy against weeds in winter oilseed canola (Miklaszewska et al., 2000; Murawa and Warminski, 2005; Delchev, 2018).
Cruciferous weeds are the most dangerous weeds in canola because of their difficult control in its crops. *Sinapis arvensis* L. and *Raphanus raphanistrum* L. are among the dominant species. Studies herbicides provide secure control over them is still very limited. Little attention is paid to the stability of herbicides in different in climatic terms years.
The aim of this study is to explore the possibilities to use the new vegetation herbicide Salsa 75 WG in combination with other partner products - herbicides and adhesives, to ensure continuous and stable over the years herbicidal effect.

MATERIAL AND METHODS

In the period 2012 – 2014 a field experiment was carried out with the conventional Maximus hybrid PX113 (*Brassica napus* L). The experiment was started by the block method, in 4 repetitions, with size of the crop plot 15 m^2, on leached vertisol soil type, after predecessor durum wheat. A total of 16 variants were tested. The active substances, doses and periods of treatments of the studied herbicides and herbicide combinations are given in Table 1. All variants are applied with working solution consumption 200 l/ha. By including a canola hybrid from the Maximus product line the need of growth regulators is eliminated. That made it possible to study only the effect of herbicides, adjuvants and mixtures of these.
Selectivity of herbicides and herbicide mixtures has been found through their effect on seed yield.
Statistical evaluation to rate the representativeness and reliable effect of the studied parameters has been applied through dispersion analysis and Fischer's parametric criterion F (Shanin, 1977; Barov, 1982). In the variance analysis the ANOVA123 software is used for calculation (Lidanski, 1988).
The selectivity of herbicides was established through their effect on grain yield and the following variances have been calculated:
Shukla, (1972) stability variance (σ_i^2)

$$Sh-\sigma_i^2=[1/(e-1)\,(t-1)(t-2)]\times\left[t(t-1)\sum_{j=1}^{s}\left(u_{ij}-\bar{u}_i\right)^2-\sum_{i=1}^{t}\sum_{j=1}^{s}\left(u_{ij}-\bar{u}_i\right)^2\right],$$

where $u_{ij}=X_{ij}-\bar{X}_{.j}$, $X_{ij}=$ observed trait value of i^{th} cultivar in j^{th} environment, $\bar{X}_{.j}=$ mean of all cultivars in j^{th} environment, $\bar{u}_{i.}=\sum_{j=1}^{m}u_{ij}/e$, $e=$ number of environments, and $t=$ number of cultivars.

In this study, calculation of adjusted stability variance (Sh-Si^2) was necessary, because the heterogeneity term was significant ($P < 0.01$). The stability statistic Sh-Si^2 calculated following removal of heterogeneity due to environmental index ($Z_j = \bar{X}_{.j} - \bar{X}_{..}$) as a covariate from GE interaction variance, where $\bar{X}_{.j}$ = mean of all cultivars in j^{th} environment and $\bar{X}_{..}$ = mean of all cultivars across all environments, using the following equation (Shukla 1972):

$Sh - S_i^2 = [t/(t-2)(e-2)] \times \left[s_i - \sum_{i=1}^{t} s_i/t(t-1)\right]$,

where $s_i = \sum_{j=1}^{s}\left(u_{ij} - \bar{u}_{i.} - b_i Z_j\right)^2$, and $b_i = \sum_{j=1}^{s}\left[\left(u_{ij} - u_{i.}\right)Z_j\right]/\sum_{j=1}^{s} Z_j^2$.

Cultivar stability across multiple years and locations also was evaluated using the ecovalence (W_i) (Wricke 1962):

$$W_i^2 = \sum_{i=1}^{t}\left(X_{ij} - \bar{X}_{i.} - \bar{X}_{.j} + \bar{X}_{..}\right)^2$$

Greatest stability is when $W = W_i^2 = 0$.

For grain yields stability parameters have been calculated. Stability variances (σ_i^2 and S_i^2) by Shukla (1972) and ecovalence W_i by Wricke (1962) show what portion of variation related to interaction of the preparations and years are accounted by the specific variant.

Through the stability criterion (YS_i) of Kang (1993) the value of each variant has been shown by simultaneous taking into account the parameter value and the stability of the variant. The value of that criterion is that by using non-parametric methods and statistical reliability of differences we obtain a combined valuation ranking variants in a descending order according to their economic value.

To calculate these parameters the STABLE software of Louisiana State University Agricultural Center, Baton Rouge, USA (1993) was used. The following model was applied to assess the stability of various variants in their interaction with years:

$X_{ij} = m + N_i + Y_j + NY_{ij} + L_{ij}$; where:

X_{ij} – grain parameter (yield, mass) of the i-th variant with j-th environment (year),

m – general mean,

N_i – effect of the i-th variant,

Y_j – effect of the j-th environment (year),

NY_{ij} – effect of interaction of the i-th variant with the j-th environment (year),

L_{ij} – error relating to the i-th variant in the j-th environment (year).

RESULTS AND DISCUSSION

The highest seed yield is obtained in treatment with the combinations Salsa + Butisan max + Codacide и Salsa + Butisan duo + Codacide, with 21.1 % and 20.0 % above the weeded control, respectively (Table 2). Good results are also obtained in the tank mixtures Salsa + Butisan max + Trend и Salsa + Butisan duo + Trend. The high yields in these variants are accounted for by both the good herbicide efficacy of Salsa and the prolonged aftereffect of Butisan max and Butisan duo for control of the secondary emerged weeds. In all three years of the experiment the addition of the adjuvant Codacide to tank mixtures Salsa + Butisan max and Salsa + Butisan duo results in obtaining higher seed yields compared to adding the adjuvant Trend.

In the herbicide mixture Salsa + Select super + Codacide due to the synergism among the preparations, seed yields are equal to those of the above combinations with Butisan max and Butisan duo. When mixing Salsa with Select super and Trend and with Targa super, Codacide and Trend seed yields are lower due to the weaker control of the secondary weed infestation in a more humid and warmer autumn.

Seed yields are the lowest in single use of Salsa without adjuvant – 10.6 % above the weeded control. When adding the adjuvants Trend, Codacide and Silwet and the herbicide Galera super to the herbicide Salsa, seed yields increase and are equal to the herbicide combination Modawn + Aramo, but still lower than those of the variants of Salsa with Butisan max, Butisan duo, Select super and Targa super due to lack of control on graminaceous weeds.

Through the applied variance analysis concerning seed yield (Table 3) it was found that the effect of the tested variants is 89.8 % of the total data variation, proven at $r \leq 0.1$ %. Years have the strongest effect on seed yield - 79.0 % of that of the variants. It is accounted for by the unequal response of variants to changes in environmental conditions. The reason for that are the big differences in weather conditions over the three years of the study. The power of influence of herbicides and herbicide combinations is 7.2 %. The effect of years and herbicides is very well proven at $r \leq 0.1$ %. There is interaction of herbicides with year conditions (A x B) - 3.6 %. It has been proven at $r \leq 5$ %.

Based on the proven interaction year x herbicide the stability of the manifestations of each variant have been evaluated in terms of seed yield of winter oilseed canola (Table 4). Stability variances σ_i^2 and S_i^2 have been calculated by Shukla, W_i ecovalence by Wricke and stability criterion YS_i by Kang.

Stability variances (σ_i^2 and S_i^2) by Shukla accounting for the linear and non-linear interactions, respectively, unidirectional assess the stability of

variances. Those variants that show lower values are assessed as more stable because they interact less with the environmental conditions. The negative values of parameters σ_i^2 and S_i^2 are considered to be 0. In reliably high values of any of the two parameters - σ_i^2 and S_i^2, the variants are considered unstable. In the ecovalence W_i by Wricke, the higher the values of the parameter, the more unstable the respective variant.
On this basis, using the first three stability parameters, it has been established that the most unstable is the untreated control, followed by the variants with single use of the herbicides Salsa, Galera super and the herbicide combination Modawn + Aramo. In these four variants the values of the stability variance σ_i^2 and S_i^2 by Shukla and the ecovalence W_i by Wricke are the highest and mathematically proven. In the weeded control and the herbicides Salsa and Galera super instability is of linear type - proven values of σ_i^2. The reason for this instability is the wide variation in seed yields throughout the years of the experiment as a result of differences in the efficacy of herbicides. In the herbicide combination Modawn + Aramo instability is of non-linear type - proven values of S_i^2. The reason for this high instability is the high phytotoxicity of the herbicide Modawn in 2012. The other variants show high stability during the three years of the study.
In order to fully assess the effectiveness of each variant one should take into consideration both its impact on seed yield of winter oilseed canola and its stability - the reaction of the crop to that variant throughout the various years. Very valuable information on the technological value of the variants is provided by the parameter YS_i by Kang for simultaneous assessment of production and stability, based on the reliability of differences in yield and the variance of interaction with the environment. The overall criterion for stability YS_i by Kang, taking into account both the stability and value of yield gives a negative assessment solely of the weeded, untreated control, characterizing it as the most unstable and low yielding.
According to that criterion technologically the most valuable are the tank mixtures of Salsa with Butisan max and Butisan duo with the participation of the adjuvant Codacide, followed by those with the participation of the adjuvant Trend. These combinations combine high seed yields and high stability of that parameter over the various years.
In terms of technology for growing winter oilseed canola, tank mixtures of Salsa with herbicides Select super and Targa super are rated high, followed by that of Salsa with Galera super. In these combinations relatively good seed yields are combined with high stability throughout the years of the study. Regardless of the higher herbicide efficacy against graminaceous weeds, these variants have lower ratings compared with the variants with the participation of Salsa and Butisan max and Butisan duo, due to the weaker

aftereffect in 2013 and 2014, when there were conditions for secondary weed infestation in autumn. The combination Salsa + Galera super receives lower rating because of its inefficiency against graminaceous weeds.
The combinations of the herbicide Salsa with the adjuvants Trend, Codacide and Silwet, but without the participation of a partner herbicide receive low ratings because of their inefficiency against graminaceous weeds and the lack of soil effect, resulting in secondary weed infestation in the late autumn of 2013 and 2014. The herbicide combination Modawn + Aramo receives low rating because of the high phytotoxicity of the herbicide Modawn in 2012.

CONCLUSIONS

The efficacy of the herbicide Salsa, when used separately, is higher than its application with the adjuvant Trend, compared to the adjuvants Codacide and Silwet.
In tank mixtures of Salsa and Butisan max and Butisan duo herbicide efficacy and aftereffect against graminaceous weeds are higher when applied with the adjuvant Codacide.
In concomitant use of Salsa and Select super with the adjuvant Codacide synergetic effect is accounted for.
In the tank mixture of Salsa + Targa super greater efficiency is reported in its joint use with the adjuvant Trend.
Technologically the most valuable are tank mixtures of herbicides Salsa, Butisan max and Butisan duo with the participation of the adjuvant Codacide, followed by those with the participation of the adjuvant Trend.
In terms of technology for growing winter oilseed canola tank mixtures of Salsa with herbicides Select super and Targa super are rated high, followed by that of Salsa with Galera super.
The combinations of Salsa with adjuvants Trend, Codacide and Silwet, but without the participation of a partner herbicide and the herbicide combination Modawn + Aramo get low rating.

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Table 1. Investigated variants

№	Variants	Active substance	Doses	Treatment period
1	Control	-	-	-
2	Salsa 75 WG +	ethametsulfuron-methyl *	20 g/ha	2-4 leaf

	Trend 90		0.1 %	
3	Salsa 75 WG + Codacide	ethametsulfuron-methyl **	20 g/ha 1.5 l/da	2-4 leaf
4	Salsa 75 WG + Silwet L-77	ethametsulfuron-methyl ***	20 g/ha 100 ml/ha	2-4 leaf
5	Salsa 75 WG	ethametsulfuron-methyl	20 g/ha	2-4 leaf
6	Salsa 75 WG + Butisan duo + Trend 90	ethametsulfuron-methyl metazachlor + dimethenamid *	20 g/ha 2 l/ha 0.1 %	2-4 leaf
7	Salsa 75 WG + Butisan duo + Codacide	ethametsulfuron-methyl metazachlor + dimethenamid **	20 g/ha 2 l/ha 1.5 l/ha	2-4 leaf
8	Salsa 75 WG + Butisan max + Trend 90	ethametsulfuron-methyl metazachlor + quinmerac + dimethenamid *	20 g/ha 2 l/ha 0.1 %	2-4 leaf
9	Salsa 75 WG + Butisan max + Codacide	ethametsulfuron-methyl metazachlor + quinmerac + dimethenamid **	20 g/ha 2 l/ha 1.5 l/ha	2-4 leaf
10	Salsa 75 WG + Select super 120 EC + Trend 90	ethametsulfuron-methyl clethodim *	20 g/ha 800 ml/ha 0.1 %	2-4 leaf
11	Salsa 75 WG + Select super 120 EC +	ethametsulfuron-methyl clethodim **	20 g/ha 800 ml/ha	2-4 leaf

	Codacide		1.5 l/ha	
12	Salsa 75 WG + Targa super 5 EC + Trend 90	ethametsulfuron-methyl quizalofop-P-ethyl *	20 g/ha 1.2 l/ha 0.1 %	2-4 leaf
13	Salsa 75 WG + Targa super 5 EC + Codacide	ethametsulfuron-methyl quizalofop-P-ethyl **	20 g/ha 1.2 l/ha 1.5 l/ha	2-4 leaf
14	Galera super	clopyralid + picloram + aminopyralid	200 ml/ha	2-4 leaf
15	Salsa 75 WG 75+ Galera super + Trend 90	ethametsulfuron-methyl clopyralid + picloram + aminopyralid *	20 g/ha 200 ml/ha 0.1 %	2-4 leaf
16	Modawn 4 F + Aramo 50	bifenox tepraloxydim	1 l/ha 1.5 l/ha	4-6 leaf

Table 2. Influence of some herbicides and herbicide combinations on seed yield of canola (2012 - 2014)

Factor A Factor B	2012		2013		2014		Mean (Factor B)	
	kg/ha	%	kg/ha	%	kg/ha	%	kg/ha	%
Control	2110	100	3430	100	3822	100	3121	100
Salsa + Trend	2439	115.6	3917	114.2	4494	117.6	3614	115.8
Salsa + Codacide	2405	114.0	3869	112.8	4444	116.3	3570	114.4
Salsa + Silwet	2410	114.2	3876	113.0	4444	116.3	3574	114.5
Salsa	2344	111.1	3756	109.5	4248	111.1	3452	110.6

Salsa + Butisan duo + Trend	2507	118.8	4047	118.0	4613	120.7	3720	119.2
Salsa + Butisan duo + Codacide	2521	119.5	4065	118.5	4663	122.0	3745	120.0
Salsa + Butisan max + Trend	2515	119.2	4047	118.0	4640	121.4	3730	119.5
Salsa + Butisan max + Codacide	2534	120.1	4102	119.6	4697	122.9	3780	121.1
Salsa + Select super + Trend	2469	117.0	3975	115.9	4548	119.0	3661	117.3
Salsa + Select super + Codacide	2507	118.8	4034	117.6	4651	121.7	3726	119.4
Salsa + Targa super + Trend	2464	116.8	3975	115.9	4548	119.0	3661	117.3
Salsa + Targa super + Codacide	2448	116.0	3948	115.1	4506	117.9	3630	116.3
Galera super	2321	110.0	3746	109.2	4281	112.0	3446	110.4
Salsa + Galera super + Trend	2437	115.5	3920	114.3	4506	117.9	3620	116.0
Modawn + Aramo	2344	111.1	3962	115.5	4541	118.8	3595	115.2
Mean (Factor A)	2423	-	3917	-	4194	-	-	

LSD, kg/ha:

F. A	$p \leq 5\% = 66$	$p \leq 1\% = 85$	$p \leq 0.1\% = 107$
F. B	$p \leq 5\% = 124$	$p \leq 1\% = 164$	$p \leq 0.1\% = 214$
A x B	$p \leq 5\% = 213$	$p \leq 1\% = 283$	$p \leq 0.1\% = 367$

Table 3. Analysis of variance for seed yield

Source of variation	Degrees of freedom	Sum of squares	Influence of factor, %	Mean squares	Fisher's criterion	Level of significance
Total	143	947965	100	-	-	-
Tract of land	2	49941	5.4	25871.0	8.2	***
Variants	47	888075	89.8	19108.8	27.5	***
Factor A - Years	2	854169	79.0	42208.5	19.6	***
Factor B - Herbicide combinations	15	29785	7.2	2552.5	2.8	***
A x B	30	3020	3.6	104.6	0.4	*
Pooled error	94	8850	4.8	95.1	-	-

*$p \leq 5\%$ **$p \leq 1\%$ ***$p \leq 0.1\%$

Table 4. Stability parameters for the herbicides for seed yield with relation to years

Variants	$\bar{x}$	σ_i^2	S_i^2	W_i	YS_i
Control	3121	851.9**	99.8	1563.1	-9
Salsa + Trend	3614	-5.8	-1.1	1.5	6
Salsa + Codacide	3570	33.5	48.1	73.1	3
Salsa + Silwet	3574	27.5	40.2	60.9	4
Salsa	3452	283.2*	64.1	1330.8	0
Salsa + Butisan duo + Trend	3720	17.7	4.1	43.9	14+

Salsa + Butisan duo + Codacide	3745	52.5	-0.6	104.3	16+
Salsa + Butisan max + Trend	3730	32.8	-0.8	70.1	15+
Salsa + Butisan max + Codacide	3780	88.8	-0.5	167.0	17+
Salsa + Select super + Trend	3661	-2.8	-1.1	8.4	11+
Salsa + Select super + Codacide	3726	60.2	8.0	117.7	14+
Salsa + Targa super + Trend	3661	-0.6	-1.1	12.2	10+
Salsa + Targa super + Codacide	3630	-6.0	1.5	2.9	9+
Galera super	3446	263.5*	60.6	923.4	2
Salsa + Galera super + Trend	3620	-4.9	1.4	4.8	8+
Modawn + Aramo	3595	174.6	1144.8*	1143.6	7
Mean	3603				7.9
LSD (p=0.05)	213				

PRIMARY METABOLITES IN FRUIT OF CURRANTS FROM WESTERN SERBIA

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ABSTRACT

The objective of this study was to identify and quantify primary metabolites in berries of six black ('Ben Lomond', 'Ben Sarek', 'Titania', 'Čačanska Crna', 'Tisel', and 'Tiben'), one red ('Jonkheer van Tets') and two white currant cultivars ('Weisse aus Juteborg' and 'Primus'). High-performance liquid chromatography (HPLC) was used for the identification of sugars (glucose, fructose, sucrose) and organic acids (citric, malic, quinic, shikimic and fumaric acid). Significant differences in the tested parameters were detected among the cultivars. Fructose was the dominant sugar, followed by glucose and sucrose in all tested cultivars. 'Titania' was the cultivar with the highest content of all individual and total sugars, while the cultivars 'Ben Sarek', 'Tisel', and 'Tiben' had a low content of sugars.'Ben Lomond' was the cultivar with the highest content of all examined acids and total acids, except shikimic. The best sugar and acid ratio was recorded in the berries of 'Titania' and 'Čačanska Crna'. Wide variations in major taste compounds of the target analytes examined were observed, and thus, it is clear that the differences in currant cultivars play an important role in determining fruit composition. The most suitable currant cultivars for grown in Western Serbia proved to be 'Titania' and 'Čačanska Crna'.

Keywords*: black currant, red currant, white currant, sugars, organic acids*

INTRODUCTION

Small fruits are widely recognized for their nutritional quality and potential health benefits. Currants (*Ribes* sp.) are an important small fruit crop due to its good fruit colour and organoleptic properties. Its berries provide a rich source of sugars and organic acids as important primary metabolites. Fructose and glucose are the major sugars that contribute to the sweetness of current berries, while the major acid is malic acid, which causes a sour and bitter taste. Generally, sugars and organic acids play a key role in cell metabolism and reproduction (Hartmann, 2007). For this reason, Kampuss (2005) pointed to the significance and the need for more intensive exploitation of this species. Therefore, an important goal in currant breeding is to improve the chemical composition of the fruit i.e. its content of sugars, acids and colorants, and create new dessert genotypes intended for fresh use (Mišić and Nikolić, 2003). According to Kafkas et al. (2006) and Tosun et al. (2009), sugars and organic acids are main soluble constituents and have a major effect on taste and fruit ripeness, or even represent a suitable index of consumer's acceptability. Also, Bordonaba and Terry (2008) reported that sugar and acid content and sugar to the acid ratio in currants and other fruits are essential in flavor formation. In this ratio, the organic acid content as well as the composition of individual metabolites play a crucial role in fruit taste perception. It is often understood that the fruits that exhibit pleasant sensory characteristics have a high sugar content and a relatively low content of acids (Zheng et al., 2009).

Therefore, the objective of this study was to examine the effect of genotype on the content of primary metabolites in berries of six black, one red and two white currant cultivars using high-performance liquid chromatography (HPLC). On the basis of the obtained results, we could recommend cultivars suitable for growing in the area of Western Serbia, all with the aim of improving the existing technology of currant production and achieving satisfactory fruit quality.

Materials and methods

The research was conducted at the Fruit Research Institute, Čačak (Western Serbia) in 2018. A currant planting was established in the spring of 2011 using two-year-old plants. Currants were grown as bushes at a spacing of 3 m between rows and 1 m in the row. Fruits of six black ('Ben Lomond', 'Ben Sarek', 'Titania', 'Čačanska Crna', 'Tisel', and 'Tiben'), one red ('Jonkheer van Tets') and two white currant cultivars ('Weisse aus Juteborg' and 'Primus') were analyzed. Each cultivar was replicated three times in a randomized complete block design. Fruits were sampled at full ripeness in June, at the stage of full development. Berries were selected from the inner and outer range of the bush. A total of 100 g berries were sampled from 5 bushes per replication.

Analyses of sugars and organic acids

Plum (2.0 g) was homogenized in 10 ml double-distilled water. After 30 min shaking at room temperature, the extract was centrifuged 10 min. at 10,000 rpm (Eppendorf Centrifuge 5810 R), filtered through a 0.20 µm cellulose ester filter (Macherey-Nagel, Germany) and poured into a vial prior to the analysis on a high performance liquid chromatography system (HPLC; Thermo Scientific, Finnigan Spectra System, Waltham, MA, USA) as it was previously described by Jakopic et al. (2016). Briefly, sugar separations were carried out using a Rezex RCM-monosaccharide column from Phenomenex (Ca+ 2%) operated at 65 °C (300 mm x 7.8 mm). The mobile phase was double distilled water, the total run time was 30 min, and a refractive index (RI) detector was used. The injection volume was 20 µl, and the flow rate was maintained at 0.6 ml/min. Analyses of organic acids were performed on the same HPLC system, equipped with a UV detector set at 210 nm, using a Rezex ROA-organic acid (H+ (8%)) column from Phenomenex (300 mm x 7.8 mm). The column temperature was set at 65 °C, the elution solvent was 4 mM sulphuric acid in double distilled water and the flow rate 0.6 ml/min.

The results are presented in milligrams per gram fresh weight (mg/ g FW) for sugars and organic acids. The content of all analyzed sugars and acids were summed and presented as total sugars and total acids. The sugar to acid ratio is defined as the proportion of total sugars and the total organic acids in the currant samples.

Chemicals

The following standards were used for determination of sugars: sucrose, fructose, glucose, sorbitol, and organic acids: citric, malic, quinic, and fumaric acids were purchased from Fluka Chemie (Buchs, Switzerland) and shikimic acid from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

Statistical analysis

The data obtained in the research was processed applying the one-way analyses of variance (ANOVA, F test) Hypotheses were rejected at $P<0.05$. The analyses were performed in four replications and the obtained values were expressed as the means ± standard error. Means were compared with the Duncan test at $P \leq 0.05$.

Results and discussion

Sugars and organic acids play an important role during maturation and senescence of the fruit, and therefore important parameters at harvest. The composition and contents of sugars and acids affect fruit organoleptic characteristics (Mikulič-Petkovšek et al., 2012). Glucose, fructose and sucrose are the major sugars, citric acid is a major organic acid, and malic acid is present in minor concentrations in the fruit of currant (Hummer and Barney, 2002; Rubinskiene et al., 2006; Milivojević et al., 2009). Individual sugars and organic acids in berry extracts were identified by the HPLC-DAD analysis, with the corresponding results presented in Tables 1 and 2.

Table 1. Content of individual and total sugars in the fruit of currant

	Sucrose (mg/g FW)	Glucose (mg/g FW)	Fructose (mg/g FW)	Total sugars (mg/g FW)
Black currant				
'Ben Lomond'	5.8±0.1 b	36.3±0.8 c	43.7±1.1 c	85.8±0.9 c
'Ben Sarek'	n.d.	12.4±1.0 e	23.8±1.8 e	36.2±2.7 e
'Titania'	8.6±0.2 a	43.9±1.1 a	55.2±0.9 a	107.8±2.2 a
'Čačanska Crna'	3.7±0.7 c	38.9±0.8 bc	50.0±0.9 b	92.6±1.8 b
'Tisel'	n.d.	10.9±0.5 e	25.5±1.3 e	36.5±1.1 e
'Tiben'	n.d.	2.4±0.1 f	24.0±1.4 e	26.4±1.5 f
Red currant				
'Jonkheer van Tets'	0.6±0.0 d	26.2±1.4 d	26.3±0.9 e	53.1±2.2 d
White currant				
'Weisse aus Juteborg'	8.6±0.6 a	41.0±0.6 ab	38.2±0.4 d	87.9±0.9 bc
'Primus'	4.6±0.6 bc	38.9±2.6 bc	42.7±1.5 c	86.2±3.0 c
ANOVA	*	*	*	*

Data represent the means of four replicates ± standard error. The different lower-case letters in the columns indicate statistically significant differences among the mean values relative to cultivars at $P \leq 0.05$ level (Duncan's test).

Sugars represent the basic components in the formation of fruit flavor. The level of sugars and organic acids in the fruits depending on the genotype and are also influenced by the environmental factors and agrotechnical practices carried out in the orchard (Colaric et al., 2005; Hudina and Štampar, 2009). The sugar content in our study was significantly affected by genotype. The dominant participation in the structure of total sugars had fructose and glucose, while sucrose is detected at low concentrations. 'Titania' was the cultivar with the highest content of all individual and total sugars, while the cultivars ‘Ben Sarek’, ‘Tisel’, and ‘Tiben’ had a low content of sugars. The content of fructose ranged from 23.8 in the fruit of ‘Ben Sarek’ to 55.2 mg/g in the fruit of ‘Titania’,while the content of glucose in the fruit of the cultivar ʽJonkheer van Tets’ (41.0 mg/g) was not significantly different from cultivar ʽTitania’ (43.9 mg/g).

Acids represent another important component that is involved in the formation of fruit flavor. The nature and the concentration of organic acids are important factors influencing the organoleptic properties of fruit and fruit products (Silva et al., 2002). The sum of all quantified acids ranged from 28.12 in the fruit of ʽJonkheer van Tets’ to 53.51 mg/g FW in the fruit of ‘Ben Lomond’. The most common acid in the fruits of the studied cultivars was malic, with the highest proportion in the total acidity, while the proportion of quinic, citric, shikimic and fumaric acids were significantly lower (Table 2).‘Ben Lomond’ was the cultivar with the highest content of all examined acids and total acids, except shikimic. As compared to the present results, under the environmental conditions of Serbia, Djordjević et al. (2015) reported similar values for content of sugars and acids, while Milivojević et al. (2009, 2013), Paunović et al. (2013) and Paunović and Mašković (2018) obtained higher values for individual sugars, but lower values for total and organic acid. The difference in the measured contents can be explained by strong variations in the synthesis and accumulation of chemical compounds under different climates.

Table 2. Content of organic acids in extracts berries of currants

	Citric acid (mg/g FW)	Malic acid (mg/g FW)	Quinic acid (mg/g FW)	Shikimic acid (mg/g FW)	Fumaric acid (μg/g FW)×10^{-3}	Total acids (mg/g FW)
Black currant						
‘Ben Lomond’	35.07±0.92 a	10.50±0.29 a	7.69±0.35 a	0.23±0.01 cd	19.13±0.89 a	53.51±1.50 a
‘Ben Sarek’	29.58±0.14 bc	6.76±0.09 b	2.70±0.68 c	0.05±0.00 f	8.98±0.14 cd	39.10±0.65 c
‘Titania’	26.10±1.91 d	4.00±0.31 c	5.63±0.50 ab	0.26±0.02 c	11.62±0.55 b	35.99±2.65 c
‘Čačanska Crna’	24.16±0.54 dc	3.68±0.11 c	6.26±0.31 ab	0.24±0.01 c	10.73±0.29 bc	34.35±0.79 de
‘Tisel’	20.57±0.16 e	5.84±0.50 b	4.29±0.58 abc	0.12±0.01 cf	6.01±0.74 e	30.83±2.55 c
‘Tiben’	23.04±0.12 de	6.92±0.43 b	5.92±0.22 ab	0.05±0.00 f	10.55±0.92 bc	35.94±2.35 cd
Red currant						
ʽJonkheer van Tets’	22.52±0.48 de	2.89±0.15 c	2.56±0.34 c	0.15±0.01 de	4.64±0.78 e	28.12±0.95 e
White currant						
ʽWeisse aus Juteborg’	32.47±1.73 ab	5.83±0.81 b	7.67±1.06 a	0.54±0.06 a	12.21±0.55 b	46.52±0.79 b
ʽPrimus’	26.38±2.02 d	6.58±0.64 b	4.14±1.12 abc	0.39±0.06 b	8.59±0.59 cd	37.49±1.45 c
ANOVA	*	*	*	*	*	*

Data represent the means of four replicates ± standard error. The different lower-case letters in the columns indicate statistically significant differences among the mean values relative to cultivars at *P*≤ 0.05 level (Duncan's test).

Generally, the ratio of sugars and organic acids is an important indicator of perceived taste, maturity/ripeness and general quality, which may serve as an index of consumer acceptance (Bordonaba and Terry, 2008). Besides sugar, an important role in the formation of fruit taste has the sugar/acid ratio. Study of Colaric et al. (2005) shows that sugars/organic acids ratio and levels of citric acid and shikimic acid have significant impacts on the perception of sweetness. The best sugar and acid ratio was recorded in the cultivars 'Titania' and 'Čačanska Crna'(Figure 1).

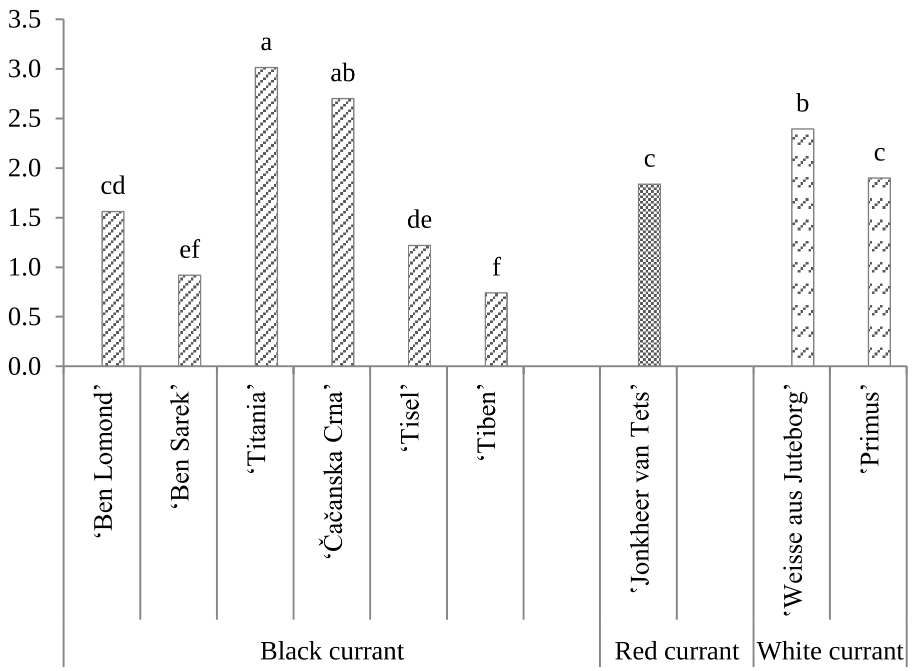

Figure 1. Sugars/Organic Acids ratio in the fruit of currant cultivars. The different small letters at the top of columns indicate significant differences in Sugars/Organic Acids Ratio at P ≤ 0.05 by Duncan's test.

Conclusion

Currants are an exceptionally rich source of sugars and acids, and an interesting nutritional alternative. Berries could be of interest to industrial applications in healthcare and can be used as a new source of natural foods.

Wide variations in major taste compounds of the target analytes examined were observed, and thus, it is clear that the differences in currant cultivars play an important role in determining fruit composition. The most suitable currant cultivars for grown in Western Serbia proved to be ‘Titania’ and ‘Čačanska Crna’.

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THE INFLUENCE OF THE ACIDIC SOIL TREATMENT SYSTEM ON SOME MORPHOLOGICAL AND PRODUCTIVE PROPERTIES OF CORN HYBRID

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ABSTRACT

The aim of this paper was to test the influence of the soil treatment system on some morphological and productive properties of some corn hybrids. The experiment was carried out in the vicinity of Požega during 2015 and 2016, on vertisol soil type. 4 soil treatment systems were included in the experiment (CT - conventional tillage: autumn plowing + disc-harrowing + seedbed preparation; RT - reduced tillage: disc-harrowing + seedbed preparation; RT1 - disc-harrowing; NT - no tillage) as well as 3 corn hybrids (ZP427, ZP555 and AS603). The lowest average plant height was recorded with the NT soil treatment system. There were no statistically significant differences in the height of corn cob among the CT, RT and RT1 soil treatment systems, while the NT soil treatment system had the lowest height of the cob. The smallest average 1000 grains weight was recorded with the NT soil treatment system, while the hybrids did not have a significant effect on this property. The grain yield significantly depended on the soil treatment system and on the hybrid. The highest average yield was with the CT soil treatment system, and the lowest with the NT soil treatment system. On soils such as vertisol, reduced tillage and direct sowing should be avoided if high corn grain yields are desired.

Key words: *Conventional tillage, corn, direct sowing, reduced tillage, yield.*

INTRODUCTION

Acidic soils due to their physical properties, belong to a group of heavy soils, and require treatment systems that ensure the preservation of the natural potentials of fertility. A properly selected soil treatment system is an important factor that greatly influences the yield of cultivated plants. The most common processing systems are classical or conventional tillage, reduced or conservation tillage and direct sowing. Dutzi (2001) points out that, based on the eleven-year study at the Giessen College, it was found that the yield in the conservation treatment system compared to the classical one, on average, increased by 7.5%. In the US, the use of conservation tillage and direct sowing is dominant, while in Europe it is expanding, taking in some countries up to 30 – 50% of the area (Martinov et al., 2005). It is to be expected that the tillage systems in Europe will occupy the largest areas, and the share of regularly plowed areas will be reduced to 10 – 20% (Đević, 2007). Kovačević et al. (2008) point out in their research that the best results of corn yield are achieved by the conventional tillage, then by reduced and the lowest by direct sowing. Analyzing corn grain yields in various soil treatment systems, Momirović et al. (2011) point out that the highest yields, in all tested years, were achieved with the conventional system and the lowest with the system of direct sowing. Videnović et al. (2011) point out that in the ten-year research, the highest corn grain yields were obtained with the conventional soil treatment system while the average with the reduced tillage and direct sowing was lower. Deprsch (2000) states that reduced soil treatment and direct sowing are not an ideology but the consequence of the economic and ecological decision-making process. Malinović et al. (2008) do not recommend conservation tillage and direct sowing for the production of seed corn. Dragicević et al. (2012) point out that in all tested years, considerably higher corn yields were achieved with the conventional system, in relation to reduced tillage and direct sowing.

Research on the impact of reduced and conservative soil treatment on the corn yield and other field crop plants was done by other researchers (Ercegović, 2009**;** Najafinezhad et al., 2005.; Saberi et al., 2014; Košutić, 2005.**;** Butorac et al., 2006.; Sabo et al., 2006).

The aim of this paper was to test the influence of the soil treatment system of the vertisol soil type on some morphological and productive properties of some corn hybrids.

MATERIAL AND METHODS

The experiment was carried out in the vicinity of Požega, during 2015 and 2016, on vertisol soil type. 4 soil treatment systems were included in the experiment (CT-conventional tillage: autumn plowing + disc-harrowing + seedbed preparation; RT-reduced tillage: disc harrowing + seedbed preparation; RT1- disc harrowing; NT-no tillage) and 3 corn hybrids (ZP 427, ZP 555 and AS 603). The following parameters were observed: plant height, the height of corn cob, 1000 grains weight and grain yield with 14% moisture. The view was set by the block system in 3 repetitions. Autumn plowing for the CT variant was performed on 29th October, 2014, disc-harrowing, seedbed preparation and sowing on 10th May. For variants RT and RT1 reduced processing and sowing was done on 10th May, 2015. For variant NT sowing was done on 10th May, 2015. The pre-culture was wheat. Mineral nutrition means the application of mineral fertilizers NPK 300 kg ha^{-1} for variants CT, RT and RT1, respectively 200 kg ha^{-1} KAN in top dressing. On NT variant, in top dressing, 400 kg of ha^{-1} KAN were applied. On the variants RT, RT1 and NT, the total herbicide *Glyphosate* was applied in the amount of 3 l ha^{-1} immediately prior to sowing, while on all variants of processing, except for variant NT, after sowing and before germination, herbicides *Acetogal* + *Rezo*n were applied in the amount of 1.5 l ha^{-1}. The results were presented as a two-year average and were processed statistically, by analyzing variance, using WASP 1.0 software, and for individual comparisons the LSD test were used.

RESULTS AND DISCUSSION

Soil and climatic conditions

Tables 1 and 2 show the chemical properties of the soil i.e. the climatic characteristics of the area.

Table 1. Chemical properties of the soil

Depth (cm)	pH		Humus	N	P_2O_5	K_2O
	H_2O	KCl	%	%	mg/100 g	mg/100 g
0-30	7.2	5.80	3.1	1.85	10.7	20.0

In a layer of 0 to 30 cm the pH of the soil solution is 5.8 and this soil belongs to a group of moderately acidic soils. The humus content is 3.1%, which is a good supplied. The nitrogen content was 1.85%. The content of phosphorus of 10.7 mg/100 g is at the margin of poor and medium security, while the potassium content of 20 mg/100 g is at the level of medium and good supplied. A very successful plant production can be organized on such a soil, with some agrotechnical measures.

Table 2. Precipitation (mm) and mean air-temperature (^{0}C) in Požega

Year		Month												Aver. Sum
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	
2015	(°C)	0.1	1.9	5.4	10	16.6	18.2	22.4	21.6	17.6	11	4.8	0.4	10.8
	(mm)	39.2	55.6	74.5	52.1	75	131.3	11,1	39.5	66.2	65.8	43.8	5.9	648.9
2016	(°C)	-1.2	6.6	6.5	12.3	14.2	19.9	21.1	18.7	15.7	9.7	4.6	-1.5	10.5
	(mm)	60.6	30.4	168.6	46.6	145.5	75.8	72.4	180.2	43.8	81.0	85.9	9.1	999.9

The table shows that during the sowing there was enough moisture in the soil, which enabled good and proper germination and spraying of corn. The total amount of precipitation during maize vegetation (2015) was 441 mm, but their arrangement was rather uneven and irregular, especially in 6th, 7th and 8th month. During 2016, during the vegetation, 643.3 mm of precipitation fell, whose schedule was much more favorable. During the vegetation of the corn, the temperatures were not a limiting factor and their arrangement was quite favorable.

Plant height

Plant height is a very important feature, both for describing new corn genotypes and for producing fresh and dry matter, and even for grain yield. Irfan et al. (2014) state that plant height is a very important component for plant production due to photosynthesis activities. Plant height expression is controlled by many genes, the interaction between these genes, the environmental factors, and the interaction of genetic and environmental factors (Radanović et al., 2015). This trait is inherited super-dominant. Table 4 shows the height of the plants depending on the soil treatment system.

Table 3. Impact of the soil treatment system on plant height (cm)

A. Hybrids	B. Soil treatment system				Average A
	CT	RT	RT1	NT	
ZP 427	270	260	254	180	241
ZP 555	290	280	272	230	268
AS 603	290	275	273	260	275
Average B	283	272	266	223	261
LSD	A 5%-14.01 1%-19.04	B 5%- 16,00 1%- 21.76	AxB 5% - 28.11 1% - 38.20		

CT-conventional tillage; RT-reduced tillage; RT1- reduced tillage; NT-no tillage

The soil treatment system had an impact on the height of the plants. The highest average plant height was determined on the CT soil treatment system (283 cm) and it was significantly higher than on the RT1 and NT systems. The lowest average plant height (223 cm) was recorded in the NT tillage system and was significantly lower than in other tillage systems. The highest plant height (290 cm) had hybrids ZP 555 and AS 603 in the CT soil treatment system. Hybrid ZP 427 had significantly lower stem height than hybrids ZP 555 and AS 606, but no significant differences in plant height were found.

Height of corn cob

Regarding the height of corn cob, from the point of view of the selection and producer, there is a tendency for the cob to be positioned as lower as possible in order to minimize the lodging and cracking of the stalk, and these trends are also observed in the studies Meghi et al. (1984) and Duvick et al. (2004). Table 4 shows the height of corn cob depending on the soil treatment system.

Table 4. Effect of the soil treatment system on the height of corn cob (cm)

A.Hybrids	B. Soil treatment system				
	CT	RT	RT1	NT	Average A
ZP 427	90	90	90	60	83
ZP 555	100	95	90	70	89
AS 603	100	100	90	90	95
Average B	97	95	90	73	89
LSD	A 5%-8.05 1%-10.95	B 5%- 9.14 1%- 12.43	AxB 5% - 11.07 1% - 15.05		

CT-conventional tillage; RT-reduced tillage; RT1- reduced tillage; NT-no tillage

The average height of corn cob for all soil treatment systems ranged from 83 cm in hybrid ZP 427 to 95 cm in hybrid AS 603. There were no statistically significant differences in the height of corn cob between hybrids ZP 427 and ZP 555, nor between hybrids ZP 555 and AS 603. The hybrid AS 603 had a statistically significantly higher height of corn cob than the ZP 427 hybrid. The average height of corn cob for all hybrids ranged from 73 cm in the NT processing system up to 97 cm in the CT processing system. Statistically significant differences of the height of corn cob were not found among the CT, RT and RT1 processing systems, while the average height of corn cob in the NT soil treatment system was statistically significantly lower than in other soil treatment systems. The highest height of corn cob (100 cm) had hybrids ZP 555 and AS 603 in the CT soil treatment system, while the lowest height of corn cob (60 cm) had a ZP 427 hybrid in NT soil treatment system.

1000 grains weight

1000 grains weight is an indicator of the size of the grain and it represents the ratio between the weight and the number of grains. It is an important component of the yield that depends on the genotype, but it is significantly influenced by agroecological and agrotechnical conditions. Table 5 shows the absolute grain mass depending on the soil treatment system.

Table 5. Effect of the soil treatment system on the 1000 grains weight (g)

A. Hybrids	B. Soil treatment system				Average A
	CT	RT	RT1	NT	
ZP 427	400	390	340	310	360
ZP 555	370	370	350	330	355
AS 603	390	390	350	330	365
Average B	387	384	347	324	360
LSD	A 5%-24.05 1%-32.70	B 5%- 26.11 1%- 35.50		AxB 5% - 31.21 1% - 42.44	

CT-conventional tillage; RT-reduced tillage; RT1- reduced tillage; NT-no tillage

The smallest average 1000 grains weight (324) was recorded in the NT processing system and was significantly lower in comparison to the CT and RT soil treatment systems, while between the NT and RT1 processing systems there were no significant differences. The largest 1000 grains weight (400 g) had hybrid ZP 427 in the CT soil treatment system. No statistically significant differences were found in the 1000 grains weight among the tested hybrids.

Grain yield

Yield is the most important agronomic feature and the main reason for growing corn, which depends on genotype and agroecological conditions. Table 6 shows the grain yield depending on the soil treatment system.

Table 6. Effect of the soil treatment system on corn grain yield (t ha^{-1}) with 14% moisture

A. Hybrids	B. Soil treatment system				Average A
	CT	RT	RT1	NT	
ZP 427	8.52	7.47	7.13	4.91	7.01
ZP 555	8.90	8.10	8.00	5.46	7.61
AS 603	9.20	7.20	7.45	5.85	7.43

Average B	8.87	7.59	7.53	5.41	7.35
LSD	A 5%-0.41 1%-0.55	B 5%- 0.51 1%- 0.69	AxB 5% - 0.85 1% - 1.15		

CT-conventional tillage; RT-reduced tillage; RT1- reduced tillage; NT-no tillage

The grain yield significantly depended on the soil treatment system. The highest average yield (8.87 t ha^{1}) was in the CT processing system, and the lowest (5.41 t ha^{-1}) in the NT soil treatment system. The average yield achieved in the CT soil treatment system was significantly higher than yields in other soil treatment systems. No statistically significant differences in corn grain yield were found between RT and RT1 soil treatment systems. The highest yield (9.20 t ha^{-1}) had a hybrid AS in the CT soil treatment system. Hybrids ZP 555 and AS 603 had significantly higher average grain yield compared to hybrid ZP 427, while there were no statistically significant differences in grain yield between them.

Our results are in agreement with the results of Kresović and Tolimir (2009), who in their research emphasize that the best results of corn yield are achieved by conventional processing, then reduced and the lowest by direct sowing. Also,Videnović et al. (2011) achieved the highest corn grain yield in the conventional soil treatment system while the average with reduced tillage and direct sowing was lower.

CONCLUSION

The lowest average plant height was recorded in the NT tillage system. Statistically significant cob differences were not found between the CT, RT and RT1 tillage systems, while the average cob height in the NT soil treatment system were the lowest. The smallest average 1000 grains weight was recorded in NT processing system and it was significantly lower compared to CT and RT soil treatment systems. The largest 1000 grains weight had hybrid ZP 427 in the CT soil treatment system. There were no statistically significant differences in the 1000 grains weight among the tested hybrids. The grain yield significantly depended on the soil treatment system and hybrids. The average yield achieved in the CT soil treatment system was significantly higher than yields in other soil treatment systems. Hybrids ZP 555 and AS 603 had significantly higher average grain yield compared to hybrid ZP 427. In soils such as vertisol, reduced tillage and direct sowing should be avoided if high corn grain yields are desired.

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EFFECTS OF ONION WATER EXTRACTS ON SOME CROPS SEED GERMINATION AND SEEDLING LEGNTH

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ABSTRACT

Water extract of aerial onion plant parts, was made by collecting all dry leafs. One hundred grams of leaf dry matter was soaked in one liter of distilled water. Obtained extract was filtered diluted in concentrations 5 ml/mg, 10 ml/mg, 25 ml/mg, 50 ml/mg dry mass/l of distilled water. Effects of onion aqueous extracts were observed on seven crops: arugula, clover, carrot, kohlrabi, lettuce, onion and savoy cabbage, in order to evaluate the effects on their seed germination and average seedling length. The effects of onion water extracts were stimulating for all crops seed germination in concentration 5 ml/mg and 10 ml/mg except lettuce. In contrast the higher concentrations 25 and 50 ml/mg had an inhibiting effect on seed germination of all crops except savoy cabbage in which the higher concentrations stimulated seed germination. Water extracts had a stimulating effect on average seedling length of all crops, but they expressed negative effect on average seedling length in clover water extract concentrations 25 and 50 ml/mg and lettuce in concentrations 10, 25 and 50 ml/mg.
Key words: *water extract, arugula, clover, carrot, kohlrabi, lettuce, onion, savoy cabbage, seed germination, seedling length*

INTRODUCTION

Onion (*Allium cepa L*), one of the most widely grown and consumed vegetables in the world, is known to contain high levels of antioxidant compounds phenolics, especially flavonoids. Flavonols and anthocyanins are the main subclasses of flavonoids present in onions, with the latter being found mainly in red onions. All types of onions (white, yellow or red) are reported to be rich in flavonols, mainly quercetin, and are recognized as the major dietary source of quercetin. In addition, onions are a source of polysaccharides and selected vitamins and minerals (Siddiq et al., 2013).

The most abundant class of allelochemical compounds in *Allium* species are carbohydrates (glucose, fructose, sucrose, oligosaccharides) and the fructans. *A. cepa* contains high levels of alliin. This allelochemical act as enzyme inhibitor for cyclo-oxygenase and lipoxygenase. However it gets converted into the anti-microbial active allicin, in the presence of enzyme allinase. The other forms of alliin are isoalliin and methiin (Yousaf et al., 2012). The definition of allelopathy was first used by Molish in 1937 to indicate all of the effects that directly and indirectly result from biochemical substances transferred from one plant to another (Cheng and Cheng, 2015). Allelopathy is a form of positive and negative interaction among organisms that is caused by the action of chemical compounds referred to as allelochemicals (Trezzi et al., 2016). It is a well-known fact that plants produce thousands of secondary metabolites and these allelochemicals are released into the environment by root exudation, leaching, volatilization, and residue decomposition (Zeng, 2014). Action of these are compounds is concentration dependent as these inhibit the plant growth at high concentrations and promote that at low concentrations (Farooq et al., 2013). Allelopathy is considered as a chemical interaction among higher plants, in which allelochemicals - released from the donor plants can greatly modify, generally inhibit the development of the recipient (test) plants (Kazinczi et al., 2013). In previous studies allelopathic effect of different onion varieties was determined by using plant parts and seeds in aqueous extracts. Allelopathic potential of aqueous extracts of onion seed on germination and growth pea was studied. Aqueous extracts at 0, 10, 30 and 60% concentrations were used. The greatest effect was observed by onion aqueous extract with concentration 60% only 40 % of pea seeds were able to germinate compared to control 98%. Onion extracts also reduced the seedling growth, the pea plants treated with onion water extract of 60%, had the shoot length of 5 cm compared to control 15 cm, the other two onion aqueous extract concentrations showed also a reducing growth effect on pea shots (El-Ghit, 2016). To investigate the effect of phenolic compounds from *A. cepa* in laboratory experiments on four weed species: *Echinochloa crus-galli*, *Portulaca oleracea*, *Sisymbrium irio* and *Solanum nigrum* at water extract concentrations (0.1 or 3% w/w). The results showed that the garlic and onion plant residues were capable of causing significant reductions in seed germination of weeds (Susan et al., 2007). The study to establish the relationship between allelopathic interactions among onion and peppers has been conducted in period 2009-2011 in laboratory conditions. Extracts were prepared from roots and vegetation parts of the plants, taking an average sample of 0.5 g or 1 g of roots or mixed vegetative mass (stems, leaves, flowers). Study the effects of extracts from plants on the growth and development of seeds of pepper. The inhibitory effect was shown by allelochemicals from onion on the root system on pepper. The concentration of 1% allelochemicals suppresses in a highest extent the development of the root system (Valcheva and Popov, 2013). Arugula comprises a number of species of the *Brassicaceae* (*Cruciferae*) family belonging to the *Eruca* (Miller) and *Diplotaxis* (DC.) genera. *Eruca sativa* (Mill.) or *Eruca vesicaria* (L.) has its origin in the Mediterranean region but is widely distributed all over the world. It is mostly harvested from the wild or cultivated as an edible vegetable for the distinct spicy flavor of young leaves. Arugula seeds are also used for the production of oil and for appreciated pungent taste sprouts (Barillari et al., 2005). Clovers are in the tribe *Trifolieae* of the subfamily *Papilionoideae*, family *Fabaceae*, *Trifolium* L. The genus contains approximately 230-250 species. Clovers are used for forage, pasture, soil improvement and silage. Besides, clovers are an important source of nutrients for livestock and are grown throughout grasslands in the world (Ates, 2012). Carrot is the most widely grown species of the genus *Daucus*, a member of the large and complex *Apiaceae* family. Because of its high yield potential and use as fresh or processed product, cultivated carrot (*D. carota*) is one of the most important vegetable plants in the world. With a current annual world production of 30 million tons and a total growing area of about 1.5 million hectares. Carrots rank among the top 10 vegetable crops with the Unites States, China, and Russia accounting for 34% of the global production (Dawid et al., 2015). Koholrabi is a cool-weather plant. It is closest in form to wild cabbage (*Brassica oleracea* ssp. *oleracea*), the progenitor of all the *B. oleracea* varieties, and grows along the coasts of Europe and North Africa. There are purple and pale green cultivars of kohlrabi. Kohlrabi is more tolerant of heat and drought than most of its cabbage relatives (Park et al., 2012). Lettuce (*Lactuca sativa* L., *Asteraceae*) is considered the most important vegetable in the group of leafy vegetables (Marcu et al., 2012). Savoy cabbage (*Brassica oleracea* var. *sabauda*) is a variety native to south-east Europe. Although not distant Savoy and plain cabbage have marked differences in most agronomic traits (Sretenović-Rajačić et al., 2006).

MATERIALS AND METHODS

Water extract of aerial onion plant parts, has been prepared by collecting all dry leafs, after additional drying to the absolute dry mass, afterwards plant material grinded to the particle size of 1 mm. One hundred grams of leaf dry matter was soaked in one liter of distilled water and shaken on room temperature during 24 h in shaker with speed 120/60s. Obtained extract was filtered with filter paper and centrifuged on 5000/60 rpm, and afterwards diluted in concentrations 50 ml/mg, 25 ml/mg, 10 ml/mg, 5 ml/mg dry mass/l of distilled water. The trial was set up in laboratory conditions, randomized block system with four replications, control consisted of distilled water treatments. Twenty seeds of each crop were placed in Petri dishes that contained sterile whatman papper. Seeds were treated with different concentrations of onion water extracts. Germination was conducted in climate chamber, conditions were 22 ^{0}C/12h day and 16 ^{0}C/12h night, air humidity 65 %, after 14 days germinated seeds were counted and length of seedlings was measured.

RESULTS

Effects of onion water extracts in concentrations 5, 10, 25 and 50 ml/mg on seven crops (arugula, clover, carrot, kohlrabi, lettuce, onion and savoy cabbage) seed germination presented in figure 1.

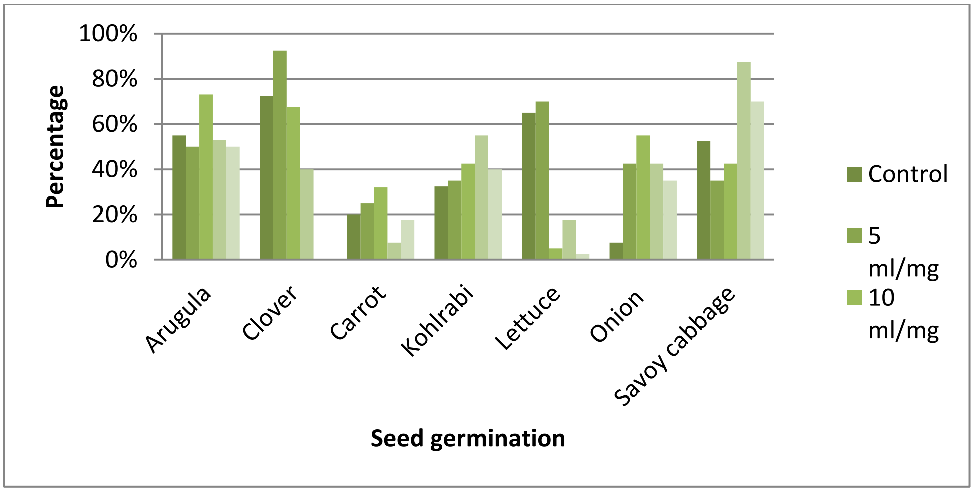

Figure 1. Onion water extracts effect on seed germination of seven crops

Effects of onion water extracts on arugula seed germination, showed that the concentration of 10 ml/mg had stimulating impact on seed germination 73% compared to control 55%, concentrations 5 ml/mg and 50 ml/mg had the greatest inhibition effect 50% of the seeds germinated, while 53% of seeds were able to germinate under the concentration of 25 ml/mg. Effects of onion aqueous on clover seed germination, results show that the concentration of 5 ml/mg had stimulating effect on seed germination 92.5% germinate compared to control 72.5%, other three concentrations had negative effects and reduced clover seed germination percentage 10 ml/mg 67.5%, 25 ml/mg 40% and 50 ml/mg 0% compared to control. Germination percentage of carrot seeds, positive effects on germination percentage were manifested by water extract concentrations 10 ml/mg 32%, 5 ml/mg 25% compared to control 20%, inhibitory effects expressed by 25 ml/mg 8% and 50 ml/mg 18% compared to control 20% seeds germinated. Kohlrabi seed had the greatest seed germination percentage when they were treated with the water extract concentration of 25 ml/mg 55% compared to control 32.5%, other concentrations showed also a positive effect on kohlrabi seed germination. Onion water extracts showed negative effects on lettuce seed germination in concentrations 5, 25, 50 ml/mg, only 2.5% of seeds germinated under 50 ml/mg concentration treatment compared to control 65%, concentration 10 ml/mg showed positive effects on germination 70% of seeds germinated. Allelopathic effect on onion was positive; all concentrations had an increasing effect on onion seed germination raging from 35% to 55% compared to the control 7.5%. Onion water extracts on savoy cabbage had stimulating germination effects in concentrations 25 and 50 ml/mg 70-85%, compared to control 52.5%, inhibiting mode of action was expressed by 5 and 10 ml/mg concentrations 35-42.5% compared to control.

Effects of onion water extracts in concentrations 5 ml/mg, 10 ml/mg, 25 ml/mg and 50 ml/mg on seven crops (arugula, clover, carrot, kohlrabi, lettuce, onion and Savoy cabbage) seedling growth shown in figure 2. It contains the average values of seedling growth expressed in millimeters

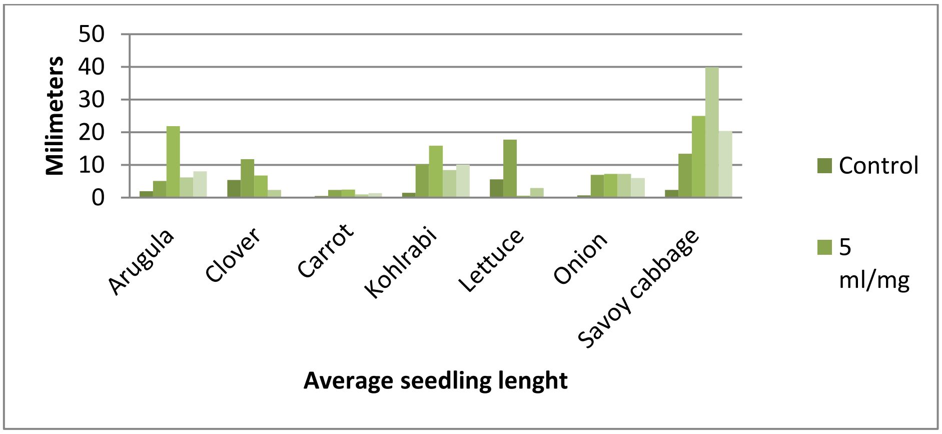

Figure 2. Effects of onion water extracts on seedling growth

Arugula seedling growth was stimulated by all four concentrations of onion water extract, the greatest effect on average length of seedlings had the extract 10 ml/mg 21.8 mm compared to control 1.98. Other three concentrations also had a positive effect on seedling growth. On clover seedling length the greatest positive impact was expressed by concentrations 5 ml/mg and 10 ml/mg 11.7 mm and 6.77 mm compared to control 5.35 mm. While the other two onion water extract concentrations 25 ml/mg and 50 ml/mg reduced the growth size of seedlings 6.77 mm and 2.35 mm compared to control 11.77 mm. All extracts had a stimulating effect on carrot seedling growth, the water extracts 5 ml/mg and 10 ml/mg had the greatest effect 2.4 mm compare to control 0.5 mm. Kohlrabi seedlings were stimulated by all four onion water extracts, but mostly by the concentration 10 ml/mg, 15.9 mm compared to control 1.43 mm. On lettuce we have observed that one concentration had stimulating effect on seedling growth 5 ml/mg 17.7 mm compared to control 5.53 mm, the other three concentrations had an inhibiting effect on seedling growth the largest had been exhibited by 50 ml/mg 0.05 mm compared to control. All water extracts had a stimulating effect on onion seedling length with the value 7.18 mm compared to control 0.65 mm. Savoy cabbage had been influenced positive by all four concentrations of onion water extracts, the concentration of 25 ml/mg had the greatest impact 39.85 mm compared to control 2.33 mm.

CONCULUSION

In this research the effect of onion leaf water extracts was studied in different concentrations on seven crops, seed germination and average seedling length. Based on previous investigations of onion water extracts that were mostly inhibiting the germination and growth of tested plants. The results of this trial showed both stimulating and inhibiting effects. From those results it can be concluded that onion water extracts have a great potential to be used in agricultural production, as a growth and germination stimulator. Further field trials are necessary in order to observe the effects of water extracts on crops in natural conditions.

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EVALUATION OF PRODUCTIVE TRAITS OF NEWLY INTRODUCED PLUM CULTIVARS IN THE WESTERN SERBIA CONDITIONS

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ABSTRACT

Autochthonous cultivars, well-known cultivar 'Stanley' and cultivars named and released in Fruit Research Institute, Čačak such as: 'Čačanska lepotica', 'Čačanska rodna', 'Čačanska rana' and 'Čačanska najbolja' dominated in plum assortment in Serbia. Introduction of new cultivars is necessary in order to fulfill different demands of fresh fruit market and achieve better results in modern plum production. Significant evaluation of these cultivars in environmental conditions of region they are being introduced is required before their growing in commercial orchards. The main objective of this study was to evaluate the most importand productive traits of six introduced plum cultivars: 'Hanita', 'Jojo', 'Jubileum', 'Presenta', 'Tegera' and 'Valor' in the Western Serbia conditions. The most important phenological (flowering and ripening time), and morphometric traits (fruit and stone weight, fruit dimensions and flash percentage), as well as tree vigour (trunk cross section area) and cropping potential (yield per tree and yield efficiency) were evaluated. Flowering phenofase in all examined cultivars lasted during the second decade of april, whereby flowering abundance was estimated as very good. The largest fruit weight, and fruit dimensions (height, width and thickness) were determined in 'Jubileum' and 'Valor', whereas these parameters were the smallest in 'Presenta'. The largest stone weight and tree vigor was found in 'Jubileum'. The largest flesh percentage was found in cultivars 'Jojo', 'Tegera' and 'Valor'. Cultivar 'Presenta' had the largest yield per tree, while 'Jojo' had the highest yield efficiency. The smallest values of yield, tree vigor and yield efficiency were determined in 'Valor', 'Hanita' and 'Jubileum', respectively.

Key words: *plum, introduced cultivars, flowering time, productive traits, yield.*

INTRODUCTION

European plum (*Prunus domestica* L.) is one of the most important species grown in Europe with total production of 2 472 603 t (FAOSTAT, 2019). Total plum production in the Republic of Serbia amounts about 425 441 t (2013-2017), which ranked third in total world production after China and Romania. Generaly, plum production in Serbia is characterized with extensive growing technology, limited utilization of agrotechnical and pomotechnical treatments (which resulted in low yields and poor fruit quality), problems caused with Sharka virus, as well as diversity of cultivars (Nenadović-Mratinić et al., 2007; Milošević et al., 2012; Milošević et al., 2013). As a result, average yield per tree is very small and amounts only 5,9 kg (Milošević et al., 2017). Increasing demands of fresh fruit market, indicate that introduction of new high quality cultivars tolerant to Sharka virus is necessary (Čmelik et al., 2007). New high quality cultivars combined with appropriate growing technology are basis of successful modern plum production. Cultivars developed all over the world must be well adapted to local conditions, therefore evaluation of introduced cultivars is very important prerequisite for improvement of plum production.

The main objective of this manuscript was to examine the most important phenological (flowering and harvest time), morphometric (fruit weight and dimensions, stone weight and flesh percentage), as well as tree vigor (trunk cross sectional area) and productivity (yield per tree and per hectare and yield efficiency) of six introduced plum cultivars: ‘Hanita’, ‘Jojo’, ‘Jubileum’, ‘Presenta’, ‘Tegera’ and ‘Valor’ in the Western Serbia conditions as one of the most important plum production areas in Serbia.

MATERIAL AND METHODS

Experiment was carried out at facility Preljinsko Brdo of Fruit Research Institute, Čačak, ten kilometers north-east from Čačak. The plant material was six introduced plum cultivars: ‘Hanita’, ‘Jojo’, ‘Jubileum’, ‘Presenta’, ‘Tegera’ and ‘Valor’ grafted on St. Julian A rootstock. Orchards was established in spring 2005 with standard plant material. Trees were planted at distance $4 \times 1,5$ m (1667 trees per hectare) and training system is slender spindle. The orchard was fertilized on the basic local empiric criterion with 500 kg of compound NPK (15:15:15) mineral fertilizer in fall and with 400 kg/ha of calcium ammonium nitrate (CAN) contained 27% of N at the onset of the growing cycle. Trees were grown under standard practices for plum, without irrigation applied. Weather conditions of Čačak area are characterized by the average annual temperature of 11.3°C and total annual rainfall of 690.2 mm. The experiment was set up as a randomized block design in three replicates with 5 trees each (total 15 trees per year was also calculated for each cultivar). Flowering phenophase was examined in accordance with methodology recommended by International Pollination Working Group (Wertheim, 1996). Records were made of dates of flowering onset (10% of open flowers on a tree), dates of full bloom (80% of open flowers on a tree) and end of flowering (over 90% of petals fell). Data was shown as a number of days from January 1. The harvest time was determined when the fruit samples were hand harvested fully mature, at commercial maturity stage in 2017 and 2018. For a period of two harvest seasons, 25 fruits from each cultivar of each of three replicates were collected and fruit and stone weight (g) were measured using an Ohaus Adventurer technical scale (Parsippany, NJ, USA). Yield per tree (kg) was measured in 2018 using an ACS System Electronic Scale (Zhejiang, China). For determining flesh percentage fruits were cut in half horizontally with a stainless-steel knife and the stones were removed and weighed. The flesh percentage (%) was calculated by subtracting the stone weight from the whole plum fruit weight. For each plum fruit, three linear dimensions, hight, width and thickness were measured by using a digital caliper Starrett, 727 Series (Athol, NE, USA) with a sensitivity of 0.01 cm. Soluble solids content was determined by Milwaukee MR 200 hand refractometer (ATC, Rocky Mount, NC, USA) at 20ºC (ºBrix). Trunk circumferences (cm) were measured at the end of growing season 20 cm above the graft union and used to calculate the trunk cross-sectional area (TCSA, cm^2). The yield efficiency was calculated as the ratio between yield per tree and trunk cross-sectional area (TCSA).

Data in the present study were subjected by analysis of variance (ANOVA) using the MSTAT-C statistical package [Michigan State University, East Lansing, MI, USA] and means were separated by LSD test at $p \leq 0.05$.

RESULTS AND DISCUSSION

Flowering onset, full bloom, end of flowering, flowering abundance and harvest time are presented in Table 1. All examined cultivars were blooming during the second decade of April in both years. The earliest flowering onset was determined in ‘Jojo’, while the latest was observed in cultivars ‘Jubileum’ and ‘Tegera’ with the difference of only three days. Full bloom started approximately 3 to 4 days, while end of flowering occurred 9 to 10 days after flowering onset depending on cultivar. Flowering abundance was estimated to be very good in both years.

Table 1. Phenological properties of studied plum cultivars

Cultivar	Flowering time			Flowering abundance	Harvest time	
	Onset	Full	End			
‘Hanita’	103	108	112	4	227	15.08.
‘Jojo’	101	103	109	4	244	2.09.
‘Jubileum’	104	108	114	4	226	14.08.
‘Presenta’	102	105	112	4	259	16.09.
‘Tegera’	103	107	112	4	199	18.07.
‘Valor’	102	107	113	4	243	1.09.

*flowering and harvest time is presented as number of days since January 1

Similar results regarding flowering phenofase were obtained by Milošević et al. (2012) in the Serbian conditions, Vitanova et al. (2007) in Bulgaria and Koskela et al. (2010) in Netherlands, while Blažek and Pištěková (2009) determined later flowering time in Czech Republic conditions due to colder climate in that country. Among examined cultivars, the earliest harvest time was found in ‘Tegera’, medium late in ‘Jubileum’ and ‘Hanita’, late in ‘Jojo’ and ‘Valor’ and very late in ‘Presenta’. Early ripening time cultivars, such as ‘Tegera’ had a shorter harvesting period in regard to cultivars with later harvest time such as ‘Jojo’ and ‘Presenta’. Harvest date of ‘Jubileum’ is not optimal due to lot of high quality cultivars with similar rippening time, especially if it is well known that cultivars with violet skin colour are not popular on markets. On the other hand, there is a great demand for cultivars with late and very late harvest time due to lack of high quality fresh fruits on the market in that period.

Fruit weight is one of the most important quantitative trait which affects yield, fruit quality attributes and consumers acceptability (Chrisosto et al., 2004). In our study, fruit weight significantly varied from 29.38±0.70 g to 48.18±0.83 g (Table 2). Cultivars ‘Valor’ (48.18±0.83 g) and ‘Jubileum’ (46.63±0.86 g) had significantly higher fruit weight compared to other cultivars. On the other hand, ‘Presenta’ (29.38±0.70 g) had the smallest fruit weight. This trait is mainly affected by genotype (Nergiz and Yıldız, 1997), as well as yield and agrotechnical and pomotechnical treatments in the orchard (Gryzb i Sitarek, 2006) which is confirmed in our study. Results obtained in this study showed higher fruit weight in ‘Hanita’, and smaller in ‘Valor’ than the results reported by Blažek and Pištěková (2009) in Czech Republic. According to Hartmann and Neumüller (2006) in conditions of Germany, fruit weight of ‘Hanita’ and ‘Jojo’ was higher than results determined in our study. These differences are probably caused with different pedoclimatic conditions and/or different orchard management. The smallest stone weight was found in ‘Jojo’ (1.48±0.03 g) and ‘Tegera’ (1.50±0.04 g), while the largest was determined in ‘Jubileum’ (2.22±0.03 g) (Tabela 2).

Table 2. Fruit and stone weight and flesh percentage of studied plum cultivars

Cultivar	Fruit weight (g)	Stone weight (g)	Flesh percentage (%)
‘Hanita’	34.39±0.74 c	1.84±0.06 c	94.65±0.16 c
‘Jojo’	36.98±1.33 b	1.48±0.03 e	95.97±0.13 a
‘Jubileum’	46.63±0.86 a	2.22±0.03 a	95.22±0.13 b
‘Presenta’	29.38±0.70 d	1.55±0.02 d	94.70±0.13 c
‘Tegera’	36.40±0.76 bc	1.50±0.04 de	95.88±0.10 a
‘Valor’	48.18±0.83 a	1.97±0.02 b	95.90±0.08 a

**The different lower-case letters assigned to columns show significant differences for $P \leq 0.05$ after applying LSD test.*

Flesh percentage represents flash and stone ratio and it is preferable to be as higher as possible (Nenadović-Mratinić et al., 2007). In our study, the highest flesh percentage was obtained in ‘Jojo’, ‘Valor’ and ‘Tegera’ (95.97±0.13%, 95.90±0.08%, 95.88±0.10%, respectively), and the smallest in ‘Hanita’ (94.65±0.16%).

Table 3. Fruit height, width and thickness of studied plum cultivars

Cultivar	Fruit height (mm)	Fruit width (mm)	Fruit thickness (mm)
‘Hanita’	45.19±0.47 bc	36.13±0.45 c	34.22±0.24 c
‘Jojo’	41.54±2.40 d	35.59±0.37 c	35.44±0.59 b
‘Jubileum’	47.33±0.59 ab	44.68±0.48 a	41.55±0.46 a
‘Presenta’	44.86±0.38 bcd	33.90±0.39 d	33.68±0.44 c
‘Tegera’	43.04±1.10 d	36.98±0.48 b	34.56±0.53 bc
‘Valor’	50.04±0.31 a	44.63±0.43 a	41.40±0.33 a

**The different lower-case letters assigned to columns show significant differences for $P \leq 0.05$ after applying LSD test.*

The fruit dimensions are important in fruit shape description which is used in pomological research, segregation of cultivars and cultivars registration (Beyer et al., 2002). In our study, the lergest values of fruit dimensions (fruit height, width and thickness) were found in 'Valor' (50.04±0.31 mm, 44.63±0.43 mm, 41.40±0.33 mm, respectively) and 'Jubileum' (47.33±0.59 mm, 44.68±0.48 mm, 41.55±0.46 mm, respectively) (Table 3). On the other hand, the smallest fruit height was determined in 'Jojo' (41.54±2.40 mm) and 'Tegera' (43.04±1.10 mm), whereas the smallest fruit width and thickness were found in 'Presenta' (33.90±0.39 mm, 33.68±0.44 mm, respectively) (Table 3). These results are very similar to results previously reported by Blažek and Pištěková (2009) in Czech Republic, whereas Halapija-Kazija et al. (2009) stated higher values of these parameters in conditions of Croatia.

Values of trunk cross sectional area, as indicator of three vigor, yield per three, yield per hectare and yield efficiency of studied cultivars are shown in Table 4. The largest three vigor was obtained in 'Jubileum', while the smallest three had cultivars 'Hanita' and 'Jojo'. The highest yield per tree and per hectare were determined in 'Presenta' (23.45±0.22 kg and 53.88±2.31 t).

Table 4. Tree vigor, yield per tree and yield efficiency of studied plum cultivars

Cultivar	Yield per three (kg)	Yield per hectare (t)	TCSA (mm^2)	Yield efficiency
'Hanita'	21.21±0.39 c	35.36±0.39 c	38.84±1.24 d	0.55±0.02 b
'Jojo'	22.85±0.25 b	38.09±0.25 b	39.05±0.82 d	0.59±0.01 a
'Jubileum'	20.60±0.35 d	34.34±0.35 d	61.14±1.14 a	0.34±0.01 d
'Presenta'	23.45±0.22 a	39.10±0.22 a	53.88±2.31 b	0.44±0.02 c
'Tegera'	20.59±0.26 d	34.32±0.26 d	45.90±1.56 c	0.45±0.02 c
'Valor'	18.37±0.42 e	30.62±0.42 e	40.39±1.43 d	0.46±0.02 c

**The different lower-case letters assigned to columns show significant differences for $P \leq 0.05$ after applying LSD test.*

Cultivar 'Jojo' had the highest yield efficiency (0.59±0.01) due to high yield per three and smallest three vigor, whereas this parameter was the smallest in 'Jubileum' (0.34±0.01) who had largest tree vigor and medium yield.

Tree vigor depends on cultivar (Nenadović-Mratinić et al., 2007), rootstock (Grzyb i Sitarek, 2006; Stefanova et al., 2010), training system, yield, as well as age and orchard condition (Vitanova et al., 2007). Orhard management and environment could also affect the tree vigor (Blažek i Pištěková, 2009). Similar to our results, Stefanova et al. (2008) and Blažek and Pištěková (2009) determined that cultivars ‘Hanita’, ‘Jojo’, ‘Tegera’ and ‘Valor’ had medium tree vigor, whereas Grzyb and Sitarek (2006) reported that plum cultivars grafted on ‘St. Julian A’ rootstock had significantly lower tree vigor compared to trees grafted on ‘Myrobalan’ or ‘Fereley’. Results obtained in our work showed that cultivars ‘Jojo’, ‘Presenta’ and ‘Hanita’ had the largest cropping potential among studied cultivars. Similar statements were reported by Blažek and Pištěková (2009), and Stefanova et al. (2008) for these cultivars, whereas Gravite and Kaufmane (2017) determined similar yield in ‘Tegera’. These authors also reported that cultivars ‘Hanita’ and ‘Jojo’ had the highest yield efficiency.

CONCLUSION

Flowering time of all examined cultivars in 2017 and 2018 occured in the second decade of April, whereby flowering abundance was very good. In western Serbia conditions, ‘Tegera’ is an early ripening time cultivar, while ‘Jojo’, ‘Valor’ and ‘Presenta’ are very late ripening time cultivars so these cultivars could be very interesting for growing in commercial orchards due to lack of high quality cultivars with similar ripening time. All examined cultivars except ‘Jubileum’ and ‘Presenta’ showed lower tree vigor so could be suitable for high density orchards. All six cultivars, especially ‘Jojo’ and ‘Presenta’ exposed high cropping potential. The largest fruit weight and fruit dimensions were found in ‘Valor’. All cultivars demands further evaluation concerning fruit quality, pest resistance and different orchard management. These cultivars combined with modern orchard management could be very important factor in plum production improvement in next period.

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RATE AND DURATION OF GRAIN FILLING IN SIX-ROWED WINTER BARLEY

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ABSTRACT

Kernel weight is a function of the rate and duration filling (GF). In order to study the relationships among grain yield and yield components and the rate and duration of GF in winter six-rowed barley, field experiments were conducted at Novi Sad during the 1985-1988 period. A quadratic polynomial was used to describe the relationship between kernel weight and time from anthesis. The rate and duration of grain filling were obtained from the fitted curve. Accumulated growing-degree days (GDD) from anthesis were used as a time scale. The rate of GF ranged from 0.058 to 0.080 mg kernel^{-1} GDD^{-1} and duration of GF from 505 to 888 GDD. Rate of GF had a positive association with yield (0.81) and kernel number per spike (0.50). Duration of GF had a negative influence on grain yield (0.69). The positive correlation of yield with kernel number and the positive correlations of kernel number with kernel weight and rate of GF suggest that it should be possible to simultaneously improve the rate of GF and kernel weight. The negative correlation between the rate and duration of GF (-0.88) was mainly due to high temperatures in the late and final grain fill phases.
Key words: *winter barley (Hordeum vulgare ssp. vulgare L.), grain filling, yield, correlation*

INTRODUCTION

After spike number and kernel number per spike have been determined during the vegetative phase, cereal grain yields become proportional to kernel weight (Wiegand, Cuellar, 1981), which is a function of the rate and duration of grain filling (GF). The GF is the result of the translocation of photosynthate from source to kernels. Rate of GF represents the rate of dry matter accumulation per kernel during the period of GF. The GF period represents the duration from anthesis to physiological maturity.

Rate of GF depends on the number of endosperm cells formed during the first two weeks after anthesis (Brocklehurst, 1977) and, to a lesser extent, on increased temperature in that period (Sofield et al., 1977a, 1977b). Duration of GF is in strong negative correlation with temperature (Spiertz, 1977; Wardlaw et al., 1980; Wiegand and Cuellar, 1981; Wych et al., 1982; van Sanford, 1985; Stapper and Fischer, 1990). Genetic factors to a large extent determine the rate of GF, and environmental factors, first of all temperature to a large extent determine the duration of GF (van Sanford, 1985; Bruckner and Frohberg, 1987; Campbell et al., 1990; Triboi, 1990; Hunt et al., 1991). An increase in temperatures up to a certain point does not negatively affect the yield, since the intensification of physiological processes can compensate for the shortening of GF (Sofield et al., 1977b). Longer duration of high temperature reduces GF period to such a large extent that faster rate of GF cannot prevent yield losses (Wardlaw et al., 1980). When duration of GF is severely limited by temperature, final kernel weight is proportional to the rate of GF (Wiegand, Cuellar, 1981).

The objectives of this study were to examine (i) effect of cultivar and year on rate and duration of GF, (ii) association between kernel growth characters and yield components and (iii) kinetics of water during the GF process in six-rowed winter barley.

MATERIAL AND METHODS

Two six-rowed winter barley cultivars, Galeb and Botond, were used for investigations. The cultivar Galeb was released by the Institute of Field and Vegetable Crops, Novi Sad and cultivar Botond by the Agricultural Research Institute GATE “Fleischmann Rudolf” Kompolt, Hungary. The trials were sown on limeless chernozem soil at Novi Sad (45° 20´ N, 15° 51´ E, 86 m asl) on 15 Oct 1994, 20 Oct 1995, 12 Oct 1996 and 17 Oct 1997 at a planting rate of 350 viable seeds per m^2, in two identical trials with three replications. Plots were 5m long and consisted of 6 rows 20 cm apart. The first trial was used for GF parameters determination and the second for yield and yield components determination. Rate and duration of GF and the yield parameters were estimated for each replication in four years. The cultural practices applied were those regularly used for large scale winter fodder barley production.

At anthesis 60 main spikes from each plot of the first trial that flowered on the same day were tagged. Samples of four tagged spikes were collected from each plot at 3-4 days intervals beginning about 5-10 days after anthesis and continuing past harvest maturity. Spikes were weighed immediately after sampling, oven-dried at 70 °C for 48h to water content determination, then hand trashed in bulk to determine average kernel dry weight. Accumulated growing-degree days (GDD) from anthesis were used as the time scale. Rate of GF was expressed as milligrams per kernel per GDD.

The relation between kernel weight and accumulated GDD from anthesis for each plot was presented by fitting a quadratic polynomial $W = a + bt + ct^2$, where W is kernel dry weight (mg), t is time (GDD) from anthesis and a, b, and c - regression coefficients. The instantaneous rate of grain filling dW/dt can be calculated from the derivative of the polynomial $dW/dt = b + 2ct$ (Nass, Reiser, 1975; Gebeyehou et al., 1982). When kernel weight has reached its maximum, then $dW/dt = 0$. Solving for t in $dW/dt = 0$ gives t_2 as the estimated point of the end of duration of GF. Anthesis, i.e. beginning of GF (t_1) calculated for $W = 0$ and predicted duration of GF obtained as $t_2 - t_1$. The average of rate of GF in the interval from t_1 to t_2 can be defined as $1/(t_2 - t_1) \int_{t1}^{t2} dW/dt)\, dt = (W_2 - W_1)/(t_2 - t_1)$ (Radford, 1967), where W_1 and W_2 are the predicted kernel weights at times t_1 and t_2. Mean GF rates were estimated as predicted maximum kernel dry weight divided by GF duration. The relation between kernel water proportion and accumulated GDD from anthesis for each plot was presented by fitting a linear equation $M = a + bt$, where M is percentage of kernel moisture content, t is time (GDD) from anthesis and a and b–regression coefficients. For percentage of kernel moisture content $\arcsin\sqrt{percentage}$ transformation was used.

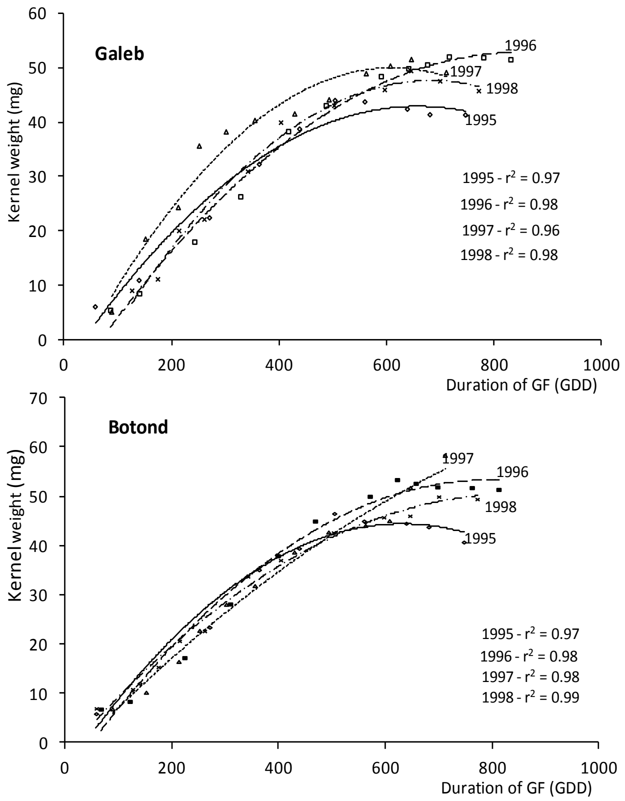
Grain yield, number of productive tillers per square meter, kernel number per spike, and kernel weight were determined for each plot and year in the second trial. Analysis of variance for each character was conducted. Simpl and path correlation coefficients among the estimated GF parameters and associated agronomic characters were calculated.

RESULTS AND DISCUSSION

The quadratic polynomial, used to describe kernel growth from anthesis to physiological maturity, provided a good description of GF for these cultivars and years. Kernel weight and GDD data have fit the model well and r^2 values exceeded 0.97 in all cases. Only for the cultivar Galeb in 1997 the linear equation gave a better description of GF for all three replications, with r^2 value higher than 0.97 (Fibure 1).

The fastest rate of GF and shortest GFD in the cultivar Botond was recorded in 1997 (0.078 mg kernel^{-1} GDD^{-1} and 505 GDD respectively). Temperatures at the late and final GF phases were moderate and did not represent a limiting factor for a longer GF. The shortening of GFD was due to high temperatures in mid-May which caused reduction of the initial and early GF phases. The slowest rate of GF (0.065 mg kernel^{-1} GDD^{-1}) and longest duration of GF in Botond was recorded in 1996 (Table 2). High temperatures in 1996 at the late and final grain filling phases did not shorten the duration of GF, so both the actual (the largest kernel weight obtained by measurement) and predicted (kernel weight in *dW/dt* = 0) kernel weight were achieved at 850 GDD. In the other two years, the rate and duration of GF were between those recorded in 196 and 1997.

Kernel water content and GDD fit the linear model well, r^2 values ranged from 0.83 to 0.94. Cultivars did not differ significantly in the rate of water release (WR); average rate of WR was 0.049% kernel^{-1} GDD^{-1} for Galeb and 0.050% kernel^{-1} GDD^{-1} for Botond. The rate of WR depended on the year and cultivar x year interaction (Table 3). The fastest WR was in 1995, 0.058% kernel^{-1} GDD^{-1} and the shortest in 1996, 0.045% kernel^{-1} GDD^{-1}.



Graph. 1. Relation between duration of grain filling and kernel weight in winter barley

The rate of GF in Galeb ranged between 0.058 mg kernel^{-1} GDD^{-1} in 1998 and 0.080 mg kernel^{-1} GDD^{-1} in 1997 were similar, while the duration of GF in 1997 was 76 GDD, or about five days, longer than that in 1995. The longer GF in 1997 was a result of lower temperatures in late May and early June, i.e. at the late grain-filling phase (period from the milk ripe phase till cessation of photosynthesis) and final grain-filling phase (period from the cessation of photosynthesis till maturity (Takahashi et al., 1993). In 1998 in Galeb, GF was the slowest and lasted the longest. This was a result of an earlier flowering (5-6 days) relative to the other years as well as of lower temperatures in the first half of GF (Table 1).

Table 1. Date of anthesis and mean 10-day temperatures during GF period

Year	Date of anthesis		1-10 May	Dev. from long-period average	11-20 May	Dev. from long-period average	21-31 May	Dev. from long-period average
	Galeb	Botond						
1995	May 16	May 13	19.2	-3.1	24.1	+1.2	19.0	+0.6
1996	May 17	May 16	20.0	+3.6	16.3	-0.8	22.5	+4.1
1997	May 16	May 16	21.2	+4.9	14.9	-2.2	17.6	-0.8
1998	May 11	May 11	15.8	-0.5	16.8	-0.3	23.0	+4.6

The fastest rate of GF for both cultivars and the shortest duration of GF for Botond were recorded in 1997. That year mean daily temperatures at the early GF phase were very high and those at the late and final GF phases below average. The significant contribution of the year to the rate of GF is supported by the variance analysis data as well (Table 3). The influence of the cultivar on rate of GF was manifested trough significant genotype x year interaction. The duration of GF depended on all three factors – cultivar, year, and their interactions (Table 3). A large number of authors (Bruckner and Frohberg, 1987; Campbell et al., 1990; Hunt et al., 1990), however, report finding the effect of the genotype to be the most important in the variance of rate of GF and that of year, i.e. environmental factors, in the case of duration of GF. The variation of spike number per m^2 depended on the year and cultivar x year interactions and that of kernel weight on the cultivar and year. Kernel number per spike and yield depended on the main factors and their interactions.

Table 2. Means of yield components and grain filling parameters in four years

Year	SN		KN		KW		Y		GFR		GFD	
	Galeb	Botond	Galeb	Botond	Galeb	Botond	Galeb	Botond	Galeb	Botond	Galeb	Botond
1995	409	432	50	46	41.5	40.3	7.20	6.75	0.079	0.072	567	726
1996	438	352	55	52	46.1	45.1	9.00	6.62	0.071	0.065	756	853
1997	459	467	61	56	47.1	43.1	11.87	11.37	0.080	0.078	643	505
1998	462	463	48	50	43.9	41.5	9.33	9.29	0.058	0.071	888	673
LSD_Y	25*	34	2.68*	3.71**	.96*	1.33**	0.76*	1.01**	0.0031*	0.0043**	19*	27**
LSD_{GxY}	35*	49	3.78*	5.25**	135*	1.88**	1.07*	1.49**	0.0044*	0.0061**	27*	38**

*0.05 **0.01

SN- spike number per m^2, KN- kernel number per spike, KW- 1000-kernel weight (g), Y- yield (t ha^{-1}), GFR- rate of grain filling (mg $kernel^{-1}$ GDD^{-1}), GFD- duration of grain filling (GDD)

Table 3. Square means for yield components, rate of GF, duration of GF, and rate of water release (WRR)

Source of variation	Degre of freedom	SN	KN	KW	Y	GFR	GFD	WRR
Replication	2	692	11.38	0.28	139212	0.0000032	460	0.0000006
Cultivar	1	1053	42.67**	27.74**	4275704**	0.0000002	3384**	0.0000015
Year	3	6691**	131.61**	28.28**	24919415**	0.000267**	72311**	0.0001952**
C x Y	3	3582**	16.33*	2.86	1640438*	0.000117**	48775**	0.0000086**
Error	14	399	4.66	0.60	383184	0.0000063	240	0.0000009

$^*P \leq 0.05$, $^{**}P \leq 0.01$

Of the three main yield components – spike number, kernel number, and kernel weight, the first two appear at the vegetative phase and the third after anthesis, at grain fill (Wiegand and Cuellar, 1981). Because of this, the rate and duration of GF and their relationship with the other yield components are of great importance in barley breeding. The relationships between the estimated parameters of grain filling, yield components, and grain fill were described by the coefficient of correlation and path coefficient (Table 4). The yield of six-rowed winter barley was positively correlated with rate of GF and negatively correlated with duration of GF. The highest yields in both varieties were obtained with the fastest rate of GF and the shortest duration of GF. The values of the indirect effects of rate and duration of GF on yield via the components of yield were low and had the opposite signs. Sofield et al. (1977a), Gebeyehou et al. (1982), van Sanford (1985) and Darroch and Baker (1990) pointed out that high kernel weight is associated with rapid rate of GF, while Nass and Reiser (1975), Gebeyehou et al. (1982) and Wong and Baker (1986), reported positive correlations between effective filling period and grain yield. On the basis of the simple coefficient of correlation, yield was found to be significantly correlated only with kernel number, while path coefficient analysis showed a significant direct influence of spike number and kernel weight. The significant positive correlation of yield and rate of GF with kernel number per spike as well as that of kernel number per spike with kernel weight and duration of GF enable a more successful selection for yield through breeding for a larger kernel number.

Table 4. Correlations (*r)* between yield and yield components, and path coefficients for direct effects of yield components on yield and for indirect effects of rate of GF and duration of GF on yield via other yield components
(Data shown are for two cultivars, four years, and three replicates with the total *n* = 24)

Yield component	KN	KW	GFR	GFD	Y	Direct effect of yield components on yield	Indirect effect of GFR and GFD on yield via yield components	
							GFR	GFP
SN	0.16	-0.08	0.17	-0.30	0.36	0.29**	0.05	-0.09
KN		0.72**	0.50**	-0.36	0.61**	0.01	0.00	-0.00
KW			-0.03	0.24	0.24	0.68**	-0.02	0.17
GFR				-0.88**	0.81**	0.46*	-	-0.40
GFD					-0.69**	-0.18	0.16	-

$^{*}P \leq 0.05$, $^{**}P \leq 0.01$

The correlation between the rate and duration of GF was negative, although most other authors (Sofield et al., 1977a; Wardlaw et al., 1980; Sayed et al., 1983; van Sanford, 1985) report a lack of any association. Brukner and Frohberh (1987) found a strong negative correlation between these two traits, which indicates that the environmental conditions favor a high rate of GF and short duration of GF. Consequently, the relationships we obtained in the present study can be attributed to the strong negative environmental correlation. During the latter part of grain filling, genotypes with a long GF duration may enter a period of high temperatures (Przulj and Momcilovic, 1998), which may significantly reduce yields and grain quality. The duration of the vegetative and generative phases should be balanced, since neither too early nor too late a flowering will bring maximum yields.

Conclusions

Earlier flowering results in longer period of grain filling. Higher temperatures in the initial and early grain filling phases reduce duration of grain filling, while temperatures at the second half of grain filling don't significantly short duration of grain filling. Rate of grain filling is under stronger influence of environmental factors and duration of grain filling under genotype, environment and their interaction.

Yield of six-rowed winter barley is positively correlated with rate and negatively with duration of grain filling. The choice of genotypes with a high rate of GF whose developmental dynamics are suitable for particular environmental conditions represents a more certain way of developing stable, adaptable and high-yielding varieties.

The breeding program on six-rowed winter barley for the environmental conditions of the Southeast Europe should favor genotypes with a larger kernel number per spike and those with a large kernel size, a high rate of GF and a moderate GF duration.

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EFFECT OF PGPR TREATMENTS ON SOYBEAN [*GLYCINE MAX* (L.) MERR.] GROWTH AND YIELD UNDER DROUGHT STRESS

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ABSTRACT

The effects of plant growth promoting rhizobacteria (PGPR) on soybean morphological parameters were tested in field conditions during 2015 using seed co-inoculation treatments: A - Bradyrhizobium japonicum 526+ Bacillus sp. Q10 + Pseudomonas sp. Q4 and B - B. japonicum + Bacillus sp. Q10 + Pseudomonas sp. M124. Comparing to the control (commercial fertilizer that contain B. japonicum), both treatments showed positive effects on soybean growth parameters and nodulation under drought conditions. The yields of 2740, 2750 and 2581 kg ha^{-1} were measured for variants A, B and control, respectively. These results have practical relevance, indicating the great importance of PGPR in soybean production, especially in drought conditions.

Key words: *PGPR, co-inoculation, soybean, field study, drought*

INTRODUCTION

Plant Growth Promoting Rhizobacteria (PGPR) are root associated bacteria with important role in agriculture production. These bacteria colonize the roots of plants (rhizosphere) that enhance plant growth (Vejan et al. 2016). Rhizobacteria stimulate plant growth by nitrogen fixation, solubilization of mineral nutrients, production and regulation of phytohormones, mineralize organic compounds, act as biocontrol agents and biofertilizers (Martínez-Viveros et al. 2010; Gupta et el. 2015). Among bacteria in the rhizosphere of many leguminous and nonleguminous crops that belong to genera *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus, Burkholderia, Enterobacter, Klebsiella, Pseudomonas, Rhizobium* and *Serratia*, the most extensively studied rhizobacteria that promote plant growth and development are *Pseudomonas, Rhizobium* and *Bacillus* species (Kumar et al. 2011; Bouizgarne, 2013). Various field and laboratory studies worldwide demonstrated positive influence of PGPR on the cultivated plants (Sharma and Johri, 2003; Erkurk et al. 2011; Marinković et al. 2016; Jošić et al. 2016; Stanojković-Sebić et al. 2017; Iličić et al. 2017; Marinković et al. 2018).

Soybean is one of the most important leguminous crops used for human and animal nutrition, with the chemical composition of the grain, about 40% protein and about 20% of the oil (Marinković et al. 2016; Jeločnik and Zubović, 2018). The advantage of soybean production in Serbia is in GMO-free production, which represents the security of exports to the world (Živanović and Popović, 2016). Vojvodina district is very suitable for soybean production, because of its favorable geographic location, climate and natural characteristics (Jeločnik and Zubović, 2018).

High temperature stress and unpredictable rainfall pattern caused by global climate change impact sustainable food production. Drought stress is the most prevalent environmental factor that limits growth, survival, physiology, nutrient and water relations, photosynthesis and productivity of plants (Yordanov et al. 2000). Drought also influence the growth of microorganisms and their ability to survive. Many bacteria are able to survive under stressful conditions due to the production of exopolysaccharide in high quantities (Putrie et al. 2013). *Bradyrhizobium*, as seed inoculant and symbiont of soybean, is capable to fix atmospheric nitrogen and reduce it to ammonia leading to improvement of soybean production, even in the drought stress. PGPR other than *Bradyrhizobium* also contribute to the tolerance and quick adaptation and adjustment of plants to drought stress (Vurukonda et al. 2016).

The aim of this study was to evaluate the effect of soybean seed co-inoculation with addition of *Bacillus* sp. and *Pseudomonas* spp. to *B. japonicum* 526 on main morphological parameters and yield in field conditions under drought stress.

MATERIAL AND METHODS

Bacterial strains

PGPR were isolated from different host plants as a part of project III46007 (Ministry of Education, Science and Technological Development of the Republic of Serbia). They PGPR properties were described earlier (Jošić et al. 2012; Poštić et al. 2013; Pivić et al. 2015; Jošić et al. 2015).

Pseudomonas strains were grown on King B liquid medium, while *Bacillus* sp. on TSB (Tripton Soy Broth) at 26 °C for 72 h, before being optimized to 10^8 CFU mL^{-1}. Prepared strains (100 mL) were added to *B. japonicum* strain 526 mixed with sterile solid carrier, just before planting by soybean seed co-inoculation.

Field trial and laboratory testing of plant materials

The field experiment was conducted in 2015 in the Vajska locality, a region known for soybean production, AP Vojvodina, Serbia. The trial was performed on soil type Humogley on the 6^{th} of May, soybean cultivar Balkan. The effects of PGP bacteria were tested on 1 ha plot per each treatment:

A – *B. japonicum* 526 + *Bacillus* sp. Q10 + *Pseudomonas* sp. Q4;

B – *B. japonicum* 526 + *Bacillus* sp. Q10 + *Pseudomonas* sp. M124;

Control – Commercial fertilizer containing *B. japonicum* strain(s).

The plant materials were collected in two vegetative periods - flowering and maturity. At the flowering stage, plant height, trifoliate leaf number, root weight, root length and nodule number were measured. At the maturity stage, plant height, trifoliate leaf number, pod number per plant, and grain mass per plant were measured. The yield was measured and compared to the the control treatment. The harvest was done in October.

Meteorological data (average value of the decade temperatures and the precipitation) are taken from the nearest meteorological station, Rimski Šančevi (distance 78 km). Average values of decade air temperatures during the soybean vegetation period (May - October) were higher than the annual average temperature, except in the third decade of September (Figure 1).

Total precipitation was significantly lower compared to the perennial average for the observed soybean vegetation period. A higher amount of precipitation from the perennial average was recorded from the second decade of May to the first decade of June and in the second decade of October (Figure 2).

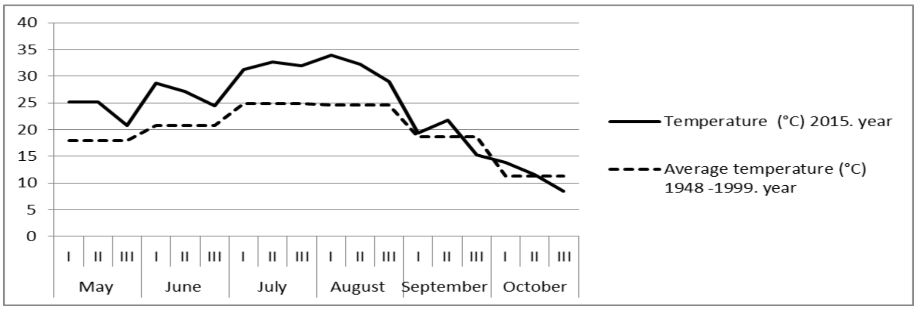

Figure 1. Average values of decade air temperatures during the soybean vegetation period with perennial average (1948 – 1999.)

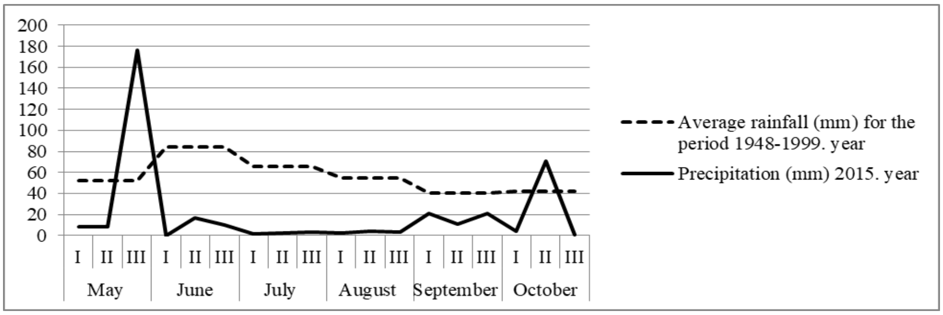

Figure 2. Precipitation during the soybean vegetation period with perennial average (1948 – 1999.)

Data analyses

For statistical analysis, Scheffe test was performed to determine the level of significance, which was accepted at $p < 0.01$ (Statistica 13.3).

RESULTS AND DISCUSSION

Soil bacteria which living freely in the soil or around the root surface may stimulate growth of the cultivated plants in different level. Selection of rhizobacteria with multiple functional traits enable their use as effective plant growth promoters. Soybean is well adapt plant, although stress conditions (drought, heat stress, plant pathogens) can lead to reduced plant growth and cause yield reduction (Hajnal-Jafari et al. 2018).

The aim of these testing was to evaluate the influence of *Bacillus* sp. and *Pseudomonas* spp. as a mixture with *B. japonicum* on soybean plants in field conditions under abiotic stress. Year 2015 was a very unfavorable for all cultivated plants due to severe drought during the whole summer. The field experiment was set in the beginning of May. The seed germination and initial growth of soybean in May were stimulated by rainfall and enable good uptake of water and nutrients, as well as metabolites produced by applied *B. japonicum*, *Bacillus* sp. and *Pseudomonas* spp. strains. During the next four month, the water capacity of soil was very poor and precipitation was lower than average values in past years (Fig. 2). In addition to high temperature in the same period (Fig. 1), it was very stressful for plant growth. In the both examination period (flowering and maturity) obtained results were significantly higher in plants treated with the mixture of *B. japonicum* with *Bacillus* and *Pseudomonas* strains than in plant treated with commercial fertilizer containing *B. japonicum* strain(s). The results are in concordance with the ability of PGP bacteria that, used as inoculant, colonize the rootadhering soil and efficiently increase resistance and adaptation of plants to drought stresses. Soil aggregates with PGPR around the root and microbe-produced capsular and slime materials enable better uptake of water and nutrients from rhizosphere and ensure plant growth and survival under drought stress. *Pseudomonas putida* strain GAP-P45 alleviate drought stress effects in sunflower seedlings by production of metabolites released into soil, such as EPS (Sandhya et al., 2009). *Pseudomonas* spp. improve antioxidant status and plant growth of maize under drought stress (Sandhya et al., 2010). Gibberellin secretion of *P. putida* strain change the stress physiology of soybean and improve the plant growth under saline and drought conditions (Sang-Mo et al. 2014). Several PGP *Bacillus* spp. are reported to have positive effect on growth and antioxidant status of maize under drought stress (Vardharajula et al. 2011). Our results suggested that applications of *Pseudomonas* spp. and *Bacillus* sp. in soybean seed co-inoculation with *B. japonicum* reduced the negative impact of drought and provides good results in terms of increasing yields on soybean cultivar Balkan.

During the flowering stage, the maximum average values of plant height was obtained in treatman A (79.90 cm). In the same treatment the maximum root length (14.50 cm) and node number (11.50) was noted. The largest number of trifoliate leaf number (11.70) was recorder in treatment B, as well as number of nodule on the root (22.40) (Table 1).

Table 1. Response of soybean to inoculation with PGPR strains during the flowering stage

Treatment	Plant height (cm)	Trifoliate leaf number	Root length (cm)	Node number	Nodule number root^{-1}
A*	79.90^{a}	11.60^{a}	14.50^{a}	11.50^{a}	21.50^{a}
B	77.90^{a}	11.70^{a}	13.80^{a}	10.90^{b}	22.40^{a}
Control	68.60^{b}	10.70^{b}	12.60^{b}	10.10^{b}	21.20^{b}

*A – *B. japonicum* 526 + *Bacillus* sp. Q10 + *Pseudomonas* sp. Q4; B – *B. japonicum* 526 + *Bacillus* sp. Q10 + *Pseudomonas* sp. M124; Control – Commercial fertilizer with *B. japonicum* strain(s). Within columns means followed by the same letter are not significantly different (Scheffe test $p<0.01$)

This results are in accordance with assertions of many authors which were studied the influence of PGPR on soybean growth parameters (Zhang et al. 1996; Cattelan et al. 1999; Marinković et al. 2016; Jošić et al. 2017; Hajnal-Jafari et al. 2018; Kumawat et al. 2019). Similar testing on soybean were performed by Marinković et al. (2016) pointing that co-inoculation of *B. japonicum* with *Bacillus* sp. resulted in significant increase in plant length, compared to variants in which these bacterial strains were applied as single-component. Hajnal-Jafari et al. (2018) also showed that application of PGP bacteria positively influenced soybean growth parameters as well as soil microbial activity. Zhang et al. (1996) demonstrated that application of PGPR increases nodulation and nitrogen fixation in soybean.

Maximum plant height during maturity stage was observed in treatment B, while between treatment A and control it was not statistically significant difference (Table 2). The maximum average root length and node number was achieved in control. Treatment A and B resulted in an increase of pod number per plant and in grain mass per plant, and the maximum value was obtained for treatment B, *B. japonicum* 526 enriched with *Bacillus* sp. Q10 + *Pseudomonas* sp. M124. Maximum yield of 2750 kg ha^{-1} was in the treatment B (2750 kg ha^{-1}), than in treatment A (2740 kg ha^{-1}), while in the control yield was 2581 kg ha^{-1}. Both treatment improved soybean growth and yield under drought conditions during 2015.

Table 2. Response of soybean to inoculation with PGPR strains during the maturity stage

Treatment	**Plant height (cm)**	**Root length (cm)**	**Node number**	**Pod number plant^{-1}**	**Grain mass plant^{-1} (g)**	**Yield (kg ha^{-1})**
A*	112.70^{a}	11.80^{a}	11.80^{b}	33.20^{a}	16.60^{a}	2740^{b}
B	164.70^{b}	11.90^{a}	11.90^{b}	35.50^{a}	17.49^{a}	2750^{a}
Control	112.40^{a}	14.60^{b}	14.60^{a}	20.40^{b}	9.70^{b}	2581^{c}

*A – *B. japonicum* 526 + *Bacillus* sp. Q10 + *Pseudomonas* sp. Q4; B – *B. japonicum* 526 + *Bacillus* sp. Q10 + *Pseudomonas* sp. M124; Control – Commercial fertilizer with *B. japonicum* strain(s). Within columns means followed by the same letter are not significantly different (Scheffe test $p<0.01$)

Based on obtained results, both treatments positively influenced soybean growth, compared to control (commercial fertilizer with one or more *B. japonicum* strains) under stress conditions. An increase in morphological parameters and yield in soybean grown in Serbia has been demonstrated earlier in studies performed by several authors. Jošić et al. (2017) suggested that combination of *Bacillus* sp. Q10 and *B. japonicum* 526 could increase the productivity of soybean cultivar Galeb, which is in the line with results achieved in this study. Effects of *Bacillus* sp. and *Pseudomonas chlororaphis* on soybean seed co-inoculation were described in study Iličić et al. (2017). Authors demonstrated positive effects on pods number and grain mass per plant, as well as improvement in soybean growth and yield in the field trial achieved by addition of *Bacillus* sp. Q10 and *P. chlororaphis* Q16 to *B. japonicum* 526. Similar results reported Marinković et al. (2018) pointing that soybean seed co-inoculation with *Bacillus* species and *Azotobacter chroococcum* had a better effect on the number of microorganisms, dehydrogenase activity and yield.

In this work *Pseudomonas* spp. and *Bacillus* sp. were predominant candidate for co-inoculation with *B. japonicum* based on their PGP traits (Jošić et al. 2012; Poštić et al. 2013; Pivić et al. 2015; Jošić et al. 2015). Similar to our results, many authors worldwide reported positive effects of dual inoculation of *B. japonicum* and some of members of those genera. Dual inoculation with *B. japonicum* MN110 and *Bacillus megaterium* LNL6 increased root and shoot length, and plant dry weight as compared to *B. japonicum* MN110 alone in soybean (Subramanian et al. 2015). Petkar et al. (2018) evaluated the effect of dual inoculation of *Bacillus subtilis* and *B. japonicum* and different levels of chemical fertilizers on plant growth parameters of soybean in field experiment in Kolhapur. Two bacterial strains in dual inoculation, as well as along with 75 % nitrogen and phosphorus, improved growth parameters of soybean. Argaw (2012) reported that combination of *B. japonicum* TAL-378 and *Pseudomonas* sp. as dual inoculation increased plant height and shoot dry weight, as well as symbiotic traits as nodulation and nodule biomass at flowering stage of soybean grown in Assossa region as compared to control. Co-inoculation of *B. japonicum* and *P. fluorescens* improved germination, plant growth, symbiotic traits and grain yield in soybean as compared to un-inoculated control treatment (Pawar et al. 2018).

In our investigation, the addition of *Bacillus* sp. Q10 and *Pseudomonas* spp. strains Q4 and M124 to *B. japonicum* 526 showed positive effects and improved the soybean growth, nodulation and yield under drought conditions.

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IMPACT OF FERTILIZATION ON GRAIN YIELD IN BARLEY PLANT GROWN ON SOIL TYPE VERTISOL

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ABSTRACT

Tests were carried out on stationary field trial, soil type vertisol in the process of degradation, characterized by low pH (pH<5.0). The dose of nitrogen was 80 kg/ha N, which was administered in combination with phosphorous and potassium fertilizer. The average grain yield and 1000 grain weight of all treatments in 2010/11 growing season was significantly greater than in 2009/10, mostly as the result of highly favourable weather conditions at major stages of plant development. The yield, thousand grain weight and test weight of barley significantly varied across years and treatments. Barley yield was the highest in the NP_1K and NP_2K (4.171 and 4.318 t/ha) treatments. Variance analysis showed statistically very significant differences for grain yield and test weight between the vegetation seasons and very significant differences for grain yield and 1000 grain weight between the effects of fertilization. Variance analysis showed significant differences for yield between the interaction of the vegetation seasons and variants of fertilization.

Key words: *barley, fertilization, mineral nutrition, yield*

INTRODUCTION

Climate changes on the global level conditioning hotter summers and mild winters, which will lid to the alterations in sowing and heading dates in the future, as well as a production region of barley. In Serbia, drought is present almost every year. In years with normal spring precipitations, winter barley is finishing vegetation mostly before first severe moisture deficiency, in the opposite, it is using moisture accumulated during the winter (Pržulj, 2001; Pržulj et al., 2013; Đekić et al., 2017; Bratković et al., 2018).

Variations in the temperature, in the amount of precipitation during vegetation as well as in the moisture content in the soil are the most important factors of the instability of the barley grain yield. In ecological conditions of Serbia, high temperatures and the water deficiency during the June result in yield decreasing and deterioration of technological properties of grain and malt, so prolonged vegetation and grain filling period do not contribute to yield increasing (Popović et al., 2011; Bratković et al., 2014; Pržulj et al., 2014; Jelić et al., 2015; Đekić et al., 2019).

Vertisol soil type is distinguished by very unfavorable physical, agrochemical, and microbiological properties. The greatest problem of this soil type is low pH value and further increasing of its acidity, mostly because of the irregular application of fertilizers during the years (Jelić et al., 2012). Low production ability of pseudogley is result of poor physical-mechanical, thermal and water-air properties (Jelić et al., 2014). Hence, production of winter barley as a sensitive plant species on this soil type is low and non-profitable.

Time of application of mineral fertilizers necessary for forming of high and quality grain yield of barley as well as amounts and types of mineral fertilizers are differ depending on soil fertility (Jelić et al., 2014). Efficacy of the nitrogen utilization from mineral fertilizers is decreasing with increasing of the nitrogen fertilizing level (Đekić et al., 2014; Jelić et al., 2017; Terzic et al., 2018). Nutrient utilization from fertilizers and yield forming are under the important influence of weather conditions and specific characteristics of the location (Paunovic et al., 2007; Đekić et al., 2015; Popović et al., 2015; Bratković et al., 2018).

The basic aim of this research was a determination of the impact of nitrogen application in a dosage of 80 kg/ha N, applied in combination with phosphorus and potassium fertilizers on the yield of winter barley, during two vegetation seasons.

MATERIAL AND METHODS

Experimental design

Effects of mineral nutrition efficiency of barley have been studied at the stationary field trial of the Small Grains Research Centre in Kragujevac (Serbia) for two years (2009/10 and 2010/11). The experiment was laid out in a randomised block design with five replications and a plot size of 10 m^2 (5 m x 2 m). In all years, winter barley was sown in the second half of October at a row spacing of 12.5 cm. The rates of nitrogen application were

80 kg/ha N. The barley cultivar used in the experiment was Rekord. Six variants of mineral nutrition C (control), N (80 kg/ha N), NP_1 (80 kg/ha N and 60 kg/ha P_2O_5), NP_2 (80 kg/ha N and 100 kg/ha P_2O_5), NP_1K (80 kg/ha N, 60 kg/ha P_2O_5 and 60 kg/ha K_2O) and NP_2K (80 kg/ha N, 100 kg/ha P_2O_5 and 60 kg/ha K_2O) were tested in the experiment.

The crop was harvested at full maturity. Grain yield (t/ha) was harvested and reported at 14% moisture. Three parameters, namely grain yield, 1000 grain weight (g) and test weight (kg/hl) were analysed. Thousand grain weight was determined using an automatic seed counter. Test weight is the weight of a measured volume of grain expressed in kilograms per hectoliter.

Meteorological conditions

Kragujevac area is characterized by a moderate continental climate, which general feature is uneven distribution of rainfall by month. Data in Table 1 for the investigated period (2010-2011) clearly indicate that the years in which the researches were conducted differed from the typical multi-year average for Kragujevac region, regarding the meteorological conditions.

Table 1. Mean monthly air temperatures and precipitation in Kragujevac, Serbia

Months	X	XI	XII	I	II	III	IV	V	VI	VII	Average
Mean monthly air temperature (^oC)											
2009/10	11.7	8.8	2.6	0.9	3.2	7.2	12.1	16.5	20.2	23.1	10.63
2010/11	10.2	11.4	2.4	0.9	0.5	7.2	12.0	15.8	20.9	22.8	10.41
Average	12.5	6.9	1.9	0.5	2.4	7.1	11.6	16.9	20.0	22.0	10.18
The amount of precipitation (mm)											
2009/10	102.6	77.5	194.2	57.0	150.5	43.3	142.2	116.7	196.7	14.8	1095.5
2010/11	86.9	27.9	50.1	29.1	48.5	20.4	20.8	65.8	32.3	62.4	444.2
Average	45.4	48.9	56.6	58.2	46.6	32.4	51.9	57.6	70.4	46.6	514.6

The average air temperature in 2009/10 was higher by 0.37°C and 2010/11 was higher by 0.16°C than the average of many years. The sum of rainfall precipitation in 2009/10 was higher by 612.1 mm, where the sum of rainfall in 2010/11 was 86.2 mm lower than the average of many years and with a very uneven distribution of precipitation per months. During the April and May in 2009/10 it was 142.2 mm and 116.7 mm of rainfall, what was 90.3

mm and 59.1 mm more compared with the perennial average. During the June in 2009/10 it was 196.7 mm of rainfall, what was 126.3 mm more compared with the perennial average.

Regard the high importance of sufficient rainfall amounts during the spring months, particularly. Namely, the total amount of precipitation is reflected on the multi annual average, but the distribution, especially at critical stages of development, is significantly disturbed in the 2009/10 year. In addition to the necessary reserve for the spring part of the vegetation, winter precipitation greatly influences the distribution of easily accessible nitrogen in the soil (Paunovic et al., 2007; Madić et al., 2014; Pržulj et al., 2014; Jelic et al., 2014, 2015; Đekić et al., 2014, 2015; Popović et al., 2016; Terzic et al., 2018).

Soil analysis

The trial was set up on a vertisol soil in a process of degradation, with heavy texture and very coarse and unstable structure. The humus content in the surface layer of soil was low (2.22%). The reduced humus content in field vertisols profiles suggests the necessity of involving humification when planning fertilization systems and soil ameliorative operations to be used to maintain and improve the soil adsorption complex. Soil pH indicates high acidity (pH in H_2O 5.19; pH in KCl 4.27), nitrogen content in soil is medium (0.11-0.15%), while the content of available phosphorus ranges from very low (1.7-2.9 mg 100 g^{-1} soil) in the N and NK trial variants to very high (26.9 mg P_2O_5 100 g^{-1} soil) in the NPK variants of fertilization. Available potassium contents are high, ranging from 19.5 to 21.0 mg K_2O 100 g^{-1} soil.

Statistical Analysis

On the basis of achieved research results the usual variational statistical indicators were calculated: average values and standard deviation. Experimental data were analysed by descriptive and analytical statistics using the statistics module Analyst Program SAS/STAT (SAS Institute, 2000) for Windows. All evaluations of significance were made on the basis of the ANOVA test at 5% and 1% significance levels. Relative dependence was defined through correlation analysis (Pearson's correlation coefficient), and the coefficients that were obtained were tested at the 5% and 1% levels of significance.

RESULTS AND DISCUSSION

Grain yield, 1000 grain weight and test weight

Table 2 presents average values for gran yield, thousand grain weight and test weight significantly varied across years and treatments during the study.

Table 2. Mean values for grain yield, 1000 grain weight and test weight

Fertilization	Years								
	2009-2010			2010-2011			Average		
	x	S	Sx	x	S	Sx	x	S	Sx
Grain yield, t/ha									
C	0.605^{C}	0.260	0.116	0.933^{D}	0.255	0.114	0.769^{C}	0.298	0.094
N	1.876^{BC}	0.784	0.351	3.270^{C}	0.333	0.149	2.573^{B}	0.929	0.294
NP_1	2.221^{BC}	0.491	0.219	3.569^{BC}	0.237	0.106	2.895^{B}	0.798	0.252
NP_2	2.562^{B}	0.482	0.215	4.002^{B}	0.416	0.186	3.282^{B}	0.869	0.275
NP_1K	3.429^{A}	0.408	0.182	4.912^{A}	0.506	0.226	4.171^{A}	0.893	0.282
NP_2K	3.403^{A}	0.423	0.189	5.232^{A}	0.380	0.170	4.318^{A}	1.036	0.328
1000 grain weight, g									
C	39.30^{B}	1.288	0.576	41.06^{B}	0.773	0.346	40.18^{C}	1.365	0.432
N	40.64^{AB}	1.358	0.607	41.44^{B}	1.101	0.492	41.04^{BC}	1.239	0.392
NP_1	40.74^{AB}	0.838	0.375	42.60^{B}	1.786	0.799	41.67^{BC}	1.640	0.519
NP_2	41.02^{AB}	1.741	0.779	43.02^{B}	1.653	0.739	42.02^{BC}	1.916	0.606
NP_1K	42.64^{AB}	3.346	1.496	44.12^{AB}	1.839	0.822	43.38^{AB}	2.662	0.842
NP_2K	44.36^{A}	5.080	2.272	46.88^{A}	4.544	2.032	45.62^{A}	4.734	1.497
Hectoliter weight, kg/hl									
C	61.51^{B}	1.221	0.546	63.54^{B}	2.361	1.056	62.52^{B}	2.070	0.655
N	63.51^{A}	1.656	0.741	65.39^{AB}	2.228	0.996	64.45^{AB}	2.099	0.664
NP_1	63.95^{A}	1.493	0.668	65.73^{AB}	2.394	1.071	64.84^{A}	2.102	0.665
NP_2	64.26^{A}	1.538	0.688	67.02^{AB}	3.320	1.485	65.64^{A}	2.839	0.898
NP_1K	64.62^{A}	0.976	0.436	67.72^{A}	1.444	0.646	66.17^{A}	2.005	0.634
NP_2K	65.22^{A}	1.009	0.451	68.00^{A}	3.153	1.410	66.61^{A}	2.649	0.838

*Means within columns followed by different lowercase letters are significantly different (P<0.05) according to the LSD test

The highest grain yield had variety Rekord application of NP_2K in a quantity of 80 kg/ha N, 100 kg/ha P_2O_5 and 60 kg/ha K_2O (4.318 t/ha). The grain yield of the barley was significantly lower in control. Average grain yield of treatments ranged from 0.605 t/ha (control) to 3.429 t/ha (NP_1K) in 2009/10 and 0.933 t/ha (control) to 5.232 t/ha (NP_2K) in 2010/11. In all years, NP_1K and NP_2K treatments produced significantly higher grain yields compared in the other treatments. Considerable variation in yield depending on years of research have established Popović et al. (2011), Jelić et al. (2014), Madić et al. (2014) and Đekić et al. (2019).

During the 2009/10 and 2010/11, thousand grain weight was significantly greater in NP_2K treatment (44.36 g and 46.88 g) than in the other treatments. The average 1000 grain weight of all treatments in the 2010/11 growing season was significantly greater than in the 2009/10 year, mostly as the result of highly

favourable weather conditions at major stages of plant development. Averaged across years, significantly higher values for 1000 grain weight were found in NP_2K treatment (45.62 g). Average 1000 grain weight of treatments ranged from 39.30 g (control) to 44.36 g (NP_2K) in 2009/10 and 41.06 g (control) to 46.88 g (NP_2K) in 2010/11.
Table 2 presents average values for grain yield, thousand grain weight and test weight across years and treatments during the two vegetation sesons. The test weight varied across years and treatments. Average test weight of treatments ranged from 61.51 kg/hl (control) to 65.22 kg/hl (NP_2K) in 2009/10 and 63.54 kg/hl (control) to 68.00 kg/hl (NP_2K) in 2010/11.

Analysis of variance the analysed traits
Table 3 shows the impact of the year, fertilization and interaction of year x fertilization on yield, 1000 grain weight and test weight. Analysis of variance was found highly significant effect of year on the grain yield ($F=15.397^{**}$) and test weight ($F=15.871^{**}$) and significantly effect of 1000 grain weight ($F=5.295^{*}$). Analysis of variance was found highly significant effect of fertilization on the grain yield ($F=23.791^{**}$), 1000 grain weight ($F=5.728^{**}$) and test weight ($F=4.028^{**}$). Based on the analysis of variance, it can be concluded that there are significant differences in grain yield regard the interaction year x fertilization (Table 3).

Table 3. The analysis of variance for the tested parameters in Kragujevac, Serbia

Effect	df	Mean sqr Effect	Mean sqr Error	F	p-level
The analysis of variance for grain yield					
Year, (Y)	1, 58	25.489	1.655	15.397	0.0002
Fertilization, (F)	5, 54	16.714	0.702	23.791	0.0000
Year x Fertilization, (YxF)	5, 48	0.644	0.192	3.352	0.0112
The analysis of variance for 1000 grain					
Year, (Y)	1, 58	45.240	8.545	5.295	0.0250
Fertilization, (F)	5, 54	37.488	6.544	5.728	0.0003
Year x Fertilization, (YxF)	5, 48	0.821	6.334	0.130	0.9849
The analysis of variance for test weight					
Year, (Y)	1, 58	85.514	5.388	15. 871	0.0002
Fertilization, (F)	5, 54	21.625	5.368	4.028	0.0035
Year x Fertilization, (YxF)	5, 48	0.776	4.177	0.186	0.9666

^{ns}-non significant; *-significant at 0.05; **-significant at 0.01;

Correlations between the analysed traits

Table 4 shows the grain yield was in a positive correlation with the 1000 grain weight as well as with the test weight. Barley yield in 2009/10 was positively and highly significant correlated with 1000 grain weight (0.476**) and test weight (0.634**). Yield in the 2010/11 vegetation season grain yield in was positively and highly significant correlation with the 1000 grain weight (0.548**) and test weight (0.488**). Thousand grain weight in 2009/10 and 2010/11 was positively and significantly correlated with test weight (0.405* and 0.434*).

The positively and significant correlation with grain yield and thousand grain weight have established Đekić et al. (2014) and Terzić et al. (2018). Đekić et al. (2014) state negatively and significant correlation of thousand grain weight and test weight. Grain yield depends directly on the the thousand grain weight (Đekić et al., 2014, 2019; Terzić et al., 2018).

Table 5 shows the correlation coefficients between the studied fertilization treatments and analysed traits. Positive correlations were observed between grain yield and thousand grain weight in all treatments, except in the treatment with nitrogen. Positively and significant correlations were observed between grain yield and thousand grain weight in the NP_1 (r=0.667*). Positively and strong correlations were observed between thousand grain weight and test weight in the unfertilized control (r=0.829**) and positively and significant correlations in the treatment NP_1K (r=0.696*).

Table 4. Correlations between the traits analyzed by two vegetation seasons

Correlations between the traits analyzed in 2009/10			
	Grain yield	1000 grain weight	Test weight
Grain yield (t/ha)	1.00	0.476**	0.634**
1000 grain weight (g)		1.00	0.405*
Test weight (kg/hl)			1.00
Correlations between the traits analyzed in 2010-2011			
	Grain yield	1000 grain weight	Test weight
Grain yield (t/ha)	1.00	0.548**	0.488**
1000 grain weight (g)		1.00	0.434*
Test weight (kg/hl)			1.00

^{ns}-non significant; *-significant at 0.05; **-significant at 0.01;

The present results confirm the statement of many authors that the traits analyzed and their correlations are genetically determined but are strongly modified by the nutrient status of the environment and weather conditions (Popović et al., 2011; Đekić et al., 2017; Jamil et al., 2017; Terzic et al., 2018).

Table 5. Correlation coefficients for the traits analyzed across treatments

	Grain yield	1000 grain weight	Test weight
Correlations between the traits analyzed in the unfertilized control			
Grain yield (t/ha)	1.00	0.534	0.149
1000 grain weight (g)		1.00	0.829**
Test weight (kg/hl)			1.00
Correlations between the traits analyzed in the N			
Grain yield (t/ha)	1.00	-0.078	0.326
1000 grain weight (g)		1.00	0.444
Test weight (kg/hl)			1.00
Correlations between the traits analyzed in the NP_1			
Grain yield (t/ha)	1.00	0.667*	0.365
1000 grain weight (g)		1.00	-0.264
Test weight (kg/hl)			1.00
Correlations between the traits analyzed in the NP_2			
Grain yield (t/ha)	1.00	0.443	0. 441
1000 grain weight (g)		1.00	0.087
Test weight (kg/hl)			1.00
Correlations between the traits analyzed in the NP_1K			
Grain yield (t/ha)	1.00	0.140	0.696*
1000 grain weight (g)		1.00	0.505
Test weight (kg/hl)			1.00
Correlations between the traits analyzed in the NP_2K			
Grain yield (t/ha)	1.00	0.398	0.452
1000 grain weight (g)		1.00	0.414
Test weight (kg/hl)			1.00

^{ns}-non significant; *-significant at 0.05; **-significant at 0.01;

CONCLUSION

Effects of mineral nutrition efficiency of barley have been studied at the stationary field trial of the Small Grains Research Centre in Kragujevac (Serbia) for two years (2009/10 and 2010/11). Nitrogen had a most

significant impact on the yield of wheat. Averaged across treatment, thousand grain weight and test weight were significantly greater in 2010/11 than in the previous year. Averaged across years, grain yield and 1000 grain weight was significantly greater in NP_1K and NP_2K than in the other treatments. Regardless of year, NP_1K and NP_2K treatments had significantly higher values for 1000 grain weight compared to the other treatments.
Grain yield shows a tendency to grow in years with higher levels and better rainfall during critical stages of plant development. Analyzing variances is a very significant effect of fertilization on grain yield, 1000 grain weight and test weight barley, while the impact of growing seasons on all the characteristics of the barley was statistically significant.

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ZOOTECHNICS

ORGANIC SHEEP FARMING AS A FACTOR OF ECOLOGY AND BIODIVERSITY CONSERVATION

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INTRODUCTION

In the opinion of many authors (Van Diepen et al., 2007; Petrović et al 2016, 2017, Yilmaz et al., 2016), the current state-of-the-art technology of farm systems in livestock breeding represents an obvious risk to biodiversity and its ecological sustainability. On the other hand, despite this conclusion, the consumption of meat and milk is growing. The growing knowledge of ecosystem disorders and the threat of the living world in its entirety imposed a need to re-examine the technologies and methods so far that have been used to accelerate productive growth in all human activities, including agriculture. It began to study the influence of various external factors on animal productivity (Petrović i sar, 2011, 2015). Rodrigez et al (2017) launched a project that will assess the sustainability of the various European sheep production systems, focusing on the ecological aspect of animal welfare and the nutritional value of lamb. Preservation of native races for use in organic systems is important and can stop the extinction of races. Unique characteristics of domestic breeds can help farmers and scientists to preserve biodiversity and ecology in the future (Nauta, 20006, Petrović et al., 2013).

The basic principles of organic sheep

Organic sheep farming means the development of this branch of animal husbandry, without disturbing relations in natural communities, without the import of animals, with limited application of products accompanying animal production including therapeutic agents.

With this kind of production, with the limitation of pollution in the habitat, it ensures the preservation of the entire biological community, achieves satisfactory income, and opens the possibility for the development of additional activities, such as tourism.
Through organic production obtained highly valued products carrying the label -organic food- increasing the price of the product, reducing health risks to consumers and ensure the market penetration.
Organic production management system in sheep farming is regulated by legal acts.
In quality standards for organic products, not only the definitions of the quality and characteristics of the final product have been incorporated, but special attention has been paid to controlling each pre-production phase of obtaining the primary product until it is placed on the market.

Legislation defines control of production processes, which aim to produce chemically and biologically unpolluted food in a way that is environmentally sound, economically beneficial and meets ethical criteria for human treatment of animals.
Since one of the important prerequisites for organic sheep farming is limited the possibility of using chemicals for the protection of animal health, autochthonous and locally adapted breeds gain an advantage over imported populations.
The organic sheep farming as an economic activity is particularly suitable for eco-zones and national parks. This approach preserves and improves the natural community, reduces the level of environmental pollution, preserves the unique and endemic forms of autochthonous breeds that are the source of new variability for future generations. The trend of depopulation of the village is mitigated and the traditional values of human society are preserved. At the same time provide revenue growth and facilitates the development of other industries, especially tourism, hunting, forestry, etc.

The importance of indigenous breeds in organic sheep farming

One of the most frequently observed phenomena in the intensive breeding of domestic animals, and thus the sheep, is the weakening of the constitution. The processes that led to the loss of general and specific resistance, adaptation and acclimatization power were, unfortunately, dictated by the breeder and selector. Over the course of time, the following unilateral goal of the animal was pulled out of the natural environment, or cultivated in a modified environment in the absence of natural selection vectors such as nutrition cycles depending on the season and climate conditions, the activity of pathogenic micro and macroorganisms, etc.

As in most highly productive, specialized breeds of domestic animals, signs of genetic weakness are noticed, so the incidence of various infections and diseases increases.

The autochthonous breed, whose adaptability is influenced by the selection factors of the environment, is a unique source of genetic variants that have resistance to new conditions and can enable the survival of the population.

Whereby Pramenka as indigenous sheep population perfectly adapted to the biogeographical conditions, their cultivation in underdeveloped regions, as well as in conditions where conditions preclude intensive production can constitute a backbone of future development in the conditions of semi-elastic and extensive agriculture.

These animals can be grown traditionally in a free hold system, and how well they use natural pastures, their nutrition is based on the existing plant resources and does not require intensification of production of animal feed.

The resistance and vitality of autochthonous sheep breeds allows their growing without major investments in health care and treatment, so in this way they produce products of special quality for human consumption, which do not contain residues of various antibiotics and plant protection products.

In order to preserve and improve the indigenous population in Serbia, it is approaching systematic measures of productivity control in order to effectively select animals. In the following tables and figures we will show the results of this work.

Figure 1. Representation of autochthonous sheep breeds in productivity control ,%

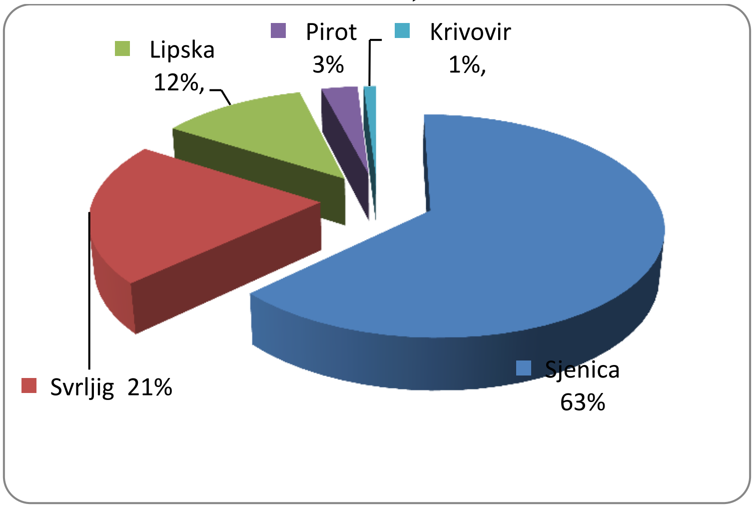


Table 1. Control of productivity of autochthonous sheep breeds by genotypes

Genotype	Number of heads	Fertility-lamb/ewe	Body weight, kg				Wool yield, kg
			BWB	BW30	BW90	BW adult	
Sjenica	2.324	1.30	3.07	11.06	27.12	60.42	2.52
Svrljig	792	1.21	3.15	11.18	24.22	56.56	2.76
Lipska	446	1.31	3.07	11.95	27.90	63.90	3.40
Pirot	113	1.23	2.75	10.98	24.18	55.44	2.52
Krivovir	53	1.08	2.85	10.65	24.15	48.20	2.20
Total	3.728						

In terms of breed structure (Figure 1), with small variations in the representation of the same trend as in the previous year. The dominant place belongs to the Sjenica , which is represented by 63%, then the Svirljig (21%), the Lipska (12%), the Pirot breed (3%) and the Krivovir representation with 1%.

From Table 1 we see can that all breeds, except from Krivovir, exhibited high fertility, which is within the range of variability of these populations. Lipska dominated with 1.31 and Sjenica with 1.30 lambs per ewe.

Selection of breeding animals for organic sheep farming

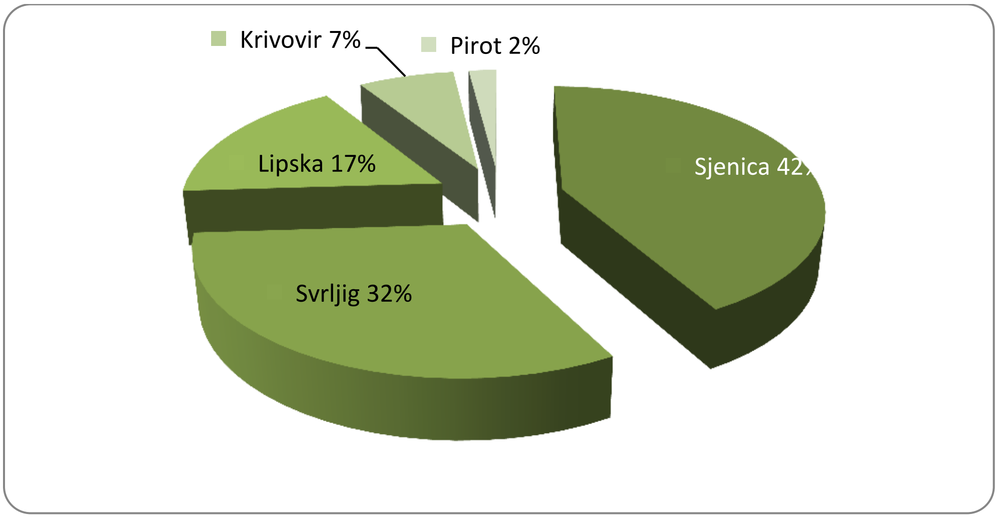
The selection of animals for organic production is a key issue for organizing successful production.
The Ordinance on Organic Production Methods emphasizes that organic production uses vital animal breeds that are adapted to local conditions of breeding and disease in order to avoid health problems.
For the successful selection of sheep, the identification and marking of the throats is necessary, as is the rule that refers to methods of organic livestock production is clearly prescribed. According to the legislation, animals and

their products must be labeled at all stages of organic production. For example, a certificate of "organic milk" can only have a product obtained from sheep farm registered for such production and for which there are certain standards.

Table 2. Milk control of sheep breeds of meat-milk-wool direction, by genotypes

Genotype	Number of heads	Duration of lactation, days	Quantity of milk, kg	Milk fat,%	Protein,%
Sjenica	1.185	97.23	62.22	6.58	5.27
Svrljig	873	90.74	62.11	6.62	4.33
Lipska	490	122.85	96.34	6.76	5.47
Krivovir	204	117.51	77.29	6.97	4.63
Pirot	66	121.96	73.03	7.32	5.05
Total	2.818				

Figure 2. Representation of indigenous sheep in the control of milk yield,%



Of the total number of throats in percentages, the Sjenica was represented by 42%, Svrljig (32%), Lipska (17%), while the rest of about 7% constituted Krivovir and 2% Improved Pirot sheep (Figure 2).
If we look at the data in Table 2, we see that the longest lactation (123 days) and the highest amount of milk (96 kg) had sheep Lipska sheep.

The highest percentage of milk fat (7.32%) was in the milk of the sheep population Improved Pirot, and at the protein content, the best was Lipska sheep (5.47%).

Animal welfare standards in organic sheep farming

The legal regulations set high standards that determine conditions for breeding animals in organic sheep farming
Forced feeding and animal exploitation is not allowed
Interventions such as tail cutting and dampening are not allowed.
Reproduction of animals in organic production is carried out through natural regeneration or artificial insemination. Other methods of biotechnology in reproduction such as induction and synchronization of estrus and embriotransfer are not permitted.

Facilities for organic sheep breeding

The standards precisely define the conditions for the accommodation of sheep. Animals are kept in the facilities that provide sufficient fresh air and natural light, (characteristics of the facility, type of floor and rugs, insulation, ventilation, relative humidity, principles of disinfection of facilities and equipment, allowed means, etc. are defined).
Animals must have enough space for feeding, resting and moving, access to water and food must be free. An optimal density of population is prescribed, which provides sufficient space for normal standing and natural movement. Surface area of the throat is 1.5 m2 plus 0.35 m2 for each lamb. In the outskirts of 2.5m2 plus 0.5m2 for each lamb.
Animals must not be kept attached. They should have access to the drain, which must have adequate protection from rain, wind, sun and extreme temperatures.
The standards define the number of heads per hectare (maximum number of sheep, per hectare is 13).
Special regulations describe procedures related to the transport and slaughter of animals, precisely define production processes and prescribe detailed monitoring of all production phases.

Nutrition of sheep in organic farming

Animal nutrition must be adapted to physiological requirements, with the maximum use of available nutrients in rounded organic production.
Food must be prepared in a form that allows animals to show natural eating habits and meet their needs.
Standards prescribe the prohibition of the use of antibiotics, coccidiostats, medical preparations, growth stimulants or any growth-promoting substance. The use of GMO and GMO derivatives throughout the production chain is prohibited.
Nutrition of lambs in organic sheep farming is a sensitive issue and is the subject of many discussions, since it is desirable for lambs to be kept for at least 45 days in breast milk for the benefit of animal welfare.

This provision directly means that organic milk is used in the feeding of cows, which particularly burdens the commercial production of milk in sheep farming.
However, in most cases, there is a practice for young people to feed milk obtained from individuals of the same species (sheep), cultivated under conventional conditions.

Converting from conventional to organic production

The transition from conventional to organic production requires a certain amount of time, which depends on the general conditions of animal breeding. In order to obtain organic products with a certificate, animals must be kept in accordance with the policy conditions, which prescribe the conversion period for the transition from conventional to organic farming. For the transition from conventional to organic production, for small ruminants it takes at least 6 months.

Preservation of animal health in organic sheep farming

Principle of animal health control in organic sheep farming:
Measures of prevention before treatment
1. Growing of adaptive and resistant breed. Indigenous breeds have a special advantage
2. Nutrition and keeping conditions in accordance with organic sheep standards
3. Implementation of the vaccination program established by the competent authorities

3. Application of alternative therapies based on plant preparations, homeopathic agents and the like
4. If alternative therapy does not help - synthetic drugs can be used.

Where should organic sheep grow

Mountains have the best conditions for organic sheep farming. These regions are often precisely because of their underdevelopment, avoid pollution by chemical preparations that accompany development, and are preserved and represent oases of intact nature.
Organic sheep farming enables the sustainable development of villages in the regions covered by the natural resource protection regimes, allowing the proper exploitation of existing agricultural areas and preventing further degradation of natural meadows and pastures.
Organic farming and agriculture are the only possible way to develop in the zones of national parks, where every intensification of production is a factor of degradation of the natural environment. In this sense, breeding of autochthonous sheep contributes to the preservation of biodiversity.

CONCLUSION

Preservation of biodiversity is a complex problem of modern science. In this chain of influences, agriculture plays an important role. Growing livestock production is achieved the necessary food for human consumption. However, due to increasingly intensive technologies and production systems, ecology is endangered. The outcome of this situation in sheep production is the raising of ecological awareness and access to organic farming. For that purpose are the best indigenous populations in the mountainous areas. This contributes to the preservation of biodiversity and provides healthier food for people.

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FUNCTION AND IMPORTANCE OF HSP 70 IN METABOLIC STRESS IN DAIRY COWS IN PERIPARTAL PERIOD

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ABSTRAKT

Peripartal period in dairy cows includes 3 weeks ante partum and 3 weeks post partum (transition period). The transition period represents the most challenging phase in the life of dairy cows, because the body occurring metabolic, endocrine, immune and reproductive changes that affect the animal health and production efficiency. During the transition period (the late gestation and early lactation) in response to metabolic stress, in dairy cows can develop numerous pathophysiological mechanisms (inflammation, insulin resistance and metabolic adaptation). Numerous mechanisms are activated in the organism of dairy cows, the main role of which is maintenance of homeostatic and homeostatic functions of all tissues, organs and organ systems. In regulation of these processes, heat stress proteins HSP70 play a key role.

Key words: *dairy cows, peripartal period, metabolic stress, HSP 70.*

INTRODUCTION

Peripartal period includes 3 weeks ante partum and 3 weeks post partum (the so-called transition period). It represents a period in which the metabolism of dairy cows faces a series of changes in the homeostasis with endocrine changes, metabolic stress, and many pathophysiological mechanisms (inflammation, insulin resistance and metabolic adaptation) that develop during metabolic stress in peripartal period in dairy cows (Petrović i sar., 2018a). In this for cows during the difficult period around the partus there are numerous metabolic adaptations that arise as a result of gravity, partus and beginning of lactation (homeoretic processes), as well as the tendency of the organism to maintain homeostasis. All these processes inevitably lead to stress in the cows. The emergence of stress in cows is a consequence of the strengthening of homeotrophic, catabolic processes and the negative energy balance of cows at the beginning of lactation (Cincović 2013a).

The biggest problem with postpartum dairy cows is the imbalance between body reserves and milk production (Petrovic i sar., 2019a). The metabolic processes in the transition period are adapted to provide a sufficient amount of energy required as well as precursors for the synthesis of dairy compounds (Grummer 1995.; Overton and Valdron, 2004). Consequently, the main feature of early lactation in dairy cows is the negative energy balance (NEBAL) and the state of metabolic stress that results from reduced intake of food, calving and starting lactation. Due to the negative energy balance, the organism increases its own reserves and enters the catabolism phase.

As a result, lipomobilization intensifies ketogenesis and lipogenesis in the liver, and as a consequence, the concentration of glucose, triglycerides and total blood cholesterol (Sevinc et al., 2003.; Đoković et al., 2007, 2009, 2010a).

In NEBAL conditions, the body consumes its own energy reserves, first of all glycogen reserves, then fat, and then protein. As a consequence, there is a faty liver, a weight loss of various degrees, a decrease in production and reproductive abilities, and in some cases a death occurs (Đoković et al., 2014a).

Especially expressed is the increased mobilization of lipids from body depots in order to use fat for energy purposes, but cows become prone to the development of ketosis and fatty liver (Cincović et al., 2012; Đoković et al., 2014b). As a consequence of lipolysis in the fat tissue, the concentration of non-esterified fatty acids (NEFA) and betahydroxybutyrate (BHB) in the bloodstream increases. This phenomenon occurs as a result of endocrine and metabolic changes, primarily due to the presence of insulin resistance (Cincović et al., 2014).

Stress resulting from a disbalance of energy metabolism with numerous endocrine, biochemical, haematological, immunological and other adaptations is called metabolic stress (Cincović 2013b). Metabolic stress in early lactation in dairy cows underlies many diseases (Cincovic et al., 2018).

The transition from the state of gravidity and drying in the period of early lactation (peripartal period) is a very strenuous process for the smooth functioning of the cow organism, and is the most critical for their health and productivity. Partus and lactation start metabolically burdening cows to the extremes, so numerous adaptations can be such that the number of cows with some of the diseases (fatty liver, ketosis, metritis, mastitis, left and **right** dislocation of **abomasum**, locomotor diseases) is increasing in this period (Cincovic 2013a; Petrović et al., 2019). There may also be a persistent decline in cow productivity through reduced milk production and poor maintenance of lactation, a significant loss of body condition after calving - BSC below 3.5 (3.25 to 3.75), the occurrence of reproductive disorders such as retained placenta and cyst ovary, causing great economic damage to farmers.

It should be noted that in all this good manufacturing practice on the farm affects the economic viability of production. Therefore, efficient reproduction on farms in dairy cows is of great economic importance. However, in modern farms, increased milk production along with poor farm management (poor nutrition or reproduction) can affect reduced fertility in animals. Namely, the selection in cattle breeding is very successful in the direction of increasing milk production. But the phenotype of milk yield is only 25%. The influence of paragenetic factors on milk properties, regardless of whether their nature is fixed (the breeding area of the years and the season of birth, season of calving, lactation in order) or continuous (age at first fertilization and calving) is very pronounced and significant, and it is necessary to include them in models for assessing the breeding value of dairy cows (Petrović et al., 2005, 2006, 2009, 2010, 2012; Bogdanović et al., 2012)

Unfortunately, with the increase in milk production, fertility decreases and, consequently, the number of animals that have partus, which is reflected in the profitability of production (Gábor et al., 2016).

The main goals of modern cattle breeding are the increase in the fertility of breeding throats, the prolongation of their exploitation time, the increase in profit and the cultivation of as many genetically high-quality offspring as possible. The achievement of these goals depends on the factors determining the genetic potential of the reproductive properties of the reproductive throat and environmental factors (paragenetic factors), which enable the phenotypic expression of reproductive characteristics (Košarcic et al., 2003; Petrović et al., 2013a, b, c.).

Therefore, it is very important to understand the reproductive cycle physiology of dairy cows and its relationship with the metabolic status of cows in early lactation (Đoković et al., 2014a).

Cellular adaptation of dairy cows to peripartal metabolic stress (HSP70)

Milk production starts after calving of cows and is maintained artificially, by husband, for the next 305 days. In this period there are very significant changes in the metabolism and nutrition of cows, and the intensity and biological basis of these changes depend on the health of cows and milk production in the next lactation. The essence of metabolic changes occurring during the transition period (21 days before and 21 days after the partisans) is based on the fact that cows are exposed to negative energy balance during early lactation, that is, they can not enter through the food the amount of energy they need for maximum milk production. There are many reasons that lead to reduced food intake and a negative energy balance. Reduced food intake is the result of adaptation to the onset of lactation. On the other hand, in the cow there is a change in the main metabolic flows to maintain the lactation that follows. These processes lead to changes in the metabolism of cows, increasing the use of fat for energy purposes, in order to keep glucose for milk production. Also, in early lactation there is a deficit of vitamins and minerals, so their role in numerous metabolic processes is absent (Đoković et al., 2014c).

Namely, the basic changes in the metabolism of carbohydrates and fats in the period around calving and early lactation are: lower glycemic levels, increased gluconeogenesis, reduced glucose consumption in peripheral tissues, normal or decreased use of acetate, increased lipid mobilization from fat stores with elevated concentrations of non-esterified fatty acids (NEFA) and their increased use in peripheral tissues (Đoković 2010b)

These changes are followed by a series of endocrine changes such as insulin resistance, decreased insulin concentration (due to reduced food intake and decreased receptor sensitivity), decreased insulin-like growth factor IG and I IGF-I (due to a reduced anabolic effect of growth hormone in the peripheral tissue cow in spite of its elevated concentration), decreased thyroid hormone concentrations, catabolic effect of growth hormone on fatty tissue - increased growth hormone concentration (which allows increased use of nutrients in the mammary gland and leads to a decrease in insulin sensitivity), elevated cortisol concentration (which helps lipomobilization and gluconeogenesis), increased glucagon levels.

During the transitional period in response to metabolic stress, numerous pathophysiological mechanisms (inflammation, insulin resistance and metabolic adaptation) are developed in dairy cows. Inflammation and insulin resistance are important pathophysiological mechanisms that develop during metabolic stress. Heat shock proteins have a significant influence in the regulation of both these processes in different animal species and humans. They help to clear the protein structure of the cell and its survival. However, if they find themselves extracellularly, they show proinflammatory effects and have to do with the development of insulin resistance and diabetes (Petrović et al., 2017).

In the organism of dairy cows numerous mechanisms are activated, the main role of which is to maintain all these processes within the physiological limits. In regulation of these processes, HSP 70 heat stress proteins play a key role.

Heat schock protein (HSP) are phylogenetically conserved and ubiquitous molecules, indicating their functional importance (Petrović et al., 2017).

Heat shock protein, HSP are chaperones necessary for the proper formation of the polypeptide chain and are responsible for its translocation in the cell. These proteins were found during exposure to heat stress, when their concentration and expression in the cells grew to explain their name (Cincović 2013a.; Petrović et al., 2016).

Heat schock proteins are synthesized in response to various forms of stress. Namely, their expression can be induced in several ways: physiological (growth factors and hormones), pathophysiological (infection, inflammation, ischemia, oxidative injuries and toxins), environmental conditions (heat stress and heavy metals) (Petrović i sar., 2018b).

They are traditionally classified according to their molecular weight. (Prohaszka and Fust, 2004.). According to the molecular mass we distinguish several types, so for example: 10 kDa (Hsp10), 20-30 kDa (Hsp27, HspB1), 40 kDa (Hsp40), 60 kDa (Hsp60), 70 kDa (Hsp70, Hsp71, Hsp72, Grp78, Hsx70), 90 kDa (Hsp90, Grp94) and 100 kDa (Hsp104, Hsp110). In cattle, four types of Hsp70 genes were identified, and IRNK for this protein was found in the tissue of different cell types and in the blood plasma (Agnew and Colditz 2008; Asea 2007).

Tavaria et al., (1996.) gave a first clarification of the nomenclature of the HSPA family and showed that the family of a human heat shock protein is composed of at least 12 members and many others agree with their allegations. Modification and extension was given by Kamping et el. (2009.), where he provided updated guidelines for the nomenclature of the human HSPA family (HSP70), as well as HSPH (HSP110), HSPC (HSP90), DNAJ (HSP40) and HSPB (smoll HSP) and human chaperone families (HSP60 and CCT). Also, Kampinga et al., (2009.) stated that the guidelines for the nomenclature of human heat shock protein are also based on systemic gene symbols assigned by the HUGO Gene Nomenclature Committee (HGNC) and used as primary identifiers in databases such as Entrez Gene and Ensemble. The best known HSPs are: stress induced form HSP70 / HSP72 (HSPA1A), constitutive forms HSP70 / HSP73 / HSS73 (HSPA8), an endoplasmic reticulum form, Grp78 / BiP (HSPA5) and a form localized mainly in mitochondria HSP75 / mtHSP70 / mortalin / TRAP-1 (HSPA9) (Petrović et al., 2018c). In addition to them, and less familiar localization, there are: Hsp70-2 (HSPA1B); Hsp70-Hom / Hsp70t (HSPA1L); Hsp70-3 (HSPA2); Hsp70-6 / Hsp70B '(HSPA6); HSP70-7 / Hsp70B (HSPA7), FLJ13874 / KIAA0417 (HSPA12A), RP23-32L15.1 / 2700081N06Rik (HSPA12B), Stch (HSPA13), HSP70-4 / HSP70L1 / MGC131990 (HSPA14) (Petrović et al., 2018a).

In the cells, the HSP70 family is the most induced HSP family in response to stress. HSP72 molecular weight of 72 kDa can represent up to 20% of the total cell protein and is very rapidly induced during cell stress after appropriate stimulation (Noble et al., 2008), especially in skeletal muscle cells (Madden et al., 2008).

Namely, the two most studied proteins in the HSP70 family are HSC73 and HSP72 (Beckmann et al., 1990). Sorger and Pelham (1987) have shown that HSC73, a heat shock protein molecule mass of 73 kDa, is synthesized in most cellular organisms and is only slightly inducible. Unlike HSC73, HSP72 is present in small amounts in ungraded cells, and is thought to be primarily stress-induced (Kiang and Tsokos 1998; Hartl 1996). During the action of various stress stimulus, the organism strives to meet increased demands during stress-related events in HSP72 synthesis (Black and Subjeck 1991).

HSP70 has the ability to exert completely opposite effects depending on its localization (Rodrigues-Krause et al., 2012). Namely, heat shock proteins have long been considered exclusively cytoplasmic proteins with certain functions that are limited to the intracellular part of the cell. However, an increasing number of studies have shown that they can be released into extracellular space (eHSP72) and have different effects on other cells (Titell 2005).

A high level of intracellular HSP72 synthesized in response to stress, occupies the cell and protects it through the role of molecular chaperon (Lindquist and Craig 1988). Said cytosolic inducible HSP70 can mediate through cytoprotective, antiapoptotic and immunological regulatory effects, and is most studied.

The protective role of HSP70 is well documented, and it is interesting that HSP70-induced hyperthermia can provide protection against myocardial ischaemia, suggesting that HSP70 can be protected through cross-care (Cornelusson et al., 1994). Increased HSP70 expression in experimental models of stroke, sepsis, acute respiratory distress syndrome, renal insufficiency and myocardial ischemia is created to reduce bodily injury and in some cases improve survival (Jo et al., 2006; Weiss et al., 2002; Chen et al., 2003; Giffard and Yenari 2004). It has been shown that embryonic HSP70 plays a role in normal development (processes such as apoptosis, cell cycle regulation) and protects against stressors in sensitive embryonic stages (Luft and Dix 1999).

When it comes to extracellular HSP70, it plays a role of cytokine, an immunostimulatory role (helps synthesize proinflammatory cytokines) and improves antitumour control.

Extracellular eHSP70 comes from cells to the bloodstream from living cells exposed to stress through vesicular secretion, exosomes or lysosomes, and through intact lipid membranes that are independent of the transport of proteins through the endoplasmic reticulum-Golgi apparatus, but also passive pathways from necrotic cells and stress-stressed cells (Molvarec et al., 2007; J. Campisi et al., 2003). In a research by Campisi et al. (2003), extracellular heat shock proteins (eHSP), such as those belonging to the HSP family of 70 kDa (for example, HSP72) were presented to act as a "signal of danger" toward immune cells, promoting immune response and improving host defense.

The eHSP72 function is generally associated with the activation of the immune system (Whitham and Fortes 2008). For example, eHSP72 has been reported as an inductor of the microbicidal capacity of neutrophils (Ortega et al., 2006) and chemotaxis (Ortega et al., 2009), participates in the recruitment of NK killers (Horn et al., 2007) as well as in the production of cytokines in immune cells (Asea et al., 2000; Johnson and Fleshner 2006).

There is still no known HSP fraction in the bloodstream coming in one or the other way, and the role of extracellular HSP is contradictory. Namely, the concentration of HSP increases in various diseases, and due to the lack of HSP, metabolic syndrome occurs in humans (obesity, diabetes, cardiovascular disease and dyslipidemia) (Asea 2007; Chung et al., 2008; Krause and Rodrigues-Krause, 2011). Also, the increased concentration of extracellular HSP means better survival (Pittet et al., 2002).

Namely, although induction of iHSP72 reduces the production of cytokines, extracellular HSP (eHSP) can significantly increase the production of proinflammatory cytokines (Breloer et al., 1999; Chen et al., 1999; Multhoff et al., 1999; Asea et al., 2000).

The concentration of HSP70 during gravidity and calving depends on numerous biological variables and physiology and pathology of calving (Molvarec et al., 2010).

Molvarec et al., (2007) found that the concentration of eHSP70 was lower in pregnancy than in non-pregnant women, which is consistent with our results (Petrović et al., 2016). Expression of Hsp72 mRNA in sheep myotomy (intracellular) was elevated during lambing (Wu et al., 1996), as well as in amniotic fluid in women (extracellularly), which were conceived and produced on time (Chaiworapongsa et al., 2008).

Kristensen et al., (2004) have shown that there are numerous factors that influence the concentration of HSP70 in serum cows, such as age and stage of lactation. Although there was no statistically significant difference, plasma concentrations of HSP72 were higher in early lactation (the first 60 days) compared to the middle part of lactation. The concentration of HSP72 is significantly lower in the cows before the partus and in the first weeks after the partys, in order to grow. In dairy cows there is a positive correlation between extracellular and intracellular Hsp 72 values (Catalani et al., 2010). However, this indicates the existence of certain specificities in the regulation of extracellular Hsp72 in cows in the peripartal period. In cows in early lactation, a lower concentration of eHSP70 was found in the first two weeks after calving compared to 4 and 8 weeks (Petrović et al., 2016). These values, as well as their trend, agree with the results of Catalani et al. (2010) and Kristensen et al., (2004).

However, some studies have shown that caloric restriction, hypoglycaemia or hyperlipidemia (which occurs in early lactation) can regulate HSP expression in different parts of the body. Eitam et al., (2009) reported that an extended low-energy diet promoted cell-specific HSP response in cattle with a significant increase in HSP90, but unchanged levels of HSP70 mRNA in leukocytes and lower expression of HSP70 in somatic milk cells. Febbraio et al. (2004) showed that maintaining glucose availability during the exercise reduces the circulation response of HSP72 to healthy people. Creation of intracellular HSP72 under the effect of heat stress reduces insulin resistance and reduces fat accumulation in hepatocytes (Morino et al., 2008). HSP72 concentrations in leukocytes and plasma increased rapidly after calving and correlated with NEFA, glucose, and TNFα (Catalani et al., 2010).

In a small number of studies, the association of peripartal metabolic stress with the values of chaperones was examined. The NEFA concentration in peripartal period shows a positive correlation with the NSP72 concentration (Catalani et al., 2010). Cincović and Belić (2014) showed that the concentration of NSP70 was significantly higher in weeks after calving compared to a week before calving. A higher concentration of NEFA and BHB (beta-hydroxybutyrate) was found in the first and second weeks after calving compared to other periods. The concentration of NSP70 positively correlates with NEFA and BHB values. Partial correlation shows that ties are stronger in the first and second weeks after calving, which is the period when lipid mobilization and ketogenesis are most pronounced. The concentration of NSP70 in the first two weeks after calving is dependent on the level of lipid mobilization and ketogenesis. Metabolic stress, characterized by lipid mobilization and ketogenesis, increases the blood NSP70 concentration during early lactation.

CONCLUSIONS

Hsp70 shows significant relationships with the pathophysiological mechanisms dominant in cows in early lactation, such as inflammatory response and insulin resistance.

Consequently, there is a presumption that the indicators of metabolic stress can affect the concentration of Hsp70 in serum of cows.

In the future, Hsp70 can be a significant indicator that can be used to evaluate the metabolic adaptation of cows in the peripartal period.

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ECOLOGICAL ACCESS IN FEEDING OF DOMESTIC ANIMALS

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ABSTRACT

Healthy food, or more accurately called functional food, is increasingly becoming subject of research by many institutions and individuals from rich countries, who achieved, through the well-organized production provide the necessary amount of food for the basic diet of the population.

The so-called functional food should be in addition to the biological needs of the organism, has a role in preventing the organism from the disease, and thus raising the quality of life.

When we talk about functional foods, that primarily related to the products of animal origin, or to meat and milk. The use of these products is very efficiently introduced into the human organism of a significant substance, and above all antioxidants such as provitamins, vitamins and chelate forms of microelements. Many studies have pointed out that if these substances are introduced into the organism through food, and then in that case are also influential.

Today's level of technological development has created a number of habits in the vast majority of people, which represent a latent danger to their health. Foods and habits related primarily to nutrition, the presence of various harmful substances in the air, various types of radiation and a way of life that abounds in stressful situations, lead to an increasing incidence of very serious diseases. All this is the reason for more, to pay more attention to the production of healthy food in the period ahead, as it can be expected that its production and use can be solved at least part of a number of problems related to the health of a large number of people.

Key words: *domestic animals, new nutrients, functional foods*

INTRODUCTION

Today, in most cases, the accepted way of life in urban areas has some advantages and benefits, but at the same time it carries a considerable number of latent hazards that are manifested through food as risk factors and affect the deterioration of quality of life. The use of unauthorized additives and other food additives, air pollution, radiation and permanent stress as an integral part of everyday life, leading to increasing incidence of disease from various types of cancer, followed by heart and blood vessels. Consuming healthy food would have an effect on the prevention of these diseases and their cure.

Here, first of all, is meant products of animal origin, meat and milk, through which it is possible, in the most efficient way, to bring useful substances into the human organism, primarily antioxidants (provitamine, vitamins and chelate forms of microelements), because it is known that natural or food intake is most widely used. In this way, prevent the adverse effects of free radicals which are a common cause of various diseases, and were formed by the interaction of the above mentioned agents. Functional foods must remain foods and they must achieve their effects in amounts that normally consume. It does not represent pills or various supplements.

In addition (Sretenović and Ilić, 2003) and Ilić et al. (2007) emphasize the importance of preserving a healthy environment, because it is known that animals are big eco-system pollutants, and especially land, as the excrements of large amounts of undigested substances and drugs that disrupt the balance of the soil microclimate, and it is worryingly endangered. A significant number of studies are conducted in the direction of reducing the pollution of the environment. Animals produce 130 times more waste products than humans. The entire modern biotechnology (production of enzymes, probiotics, chelated trace elements, organic selenium and chromium, oligosaccharides, etc.) is realized in order to maximize the use of nutrients in the organism and to minimize unhealthy foods in the environment.

In this sense, the orientation of countries with highly developed agricultural production in the future is the reduction of commercial production, and the promotion of the production of quality seed material both in the field of production and in the semen of tested animal heads selected for high production. High-quality seed material from plants and animals is obtained indebted to the development of molecular biology and genetic engineering.

The influence of proper nutrition on the preservation of health and reproductive ability of domestic animals

Good production and reproductive performance, then good health of dairy cows are primarily the result of proper nutrition. Proper nutrition involves balance of nutrients to satisfy the basic needs of animals and their production. The importance of proper nutrition and the effects that are achieved in this way is illustrated by the example of research carried out with high milking cows.

Namely, in the experiment carried out, the method of feeding cows usually used on a given farm is compared with the one in which nutrition is completely balanced in all relevant indicators (NRC 2007). The study was conducted on cows in the first 100 days of lactation, in the same facilities, under the same ambient conditions, with the same quality of feed used, the only difference in their balance, or in concentrate mixtures of different percentage shares of the individual components, , which are represented depending on the amount of milk produced.

The results achieved in the experiments indicate that the diet of dairy cows reserves lie in the production of milk. In the experimental group of cows in comparison with the control, it was produced even with a smaller number of nutrients 26.74% obtained milk, respectively 25.56%, up to 4% on fat of corrected milk. The results of the research (Pavličević et al 1995) are presented in Table 1.

Table 1. Production parameters of cows in trial

Indicator	Control	Experiment
Total milk for 100 days of lactation	2687.5[a]	3406.2[b]
Total 4% MKM for 100 days of lactation	2510.3[a]	3152.0[b]
Total ml produced. m. for 100 days of lactation	95.7[a]	119.3[b]
Consumption of SM / kg of milk, g	629	623
Consumption uk. prot. per kg. milk, Mr	99	113
Consumption of concentrate per kg of milk, g	292	270

a, b are statistically significant at the level P <0,01

In the intensive cattle production, the problem of reproduction is one of the highest priorities. The reduced fertility in cows, most often those with the highest milk production, as in modern farms, has been a serious economic problem for a long time, which was reflected in the reduced number of calves and, consequently, the smaller amount of milk. In fact, until recently, milk production was considered to be associated with poor reproductive performance of cows. But today, thanks to new knowledge in the field of nutrition knows with certainty that it is possible to maintain a high milk production and preserve good reproduction. In addition to a number of factors affecting the production and repair of reproductive performance, is also Vitamin A in combination with beta-carotene.

Synthetically produced as a result of new biotechnologies, vitamin A and beta-carotene have an extremely important role as components of mineral vitamin pre-mix. It is well known that the role of beta-carotene as a pro-vitamin in reproduction is completely independent of vitamin A. In the summer period of nutrition diet with green food, it brings sufficient quantity to animals, however, in the winter period, it is lacking due to its oxidative decomposition when it forages hay and silage its quantity is reduced due to long storage, so in this period, intervention with this pro-vitamin is justified, because the liver deposits are almost empty. Beta-carotene accumulates in a yellow body and causes the yellow color, which all leads to the conclusion that it has importance in its function. It is believed that the yellow body cannot be fully developed if there is insufficient amount of carotene, and in its lack of conception, the embryo mortality and early abortion increase. Calves from cows that were not supplied with sufficient beta-carotene are non-vital and easily suffer from inflammation of the intestines.

In the absence of beta-carotene, vitamins A and E in cows immediately after calving, the activity of the immune system decreases, the amount of cortisol in the blood plasma increases, and diseases such as fatty degeneration of the liver and ketosis can develop. Also, as a result of decreased immune system activity or lymphocytes, there is an increased chance of retentio secundinarum and mastitis occurring (Kolb and Seehawer, 1998). The same authors point to the importance of introducing beta-carotene, vitamin E and selenium during the period of drying, which significantly reduces the degree of immunosuppression.

Indicator with a sufficient supply of the beta-carotene is its content in the blood plasma. A 400 mcg / 100 mL is considered to be sufficient supply. Since it is well known synergistic effect of beta-carotene, vitamin A and vitamin E are usually given in combination.

Although in the literature there are also different opinions, most researchers agree with the fact that the role of beta-carotene in the reproduction of cattle-breeding cows is irreplaceable and that with its application it should be started even in the period of drying. Some results indicate that if the beta-carotene application starts in the dried period, the service period is sharply reduced and at the same time it reduces the insemination index.

The use of some new nutrients in the feeding of reared ruminants

During the last ten years in crop production have created a new culture, which are very present in the diet of dairy cows, and other kinds and categories of domestic animals. Their use has allowed better results in livestock production.

Especially in high milk production, the use of these nutrients provides high-quality sources of energy and protein. By introducing these nutrients in the nutrition of the cow fodder, significant economic effects can be achieved.

High-protein pea is a relatively new nutrient in our country and is considered a good substitute for soybean meal taking into account the yield of grains and nutrients per hectare. Thanks to the selection measures, varieties with protein content ranging from 26-28% have been created recently, and with the variety "Bohatyr" a visit was performed with high-fat cows during the first 100 days of lactation in which it is partly (in volume up to 50% net protein values) or completely substituted soybean. (Sretenović et al.1996).

Peas is a very good yield of 4-4.5 culture gives t / ha means more than soybean, there is a short period of vegetation (100 days). Peas have less anti-nutritive substances compared to soybeans and does not require a prior heat treatment before inclusion in diets for cows. As a source of protein, peas alone or in combination with soy sauce is a good nutrient in the feeding of dairy cows. It can partially or completely replace the expensive soy sauce that is mainly imported. The biological and nutritive value of peas is not less than soybean. Table 2 shows the results of experiments, which were performed with the protein peas was determined and compared with soybean meal at the cows in the first 100 days of lactation. These two nutrients were fed through a mixture of concentrates. There was no difference in the quantity and quality of milk among the examined groups of cows, and it can be concluded that the peas give the same or similar results as the soybeans.

Table 2. Effects of high protein pea in milk production

Indicator	Group		
	K	I	II
Milk production, kg	27,22	26,02	26,22
Quantity 4% MKM, kg	26,43	23,87	25,17
Milk Fat, %	3,74	3,48	3,42
Protein, %	3,25	3,27	3,22
Lactose, %	4,71	4,58	4,65

K- Soya Sauce; I- soybean meal + peas; II - peas;

Recently, varieties of sunflowers with increased content of unsaturated fatty acids and reduced cholesterol content have been created, and which have great importance in proper nutrition. Sunflower seed contains protein; energy (contains about 50% of oil) as well as cellulose. The beans are contained all the components that are necessary in the diet. The sunflower grain should be used as an integral grain that has been harvested. n cows in the first 100 days of lactation, it is recommended to include meals in the amount of about 1.5 kg, in the other 100 days about 1 kg and at the end of lactation about 0.5 kg. Extremely high quality and insufficiently used nutrients are also maize crops as a high-energy source of energy that is obtained as a by-product of the starch industry. They contain about 50% oil with an extremely favorable ratio of saturated and unsaturated fatty acids as well as vitamin E, which is of great importance for the reproduction of the animal. This means that the growing of hybrids with higher oil content, which is concentrated in the germ, can get higher yields of energy, which is very important in the diet of cows shortly after calving.

Bearing in mind the fact that diet can influence the composition of fat tissue as well as milk fat, or preferably the presence of polyunsaturated fatty acids that are necessary in terms of healthy nutrition, this and similar nutrients should find a significant place in the nutrition of cows. Of course that sprouted grains significantly improving production and reproductive performance of high production throats, their use in an early stage of lactation also recommended.

The use of full fat soy is irreplaceable in the diet of high-fat cows, especially in the early stage of lactation. The whole soybean is used as a heat-treated (toasted, micronized, extruded), although it is possible to use it in its raw state, but not more than 3 kg per head per day. The reason why it is not recommended to give raw soy is that by thermal treatment the content of the non-degradable protein that is necessary in the diet of high-milking cows is increased.

Genetic improvement has been produced in the United States of the soybean variety Kunitz - selected to reduce the content of antinutritive substances (trypsin inhibitors, ureases, etc.). This variety can be included in the concentrate mixtures without prior heat treatment thus saving substantial amounts of electricity.

As a result of genetic engineering produced is a variety of rapeseed oil with low glucosinolate content, which adversely affect the operation of the thyroid gland, this was the technological limit of its inclusion in the mixtures of the concentrate is not more than 10-15%.

Application of Contemporary Biotechnology Products to Domestic Animal Nutrition

Microelements as essential additives to all kinds of domestic animals are traditionally used in the form of their inorganic salt most commonly sulphate, chloride, oxide or carbonate. However, as most of the factors that affect their utilization are known, there are constant efforts to improve their utilization in humans and animals. It is known that metals such as Cu, Zn and Mn in the form of a chelate, i.e. associated with amino acids and peptides can increase the utilization of these microelements and improve parameters such as growth, reproduction and health status.

In numerous studies carried out over the past years, it has been found that the use of chelate forms of microelements (Zn, Cu and Mn) in addition to improving reproduction yield positive effects in milk production, decrease the number of somatic cells in milk and the occurrence of mastitis, raise the immunity of the organism, better feed efficiency, contribute to easier overcoming stressful situation. Organic bound microelements that are not chelates include selenium and chromium. They are obtained by growing yeast on the surface enriched with selenium or chromium.

Organically bound trace elements usually found in the complexes in which carriers or bearing media of organic molecules. The trace elements in the complex are linked via a covalent bond and have no electric charge, ie are electrically neutral, and in the gastrointestinal tract do not participate in the interactions, and therefore do not form insoluble complexes with the other food ingredients, have greater solubility, they are easier absorbed and better used. It is known that chelating microelements have a higher percentage utilization compared to the inorganic sources of sulphate and oxide forms. Explanation should be sought in a more efficient absorption of the micro-nutrient, which is bonded to the organic molecule (amino acid or a short peptide). The fact that microelements are highly enzyme cofactors and participate in many metabolic processes can explain the multiple effects. For all of the above reasons it is recommended that 30-50% of the animals' need in microelements could be settled from organic sources and the rest from inorganic compounds.

Microelements such as Mn, Cu and Zn are produced in the form of a chelate. Helatization refers to a specific type of formation of the complex between metal ion and ligand. Ligands can be defined as molecules that contain an atom with free electronic pairs. The ionic metals are bound in the ligand complex through donor atoms such as oxygen, nitrogen or sulfur. Helatization represents a bond of such ligands with a metal ion through two or more donor atoms which form a complex that contains one or more heterocyclic rings (bond) containing a metal atom.

It should be noted the importance of vitamin E and selenium in the diet of dairy cows. It is known that Se and vitamin E work synergistically as components of an antioxidant system that protects the animal from various diseases. An adequate amount of vitamin E must be added to the meals to ensure the utilization of selenium. Several authors present positive results of the use of organic selenium on the production, reproduction and health condition of cows that are associated with better absorption and utilization in the body. The primary organic form of selenium is selenomethionine, which is incorporated into the selenoprotein via the amino acid mechanism. Selenomethionine is the main form of selenium present in grain, oilseeds and other plant materials. The concentration of selenium in nutrients varies widely according to the content of selenium in the soil.

Sodium selenite as the main source of selenium in animal feed is entered through the premix in the rumen by means of microflora converted to insoluble compounds of passively absorbed from the intestine, chemically reduced to selenide, and transported to the liver, where it is synthesized in the selenomethionine biologically active form of selenium, or to the kidney where it is excreted in the urine, so that the ruminants use it poorly.

If selenium is not available in sufficient quantities in foods that are part of the meal, it is mandatory to add it through the premixtures to the concentrate mixtures, but take into account the chemical form of the compounds by which it is introduced because the absorption depends on it (the actual absorption is in the case of dairy cows is 10- 15% of consumed). Peter et al. (1982) stated that the absorption of Se in the form of selenomethionine is 12-13% higher than selenite (inorganic form).

That the level of selenium in the blood may be a good criterion to supply the organism with the microelement results indicate Žust et al. (1998). They point out that selenium concentration below 30 µg / l points to the deficiency of this micro-organism in the body and may be the cause of muscular dystrophy in calves. If it is not available in sufficient quantities in used nutrients, it is required to add it through a mineral-vitamin pre-mix, in the amount of 300 µg / kg SM. The most effective application of selenium is if it starts to dry period and continue during lactation.

Table 3. Average daily milk production and milk fat content

Indicator	Treatment I	Treatment II
Quantity of milk yield per cow, kg	1876.5	2141.6
Daily amount of milk per cow, kg	24.65	25.17
Amount of corrected milk (4% mm), kg	23.69a	24.52b
Milk fat, %	3.80	3.87
Milk fat, kg	71.31	82.88

a, b are significantly different at the level $P<0.05$

Considering that it has been proven that the content of selenium in milk increases by 4-5 times if it is introduced into the organism of animals in chelatable form it has much justification to engage through mineral-vitamin premixes into concentrate mixtures for all kinds of domestic animals.

The introduction of selenium in most of the world is below 200 μg per day. One of the better ways is to bring selenium through meat and milk. Since pork and chicken meat is consumed in large quantities, it is considered to be one of the best ways to bring selenium. Multi-year research Clark et al. (1998) show that by entering 200 g of selenium daily, the possibility of cancer is reduced by 50%.

Since it is known that our land is poor with selenium and that it is best adopted by the organism when it is introduced through meat, milk and eggs, it is certainly necessary to include it in animal meals. There are projects where plants are treated with selenium, they translate it into an organic form and in this way the animals are best adopted.

Also it is known that selenium is involved in the formation of tocopherols, which plays a major role in redox reactions which clearly indicates the importance in organisms (Nicholson et al. 1991). Organically bound microelements have no electrical charge so that the other ingredients of the food do not create insoluble complexes. Thanks to this fact they are more easily absorbed in an unchanged form and thus more efficiently exploit.

In the study of Beale et al. (1990) indicate that the addition of inorganic selenium to animal feed did not lead to significant residues of selenium in meat, milk and eggs. In the same paper pointed out that the accumulation of selenium in the muscle is relatively low.

The research of Ilic et al. (2012) were carried out to determine whether the addition of the bioactive substance "Bioril" in various concentrations in the concentrate (0.3% and 0.6%) has an effect on the growth and conversion of food in the lamb.

Table 4. Performance of fattening lambs

Criterion	Groups		
	K	I	II
Initial body weight, kg	18.39	18.40	18.38
Initial age, d	50	50	50
Final body weight, kg	31.75	33.37	33.62
Total gain, kg	13.36	14.97	15.24
Average daily gain, g	318	356	362

In the result shows that have achieved the biggest daily gain in lambs of the experimental group II. The difference between body weight K and I was 1.62 kg and was statistically significant (P <0.01). The difference between body weight K and II group was 1.87 kg and was also statistically significant (P <0.01). The difference between body weight I and II group was 0.25 kg and was not statistically significant (P> 0.05).

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VARIABILITY OF CALCIUM AND PHOSPHORUS IN THE BLOOD SERUM OF SHEEP

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ABSTRACT

The large number of sheep, especially the ones which spend the majority of time on pastures, is being fed with the meals which do not contain all the neccessary mineral substances. The level of calcium and phosphorus on natural pastures is too low in relation to the neccessities of sheep. So, the irregularity in the feeding of sheep occurs because of the absence of the other food sources. These irregularities are in the range from the acute mineral deficite or illness, to the mild temporary forms which can hardly be diagnosed, but they affect the level of production. The contents of calcium and phosphorus in the blood serum of sheep, as one of the indicators of the supplementation of sheep with these substances, is given in this paper. The average level of calcium in the blood serum of the control group of sheep was 2.58 mmol/l and phosphorus 1.05 mmol/l. In the blood serum of the tested group, the average contents of calcium was 2.35 mmol/l and phosphorus 0.90 mmol/l.

Key words: *sheep, calcium, phosphorus, serum*

INTRODUCTION

The knowledge of the nutritive values of the grass mixture, with which sheep meet their needs during nearly one half of the year, is very important from the standpoint of the evaluation of the food quality. Pasture is very important as the full-value source of food in the feeding of sheep, which could not be easily substituted in the extensive breeding of ruminants. The quality of the pasture and its nutritional value depend on many factors but primarily on the quality of the soil and the climatic factors. In the conditions of breeding sheep in the Sar Mountains, learning about the quality of pasture gives us the possibility of taking appropriate measures in its improving. It would be a starting point for resolving issues of proper nutrition of sheep during the summer, because that period represents an important phase of a changing in physiological states (lactation, and preparation for successful impregnation).

Certainly, these tests are not able to discover the genesis of their foundation, on which we can derive the conclusions for guidance of the direction of the further development by applying Hemo - Agro - Hido and other measures, which are dictated by the ecological factors. Because these researches do not have pretensions of such a capacity and profile, their goal is to show the picture of the chemical composition of the grass mass of the tested pastures, as a very useful component of the main research, which is related to the dynamics of calcium, phosphorus and magnesium in stages of vegetation, or the exploitation of the pastures. In many areas of the Sar Mountains, the content of stodgy cattle food is not tested enough. From the results obtained on the basis of our previous researches of mineral substances in stodgy food, it is established a low content of phosphorus in them, who appeared much more frequently than it is the case with calcium. This phenomenon is especially present during dry years and certain periods which often occur at the Sar Mountains where the testing was performed. To this should be added the fact that at the Sar Mountains, there are lands in which the content of physiologically active phosphorus (P_2O_5) is extremely low, as was found by *Stojkovic (2014)*, and a low content of this element in the soil adversely affects its content in plants. To this we should add the statement of *Stojković (2011)* that a substantial number of plant species from these areas has a low content of phosphorus.

MATERIALS AND METHODS

The testing of the influence of the physiological conditions and the seasons on the dynamics of the content of calcium and phosphorus in the blood serum of the atado sheep in Strpce 2017/2018 was conducted. There were 60 sheep in the experiment, 30 of which were in the control group – the futile sheep (treatment 1, 2, 3, 4 and 5) and 30 in the experimental group - the fertile sheep (treatment 1, 2, 3, 4 and 5). At the end of each treatment, the blood was taken from the jugular vein of the sheep for the preparation of the serum. The testing of these mineral elements was carried out in the blood serum of the sheep and the food used in their meals. The sheep were staying in a cottage at the Sar Mountains from the late May until the early October (2017). The main meal was their pasture with the addition of 200 g of corn grits. And from October until May (2018), the sheep were in their home

environment near Štrpce. The main meal was their meadow hay from 1.5 to 2.0 kg and corn grits from 200 to 300 g per day. The cattle salt in the form of the mineral plates was also available to the sheep.
Statistical analysis was performed using Statistica, version 6, SatSoft. Inc. (2003).

Table 1. The contents of nutrients in the feed for feeding sheep

Type of food	Chemical composition, (%)					
	Water	Protein	Cellulose	Ash	Ca/g	P/g
Meadow hay	11.27	8.45	30.61	7.81	0.77	0.24
Lucerne hay	11.20	9.90	28.95	8.54	0.96	0.13
Corn grits	12.95	8.50	1.89	1.57	0.11	0.30
Spring grass	21.71	9.69	24.10	6.74	0.70	0.14
Summer grass	19.68	6.75	30.230	5.50	0.87	0.17
Autumn grass	18.13	5.69	28.4056	8.234	0.71	0.32

RESULTS AND DISCUSSION

The data about the dynamic of the content of calcium in the blood serum of the sheep are shown in table 2.

Table 2. The contents of calcium in the blood serum of sheep (mmol/l)

The of taking blood	X±SD	SG
Control group – futile sheep		
Tretment 1 - August	2.53±0.18	0.48
Tretmenrt 2 - November	2.61±0.19	0.46
Tretment 3 - January	2.59±0.14	0.36
Tretment 4 - July	2.57±0.23	0.65
Tretment 5 - September	2.61±0.18	0.48
Average	***2.58±0.18***	***0.48***

Tested group – the fetile sheep		
Tretment 1 – first half of the pregnancy	2.55±0.26	0.56
Tretment 2 – second half of he pregnancy	2.36±0.12	0.47
Tretmen 3 - the beginning of lactation	2.03±0.14	0.50
Tretment 4 – the end of lactation	2.30±0.15	0.61
Tretment 5 – the period of becoming infertile	2.53±0.12	0.48
Average	***2.35±0.15***	***0.52***

After the fertilization of the sheep, the content of calcium in the blood serum was 2.53 mmol/l, while in the futile sheep at that time was 2.61 mmol/l. In the second half of the pregnancy, the content of calcium decreased to 2.59 mmol/l, while in the futile sheep this decrease was much lesser (2.57 mmol/l). In the phase of the most opulent secretion of the milk in the lactating sheep, the decrease in the content of calcium is most pronounced, and it was slightly below the physiological limits (2.02 mmol / l) then. At that time, the content of calcium in the blood serum of the futile sheep was at the same level (2.55 mmol / l). In the phase of reduced secretion of the milk in the lactating sheep, the increase in the content of calcium of 4.00% was noted, and later by becoming infertile, the level of calcium in the blood serum climbed to 2.36 mmol / l. At that time, in the futile sheep, the content of calcium held at the same level (2.57 and 2.60 mmol/l). By the dynamics of the content of calcium in the blood serum of the fertile and the futile sheep, the general conclusion can be made, that, normocalcemia existed in the conception of the sheep and that the changes, that occurred in the fertile sheep, the result of normal moving, were caused by the different needs of the organism in accordance with the physiological conditions. Considering the content of calcium in the blood serum of the sheep it can be concluded that calcemia was normal and that there were no significant differences ($P>0.05$) in physiological conditions, or season.

The dynamics of the content of calcium in the blood serum shows a regular rhythm when we correlate it with changes in the physiological status of the sheep, making a concave parabola with the lowest point in the time of the most opulent secretion of milk. At the same time, the contents of calcium in the serum in the futile sheep ranged slightly above average the normal values (between 2.57 and 2.74 mmol / l) and despite the season it was not subjected to changes. The percentage of variation in the content of calcium was significantly lower in the futile sheep, which is understandable.

According to the data from *Jašovic et al. (2016)*, this value was lower for 5.15% of the average content of calcium in our previous experiments, *Stojkovic (2014)*. But, in literature there are the data according to which the content of calcium in the blood serum of sheep can be much higher, Pavličević and co. (2001), as was the case when the minerals with high calcium content were added to the meals. But there are also data on cases of the expressive hypocalcimia such are indicated by *Stefanovic et al. (2015)* in whose experiments the level of calcium in the blood reach the level of only 1.98 mmol /l.

The data about the content of phosphorus in the blood serum of the sheep are shown in the table 3.

Table 3. The content of phosphorus in the blood seum of the sheep (mmol/l)

The of taking blood	X±SD	SG
Control group – futile sheep		
Tretment 1 - August	0.91±0.16	0.44
Tretmenrt 2 - November	1.02±0.15	0.38
Tretment 3 - January	1.04±0.08	0.22
Tretment 4 - July	1.08±0.06	0.17
Tretment 5 - September	1.21±0.04	0.10
Average	***1.05±0.09***	***0.26***
Tested group – the fetile sheep		
Tretment 1 – first half of the pregnancy	1.14±0.15	0.67
Tretment 2 – second half of he pregnancy	0.82±0.09	0.35
Tretmen 3 - the beginning of lactation	0.69±0.08	0.30
Tretment 4 – the end of lactation	0.94±0.08	0.34
Tretment 5 – the period of becoming infertile	0.91±0.19	0.43
Average	***0.90±0.10***	***0.41***

In the first half of the pregnancy, the content of phosphorus in the blood serum of the sheep was 1.14 mmol/l. in the second trimester the content decreased to 0.82 mmol/l. During the period of full lactation, the maximum decrease was 0.69 mmol/l. From this, along with the decrease in the secretion of the milk, the level of phosphorus increased to 0.94 mmol/l, only to reach the level of 0,91 mmol/l in the infertile sheep in September. In the futile sheep, the content of phosphorus in the blood serum in August was 0.91 mmol / l and it was constantly increasing during the year (1.02 mmol / l, 1.04 mmol / l, 1.08 mmol / l and 1.08 mmol / l) to reach in the beginning of September 1.21 mmol / l. In the fertile sheep, the smallest deviation from the average values between the certain sheep was noticed after the fertilization and they were constantly growing from the period of becoming infertile. In terms of percentage of the variations of the content phosphorus in the blood of the futile sheep, the flow is reversed. Varying is the highest at the time when sheep should be impregnated and it constantly declines until September.

From the presented data it can be seen that the content of phosphorus in the blood serum of the sheep during the year, that is in all physiological states, under the physiological limits. Hypophosphathimia reached its peak in the fertile sheep in the phase of the most opulent secretion of the milk. Concerning that, hypophosphathimia was very pronounced in the fertile sheep, and the average varying of the inorganic serum of phosphorus during the year was higher than in the futile sheep. Differences in the content of phosphorus per season and physiological condition were in the level of the statistical significance ($P < 0.01$).

The content of phosphorus for all groups in the experiment was in the average 0.98 mmol/l of the serum. With these values it was located below the physiological limits of the normal range of the content according to the data from the literature (1,45-2,00 mmol / l).

The lowest values were recorded in the most opulent stage of lactation (0.74 mmol / l) and the highest in the first half of the pregnancy (1.13 mmol / l). With lactation passing off and the arrival of the nonproductive lactation period (in autumn), the content increased to 0.97 mmol / l. In terms of variation, it is characteristic that, from the beginning to the end of pregnancy of the monitored period, the variety grew more and more (from 5.03 to 15.50%). Although, in the futile sheep, the average content of phosphorus in the serum was higher than in the fertile sheep by 11%, the general level was very low, and the changes were more subordinate to the factor of diet, which was not the same case and in the fertile sheep. The content of phosphorus in the futile sheep was constantly increasing during the year, starting from 0.98 mmol / l in August and to 1.13 mmol / l at the end of the observed period. Concerning the content of phosphorus in the serum of the fertile and the futile sheep, it can be concluded that hypophosphatemia is expressed during the whole year.

During the general hypophosphatemia, of both fertile and futile sheep, it is important to notice the appearance of a very different character of variation in the content of phosphorus per year, namely caused by the physiological state. In the futile sheep it can be seen a constant, although small increase in the content of phosphorus during the “vacation” until next season of fertilization, while at the same time narrowing of the variation span, whereas in the pregnant sheep, we have a decrease in the content of phosphorus, which goes along with the duration of the stress of the organism (increased distress) in pregnancy in the first two months of lactation, and its increase with the arrival of the period of the physiological relief, with a permanent increase in the percentage of the variations in the content of phosphorus in the blood serum. These findings supports the assumption that in the states of hypophosphatemia in the temporary futile sheep, the balance of phosphorus is gradually improving to such an extent that they can, after a one-year break, manifest the sexual passion and be fertilized. In the “fertile” sheep, although in the second half of the lactation the balance of phosphorus in average improves, it can not be improved in all animals, which is attested by the increased percentage of variation so that, slightly more than a half of the sheep came to a successful fertilization, while a smaller part remained unfertilized.

From the displayed value of the content of phosphorus, it can be seen that there was virtually a low level of this element in the blood serum of the sheep with a minimal increase in the time of their pregnancy, and with the lowest value in the stage of lactation. The reason for such a low content of phosphorus in the blood of the sheep is interpreted by its low content in food, especially hay, and the reason for the low content in hay, by the interpretation of *Miric (2000),* lies in the fact that, in this region exist large areas of land on which the content of physiologically active phosphorus (P_2O_5) is extremely low. This is confirmed by the findings of *Stojkovic et al. (2011, 2014)* in which a large number of plant species from these areas have a low content of phosphorus. A particularly low content of phosphorus in the blood serum of the sheep in lactation can be interpreted by its enhanced secretion in milk.

CONCLUSION

The content of calcium and phosphorus in sheep blood serum was examined as one of the indicators of provision of these substances for animals. The average presence of calcium in the blood serum of the examined sheep was 2.46 mmol/l and of phosphorus 0.90 mmol/l of serum. The calcium values were at the upper limit of normal content reported in the literature. It points out that there have been the provisions of the content of this element in the blood serum of the sheep and that it has been provided through feeding. The changes in the content of calcium were not significantly more pronounced in relation to the seasons and the physiological state of sheep. From the shown values of phosphorus content it can be seen that there was practically low level of this element in the blood serum of the sheep with minimal increase at the time of pregnancy, and the lowest in the lactation phase. Phosphorus values were below the deficit margin and this value decreased most at the beginning of the lactation in sheep.

The reason for this low content of phosphorus in the blood of sheep is interpreted by its low content in food, especially hay, and the reason for the low content in hay lies in the fact that in this area there are large areas of soil where the content of physiologically active phosphorus (P_2O_5) is extremely low.

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ANTIVIRAL ACTIVITY *IN VITRO* OF FIVE SELECTED INDIGENOUS PLANTS AGAINST BOVINE HERPES VIRUS-1 (BHV-1) AND BOVINE VIRAL DIARRHOEA VIRUS (BVDV)

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ABSTRACT

In this study, we investigated the in vitro antiviral activity of five selected plant extracts against Bovine Herpes Virus-1 (BHV-1) subtype 1.1 and Bovine Viral Diarrhoea Virus (BVDV) genotype 1, which with other viral and bacterial agents cause Bovine Respiratory Disease Complex (BRDC). Ethanol (alcohol) and aqueous extracts were prepared for all five plant species studied. Two prepared samples of aqueous plant extracts/macerates had antiviral effect against BHV-1 as a representative of herpesvirus (DNA virus), but none of the samples showed an antiviral effect against BVDV genotype 1, a NADL strain, that belongs to RNA viruses. In our study, Selectivity Index (SI = CTC_{50}/EC_{50}) for aqueous extracts of Matricaria chamomilla (SI = 32) and Achillea millefolium (SI = ≥ 8) indicates a significant selective inhibition of DNA virus (BHV-1, strain TN41). On the basis of the obtained results, future research is needed, which would reveal the active ingredients of the investigated plants and mechanisms of their antiviral effect.

Key words *- antiviral activity, BHV-1, BVDV genotype 1, indigenous plants*

INTRODUCTION

The reasons for restrictions on the use of antiviral drugs in human and veterinary medicine in comparison with the use of antimicrobial agents are reflected in viral mutation, new viruses, toxic effects, the severity of viral diseases, ability of virus to survive intracellularly, the high costs and the non-availability of specific antiviral chemical agents against veterinary pathogens. Deficiency of effective antiviral drugs require discoveries a new effective antiviral compounds. Most antiviral against viruses of veterinary importance are still used as animal models in the development of human antiviral drugs (e.g BVDV like surrogate model for the hepatitis C virus). Plants are naturally gifted at the synthesis of medicinal compounds, and allow creation of new, cheap drugs with high therapeutic potential. Today insists on the advancement of analytical chemistry tools, the development of standardization and extraction methods, and the standardization of virus assays (Pushpa et al., 2013; Zeedan, 2016). Herbs are rich source of phytochemicals with different biological activities including antiviral effects. Plants contain various compounds that inhibit different parts of the replication cycles of various types of DNA and RNA viruses. This antiviral activity is generally attributed to polyphenols, rosmarinic acid, chlorogenic and caffeic acid (Jassim and Naji, 2003), which interact with each other, altering the effect of a particular active compound (Gobbo-Neto and Lopes, 2007).

BHV-1 is a member of the subfamily *Alphaherpesvirinae*. Because of its importance in veterinary medicine, BHV-1 is listed on list B disease by the office International des Epizooties (OIE) (Jones, 2003). BHV-1 is the cause of many different diseases (rhinotracheitis, vulvovaginitis, balanoposthitis, abortions, conjunctivitis and generalized systemic infections) and important cofactor in the BRDC, which responsible for major economic losses in both beef and dairy production (Jones and Chowdhury, 2010; Biswas et al., 2013; Kurćubić et al., 2018). Khan et al. (2005) reported that medicinal plants are an important tools of controlling viral diseases caused by herpesviruses in both humans and animals. A large number of studies in which plant extracts were used have shown *in vitro* anti-BHV-1 activities (del Barrio and Parra, 2000; Simoni et al., 2007; Melo et al., 2008; Gupta et al., 2010; Pilau et al., 2011; Barros et al., 2012; Fernandes et al., 2012; Abdallah et al., 2013). According to our knowledge of the available literature data, antiviral activities of five different herb extracts examined in our study against BHV-1 and BVDV have never been tested.

BVDV is a non-arthropod-borne member pestivirus within the *Flavirida*e family, and causes infections of domestic and wild ruminants worldwide (Lindenbach et al., 2007). The presence or absence of visible CPE in infected cell cultures conditioned the division of BVDV into cytopathic (CP) or non-cytopathic (NCP) biotypes (Baker, 1995). Based on antigenic and genetic properties, two species of the BVDV can be distinguished, BVDV-1 and BVDV-2. Recent data (Ridpath et al., 2010) indicate that it has been identified 12 BVDV-1 subtypes (BVDV-1a - BVDV-1l) and 2 BVDV-2 subtypes (BVDV-2a and BVDV-2b). BVDV infections causes respiratory and reproductive problems: transplacental infection and fetal death, congenital malformations, neonatal and postnatal mortality, mucosal diseases, retarded growth and poor performance of surviving animals (Bolin et al., 1985; Roeder and Harkness, 1986). Also, BVDV is one of the most problematic viral agents involved in the development of BRDC. Antiviral properties of plant extracts against BVDV have been tested, and different levels of activity have been proven (Koseki et al., 1990; Ruffa et al., 2004; Pilau et al., 2011).

A commonly accepted protocol for testing of antivirus sensitivity is not available (Swierkosz and Hodinka, 1999; Khan et al., 2005). To investigate antiviral activity, the most commonly used methods are fewest different techniques based on cytopathic effect or cell viability (Swierkosz and Hodinka, 1999; Khan et al., 2005; Da Silva et al., 2006; Astani et al., 2010). Tetrazolium dye (MTT) colorimetric assay (Khan et al., 2005), measures cell viability and it has been applied to access antiviral activity against different viruses (Freitas et al., 2009). Sylvester (2011) reported that the MTT assay is rapid, convenient, and economical, and become a very popular technique for quantification of viable cells in culture.

The main objective of this study is to determine the existence and severity of the virucidal effect (anti-viral activity) of the extracts of 5 various plants obtained by 2 different extraction methods (ethanol and aqueous extracts) as well as checking viability of viral particles. The first aim in this study is to determine the concentration of plant extracts that cause cytotoxicity to 50% of the cells (the so-called 50% cytotoxic concentration - CTC_{50}). Also, the cytotoxicity of extracts of different plants obtained by different methods of extraction, as well as various solvents, was determined on the cell cultures that we later used in the experiment.

MATERIALS AND METHODS

Plant materials:

A total of 5 indigenous plants were collected from different regions of the Central Serbia, as presented in Table 1.

Table 1. List of indigenous plants (n=5) screened in the present study

No.	Plant name	Family
1.	*Anchusa officinalis* L.	Boraginaceae
2.	*Althaea officinalis*	Malvaceae
3.	*Halacsya sendtneri*	Boraginaceae
4.	*Matricaria chamomilla*	Asteraceae
5.	*Achillea millefolium*	Asteraceae

Preparation of extracts - Extraction by process of maceration (MAC):

The maceration is a one-extraction chopped prescribed drugs, prescribed solvent at room temperature. Plant samples (10.0 g) were extracted by 300 mL 96% ethanol for ethanol extracts or water for aqueous extracts as a solvents in laboratory conditions at a temperature of 22 ºC in a sheltered, dry place for seven days with occasional shaking. After seven days extract was filtered through filter paper (Whatman, No.1), then concentrated to dry mass by a rotary evaporator (Devarot, Elektromedicina, Ljubljana, Slovenia) under vacuum and dried at 60 ºC to constant weight. The dried extracts were stored in a dark glass bottle at 4 ºC to prevent oxidative damage.

Ethanol (alcohol) and aqueous extracts were prepared for all five plant species studied. In the presentation of the results (Table 2), the plant ethanol extracts (EE) are numbered from 1 to 5, and aqueous plant extracts (AE) are marked with numbers 1a to 5a.

Cell lines and Viruses:

The Madin-Darby Bovine Kidney (MDBK) cell line (ATCC, CCL-22) was used in the experiment. The cell line was grown in Eagle minimum essential medium (Eagle MEM) Hepes modification (Sigma-Aldrich, USA) supplemented with 10 % fetal bovine serum (FBS) (Capricorn, Germany), 80 µg/mL gentamicin and 100 µg/mL benzyl penicillin.

For estimation of antiviral activity of tested plant extracts, the animal pathogenic viruses Bovine herpesvirus-1 (BHV-1; IBR/IPV virus) strain TN41 (American Bioresearch, USA) as representative of DNA, and Bovine Viral Diarrhoea Virus (BVDV) strain NADL (NVSL, Ames, USA) as representative of RNA viruses, were used in the experiment. Both virus strains were growth and titrated on MDBK cell line.

To determine the titer of virus stocks, serial ten-fold dilutions were prepared in Eagle MEM medium followed by inoculation in MDBK cell monolayers contained in 96-well microtiter plates using 100 μL/well. Seven wells were used per dilution. Inoculated cells were incubated at 37 °C in up to 72 hours. The highest dilution showing CPE was considered as the end point. Virus titers were calculated as $TCID_{50}$/mL by the protocol of Reed and Muench (1938).

Cytotoxicity assay

The plant extracts obtained as water and alcohol extracts were assayed for cell toxicity prior to testing in antiviral studies. Cytotoxicity measurements were based on alteration of normal cell morphology (Benencia and Courreges, 1999; Yucharoen et al., 2011). The extracts were reconstituted by two-fold dilution with Eagle MEM Hepes modification (Sigma-Aldrich, USA) with 8% of FBS (Capricorn, Germany) and antibiotics. Serial two-fold dilutions of the plant extracts preparations ranging from concentrations from 9.77 to 1250 μg/mL were added in triplicate wells of 96-well tissue culture plate (Sarstedt, Germany) with confluent MDBK cell monolayers formed after 24h of cell seeding. Treated cultures were incubated for a 72h at 37 ^{o}C. The cultures were observed daily for evidence of cytopathic effect (partial or complete loss of the monolayer, or rounding or shrinkage of the cells). The dose, causing 50% cytopathic effect (CPE) with respect to cell control (intact cells), was estimated (50% cytotoxic concentration - CTC_{50}) and calculated according to protocol of Reed and Muench (1938).

Antiviral activity screening:

Different nontoxic concentrations (lower than CTC_{50}) of plant extracts were tested for antiviral property by cytopathic effect (CPE) inhibition assay against 100 $TCID_{50}$ virus concentrations of BHV-1 and BVDV viruses similarly as it was described by Vijayan et al. (2004). Briefly, the MDBK cells were seeded in a 96-well microtiter plate with 30.000 cells per well, incubated at 37 ºC in incubator for a period of 24h. The two-fold dilutions of plant extracts in Eagle MEM ranging from 5 mg/mL to 39.06 μg/mL were prepared in another microtiter plate. For each dilution of plant extracts six wells in microtiter plate were used. In another microtiter plate wells the prepared dilutions of tested samples in amount of 100 μL were mixed with 100 µL (100 $TCID_{50}$) of BHV-1, as well as BVDV virus in triplicate and incubated at 37 ºC for 2 hours. After incubation, the plates with MDBK cell monolayer were washed with fresh Eagle MEM and inoculated with 100 µL of prepared extracts dilutions/virus mixtures. In addition, 100 µL of Eagle MEM with 8% of FBS and antibiotics was added to each well. The final concentrations of each tested plant extract in microtiter plates were from 1250 to 9.77 µg/mL. In each microtiter plate the positive controls (MDBK cells inoculated with 100 $TCID_{50}$ of BHV-1, as well as with 100 $TCID_{50}$ of BVDV), and negative controls (only MDBK cells) in triplicate were set-up. The reactions were incubated at 37 ºC for three days. Every 24h the observation was made and cytopathic effects were recorded. Anti-BHV-1 and anti-BVDV activity was determined by the inhibition of cytopathic effect compared with control (untreated cells infected with 100 $TCID_{50}$ of BHV-1 or BVDV viruses). This inhibition of CPE, i.e. protection of the cells from the virus activity, directly connected to the antiviral activity of tested plant extracts, was scored. The concentration reducing CPE by 50% (50% effective concentration - EC_{50}) with respect to virus control was estimated. The selectivity index (SI) was determined from the ratio CTC_{50}/EC_{50}. SI ≥ 4 was considered to stand for a significant selective inhibition (Sokmen et al., 2004).

RESULTS AND DISCUSSION

The evaluation of the antiviral activity indicated that two of the aqueous tested plant extracts (4a and 5a - *Matricaria chamomilla* and *Achillea millefolium*) inhibited the replication of BHV-1 in MDBK cell line (Table 2). It should be noted that all of the plant extracts samples were toxic to the MDBK cell line used, especially in higher concentration that possibly could exhibit virucidal effect. The water extracts were less toxic than alcohol (ethanol) extracts.

In our study, selectivity index (SI = CTC_{50}/EC_{50}) for herb extracts 4a (SI = 32) and 5a (SI = ≥ 8) indicates a significant selective inhibition of DNA virus (BHV-1, strain TN41). Sokmen et al. (2004) state that significant selective inhibition of Herpes simplex virus type 1 (HSV-1) if the SI is ≥ 4. In this study, MeOH extract of herbal parts of *Origanum acutidens* have SI = 36.0, and MeOH extract from callus cultures of *O. acutidens* have SI = 15.4. In a study by Boubaker-Elandalousi et al. (2014) with non-cytotoxic *Thymus capitata* extracts in the prevention of Bovine herpesvirus-1 infection in cell cultures, the selectivity index for essential oil (EO) was 14.49, for ethanol extract 3.95 and for aqueous extract 1.81. High cytotoxic value and a low inhibitory concentration gave the highest selectivity index for EO, indicating that it is the most effective antiviral extract. Parreira et al. (2017) in his *in vitro* evaluation of extracts from leaves of *Drimys brasiliensis* (Winteraceae) against BHV-1 (Los Angeles strain - LA) exhibited strong antiviral activity.

Table 2. Cytotoxicity and antiviral effects of tested plant extracts

			Virus inhibitory effect			
No. of sample		Cytotoxicity	BHV-1 (IBR/IPV) strain TN41		BVDV strain NADL	
		CTC_{50} (µg/mL)[a]	EC_{50} (µg/mL)[b]	SI[c]	EC_{50} (µg/mL)[b]	SI[c]
1	EE	≥ 312.5	> 312.5	< CTC[d]	> 312.5	< CTC
1a	AE	> 1250.0	≥ 312.5	4	> 1250.0	ND[d]
2	EE	≥ 156.3	> 156.3	< CTC	> 156.3	< CTC
2a	AE	> 1250.0	> 1250.0	ND	> 1250.0	ND
3	EE	≥ 312.5	> 312.5	< CTC	> 312.5	< CTC
3a	AE	> 1250.0	≥ 1250.0	≥ 1	> 1250.0	ND
4	EE	≥ 312.5	> 312.5	< CTC	> 312.5	< CTC
4a	AE	> 1250.0	≥ 39.06	≥ 32	> 1250.0	ND
5	EE	≥ 156.3	≥ 156.3	< CTC	> 156.3	< CTC
5a	AE	> 1250.0	≥ 156.3	≥ 8	> 1250.0	< CTC

[a] Fifty percent cytotoxic concentration - CTC_{50}
[b] Fifty percent effective concentration - EC_{50}
[c] Selectivity index (CTC_{50}/EC_{50})
[d] antiviral activity is lower that cytotoxic concentration (< CTE) or not detected (ND)
EE - ethanol extracts
AE - aqueous plant extracts

Chiang et al. (2002) demonstrated an antiviral effect of the soluble phenolic compounds of *Plantago major*, caffeic acid and chlorogenic acid, against herpesvirus, but McCutcheon et al. (1995) reported that the methanol and ethanol extracts of *P. major* tested against herpesvirus did not exhibit antiviral activity. Variations in antiviral activity from these extracts are a consequence of differences in the structure of the viral envelope, which alters the interaction between the viruses and herb compounds.

Pilau et al. (2011) performed the MTT test (3-4,5-dimethythiazol-2yl)-2,5-diphenyl tetrazolium bromide) to determine the selectivity index (SI) of the essential oil against human and animal viruses, which was equal to 13.1, 7.4, 10.8, 9.7, and 7.2 for acyclovir resistant herpes simplex virus type 1 (ACVR-HHV-1), acyclovir-sensitive HHV-1, human respiratory syncytial virus (HRSV), bovine herpesvirus type 2 (BoHV-2), and bovine viral diarrhoea virus (BVDV), respectively. The human rotavirus (RV) and bovine herpesvirus type 1 (BoHV-1) and 5 were not inhibited by the essential oil.

Fifteen Argentine medicinal plants were tested for their antiviral activity *in vitro* against bovine viral diarrhoea virus type 1 (BVDV-1), NADL strain, ATCC VR 534. The Selective Index of the active extract ($SI_{extract} = CC_{50extract}/EC_{50\ extract}$) of *Coronopus didymus* ($SI_{extract}$=110.7), *Juglans australis* ($SI_{extract}$=8.1) and *Lippia alba* ($SI_{extract}$=19.2) against BVDV-1 justify a further analysis. The determined $SI_{extract}$ from the three plants active against BVDV-1 are higher that other eight examined plants (Ruffa et al., 2004).

Sometimes, certain compounds of a crude aqueous extract could be modified by temperature and the period of incubation during extraction, and thus altering the antiviral activity. When the crude aqueous extract of *Amaranthus spinosus* was heated for 30 min at 50-60 °C the antiviral activity against BVD was lost (Koseki et al., 1990). The result of this work may also be an explanation for the absence of BVDV antiviral activity as demonstrated in our study - antiviral activity is lower that cytotoxic concentration (< CTE) or not detected (ND).

CONCLUSIONS

The present study reports for the first time the antiviral activity of five examined herb macerates/extracts. The conducted tests and the obtained results definitely indicate the existence of differences in the virucidal effect of macerates of different plants, as well as in relation to the virus against which the antiviral effect has been tested. Namely, research has found that the antiviral effect, and without the accompanying toxic effect on cell culture as living tissue, was detected for aqueous macerate/extracts compared to ethanol macerates/extracts. Ethanol as a carrier/medium in which dilution and extraction was carried out has caused a cytotoxic effect, and probably also a virucidal but is the result of the effect of alcohol rather than extract/macerate of plants. Two prepared samples of aqueous plant extracts/macerates had antiviral effect against BHV-1 as a representative of herpesvirus (DNA virus), but none of the samples showed an antiviral effect against BVDV genotype 1, a NADL strain, that belongs to RNA viruses. In our study, selectivity index (SI = CTC_{50}/EC_{50}) for aqueous extracts of *Matricaria chamomilla* (SI = 32) and *Achillea millefolium* (SI = $\geq$ 8) indicates a significant selective inhibition of DNA virus (BHV-1, strain TN41).

We believe that it is necessary to improve the investigation of the use of herb macerate/extracts as antiviral agents in the future, and apply new approaches, such as using a low dose of synergistic antiviral combination of herb macerate/extracts. It would also be necessary to examine the mechanism of action of biologically active substances that are isolated and characterized from the examined plant macerates/extracts. The above mentioned study was carried out *in vitro* and for future research it is imperative that they be carried out on an animal model *in vivo*.

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PROPHYLAXIS OF IMMUNODEFICIENCY OF THE PIGLETS WITH ORGANIC IMMUNOSTIMULATORS

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ABSTRACT

The aim of this paper is to study the effects of organic origin immunostimulators containing polysaccharides of somatic O-antigen of *Salmonella Pullorum* bacteria and hen eggs components on parameters of cellular and humoral immunity and natural resistance of piglets – infants. In piglets of the control group with 16 to 18 days of life, an immunodeficiency condition developed, accompanied by a decrease in the number of leukocytes, lymphocytes (especially B-lymphocytes), total protein content and immunoglobulins G and A. The number of leukocytes in the blood of the piglets, treated with the immunostimulator, increased legally and was significantly higher than in the control group. An increase in the number of lymphocytes in the increase in immunoglobulin content at the expense of classes G and A of immunoglobulin is an indicator of high resistance of the organism. In the blood of the control group piglets, the percentage of phagocytosis (up to 9 - 11 days of life), the activity of phagocytes and elimination ability of the blood (EAB) decreased. The use of a new immunostimulator led to an increase in the percentage of active phagocytes, the number of affected bacteria and EAB. Accordingly, the protein-polysaccharide preparation, along with the enhancement of the specific immunity factor, increases the factors of non-specific cell protection.

Key words: natural deficiency, immunostimulators, prophylaxis, piglets.

INTRODUCTION

In modern conditions of pig breeding, technological procedures applied on farms according to many parameters do not correspond to the biological needs of animals, which negatively affects their physiological state. Therefore, a number of problems with the adaptation of animals to new conditions of keeping, feeding and exploiting, finding and applying substances that stimulate protective functions are posed to the doctors of veterinary medicine (Карпуть, 1993; Šobajić, 2002; Mroz, 2005.). Mono-, bi- and polyvalent vaccines, antibiotics and chemotherapeutic preparations, which are used for ballast prophylaxis and in the treatment of early-age piglets, still do not provide the required results. Antibiotics have been used in human and veterinary medicine for decades, both in the treatment of bacterial infections, and in livestock production as promoters of animal growth (Wegner, 2003; Line et al., 200-1; Aarestrusp, 2005; Rosengren et al., 2009.). However, due to the resistance of a growing number of micro-organisms to antimicrobials (Wray i Gnanou, 2000; Mc Ewem and Fedorka-Cray, 2002; Ozavva et al., 2012.), the following issues are increasingly posed: 1) to what extent is the effectiveness of therapy with anibiotics; 2) whether antibiotics are used in prescribed doses and are applied in the correct manner; 3) whether and how far the use of antibiotics in animals has a direct or indirect effect on humans, and 4) whether there are bioactive substances which could successfully replace the use of antibiotics with therapy and livestock production (Cromwell, 2002; Burch, 2005; Cox and Ricci, 2008.). Due to the increasing incidence of microbial resistance to antibiotics, attention is paid to the use of alternative bioactive preparations, which can increase the natural defenses of animals and reduce the massive use of conventional antibiotics (Verstegen and Williams, 2002.). One way is to add specific substances in animal feed, in order to increase their productivity and health status, primarily through the modulation of intestinal microorganisms, which play a very important role in maintaining the health of the host animal (Tuohy et al., 2005.)

The use of vaccines at the younger age is not effective enough because colostral antibodies found in piglets block antigens introduced with vaccines, enhancing the infectious process. The frequent and asymptomatic use of antibiotics and nitrofuran preparations in the therapy and prophylaxis of diarrhea in piglets, the existence of poorly toxic and toxic foods in the meals, often with nitrates and nitrites, increases the immunodeficiency of the organism of the young. Therefore, the study of immunostimulators, whose effect is directed at increasing pig resistance and specific immunostimulation, deserves special attention (Прудников, 1996; Werstegen and Williams, 2002; Tuohy et al., 2005.).

As immunostimulators, microbial products (lipopolysaccharides, glucans, nucleic acids, etc.) may be used, animal tissue preparations (blood serum, thymus, bone marrow, spleen, organic matter degradation products), synthetic substances (levomysol, tetramisol, tyloron and dr.), microelements, vitamins, hormones, etc. Starting from the specificity of the material and other properties, immunostimulators activate the immune systems of the organism: phagocytic, T and B-lymphocytes (Петрянкин et al., 1995; Yoo et al., 2001; Park and Bilkei, 2004.). Among the agents used to stimulate the immune system, most researchers prefer the most harmful biological stimulant products (Dritz et al., 1995, Karpot et al., 1999; Schoner, 2001, Šobajić, 2002; Mroz, 2005; Yuan et al., 2006; Changhua et al., 2007; Qinghua et al., 2009; Qiao et al., 2013; Vetvicka et al., 2014.).

The aim of this study is to clarify the effect of microbial origin of the iinostimulators, which contain the polysaccharides of the somatic O-antigen of *Salmonella Pullorum* bacteria and components of the hen eggs, on the indicators of cellular and humoral immunity and natural resistance of piglets - infants.

MATERIAL AND METHODS OF WORK

Pigs were used for viewing from 2 to 4 to 30 - 32 days of age, which were divided into two groups by analogy. Piglets of the first group have been administered a protein-polysaccharide preparation in a dose of 0.12 ml kg^{-1} live body (at 2 - 4 and 9 - 11 days of age). The second group of piglets was controlling, and they were not a wonderful preparation.

Of the 10 piglets of each age group (2 - 4, 9 - 11, 16 - 18, 24 - 26 and 30 - 32 days), blood samples were taken from which the serum was later separated. In the blood, the number of leukocytes, lymphocytes, T and B lymphocytes and phagocytic activity of leukocytes were determined. Blood serum was determined by the content of total protein / immunoglobulin G, A and M (Карпуть et al., 1992).

RESULTS OF RESEARCH AND DISCUSSION

In the control group piglets, during 16 to 18 days of life, an immunodeficient condition was observed, accompanied by a decrease in the number of leukocytes, lymphocytes (especially B-lymphocytes), total proteins and immunoglobulin levels G and A (tab. 1). The number of leukocytes in the blood of the piglets treated with the immunostimulator increased legally and was significantly higher than in the control group ($p<0.01$). Thus, their number 7 days after the first treatment in the second group was on average 10.21 • 10 L $^{-1}$, after another -15.10 • 10 L $^{-1}$ (for 7 days) and 16.92 • 10 L $^{-1}$ (through 21 days). The content of lymphocytes in the blood of the piglets of the given group increased at the expense of T- and B-lymphocytes. At the same time, the level of total proteins ($p<0.01$) and immunoglobulin G and A in the blood serum increased in the blood serum compared to the control group during the immunodeficiency state (16-18 days). The increase in the number of lymphocytes in the increase in immunoglobulin content at the expense of classes G and A of immunobiobulin is an indicator of high resistance of the organism (Карпуть et al., 1999; Yuan et al., 2006; Lai et al., 2007; Chen et al., 2009, Qiao et al., 2013.). Statistically significant differences in the content of immunoglobulin class M between the two groups of piglets have not been established.

Table 1. Immunological parameters in pig blood (M ± m, p)

Indicator	Groups	Age (days)				
		2-4	9-11	16-18	24-26	30-32
Leukocytes, 10 L^{-1}	Experiment Control	7.03±0.30 6.84±0.46	10.21±0.81* 7.24±0.32	15.10±0.82* 5.32±0.24	14.61±0.66* 6.65±0.48	16.92±0.59* 7.01±0.44
Lymphocytes, 10 L^{-1}	Experiment Control	4.68±0.22 4.34±0.18	6.64±0.19* 4.81±0.31	9.44±0.34 3.56±0.17	9.77±0.12* 4.50±0.13	9.86±0.21* 4.45±0.28
T-Lymphocytes, 10 L^{-1}	Experiment Control	3.43±0.26 3.47±0.15	5.01±0.47* 3.38±0.25	7.01±0.37* 2.99±0.24	7.02±0.28* 3.36±0.28	7.61±0.44* 3.44±0.33
B-Lymphocytes, 10 L^{-1}	Experiment Control	0.65±0,07 0.70±0.06	1.21±0,08 1.06±0.08	1.50±0.11* 0.42±0.03	1.77±0.13* 65±0.08	1.89±0.16* 0.66±0.04
Total protein g L^{-1}	Experiment Control	65.9±1.63 65.7±1.81	79.4±2.17* 70.4±0.93	83.1±2.56* 53.5±0.98	84.7±3.98* 61.9±2.04	8l.0±4.01* 61.6±2.84
Ig G+A, g L^{-1}	Experiment Control	21.4±1.04 21.3±1.86	20.3±1.88* 15.2±1,26	21.72±1.12* 7,92±0.57	12.64±1.13* 11.21±0.97	13.2±1.14** 10.t±0.65
Ig M, g L^{-1}	Experiment Control	2.12±0.46 2.21±0.42	2.27±0.57 2.31±0.44	2.96±0.66 2.36±0.45	2.95±0.46 3.23±0.51	2.49±0.42 2.54±0.51

Note: 1- piglets treated with an immunostimulator; 2- piglets to which no preparation was applied; * $p<0.01$; (compared to piglets control group); ** $p<0.05$ (compared with control group piglets).

In the blood of the control group pig there was a decrease in the percentage of phagocytosis (up to 9-11 days of life), the activity of phagocytes and the elimination ability of the blood - EAB (16-18 days of life) - table 2.

Table 2. Fagocytic activity of leukocyte blood of the piglets (M ± m, p)

Indicator	Groups	Age (days)				
		2-4	9-11	16-18	24-26	30-32
Percentage of phagocytosis	Experiment Control	45.6±0.89 46,8 ±1.27	45.6±1.98* 32.8±1.91	49.8±1.05* 38.6±1.76	52.4±2.63* 39.4±2.35	57.0±2.37* 44,2±1.62
Phagocytic number	Experiment Control	5.24±0.29 4.68±0.32	5.88±0.41 6.23±0.40	7.76±0.46* 3.65±0.15	5.47 ±0.27* 4.08±0.31	5.76±0.43 5.17±0.44
Phagocytic index	Experiment Control	2.39±0.11 2.19±0.14	2.39±0.18 2.04±0.22	3.87±0.23* 1.41±0.14	2.87 ±0.20* 1.61±0.16	3.28±0.26* 7.29±0.17
EAB, 10 microorganisms L^{-1}	Experiment Control	4.59±0.47 4.62±0.51	7.07±0.87* 4.08±0.4l	18.38±1.62* 2.03±0.32	11.34 ±0.99* 2.82 ±0.51	19.78±1.71* 4.90±0.51

Note: 1- piglets treated with an immunostimulator; 2-piglets to which no preparation was applied; * p <0.01 (compared with control group piglets).

An increase in the phagocytic number at 9-11 days in the piglets of the experimental group should be considered as a compensation process. The use of a new immunostimulator led to a significant increase in the percentage of active phagocytes (up to 49.8 - 16-18 days, 57.0 - 30-32 days), the number of affected bacteria (7.76 - 9-11 days, 5.47 - 24-26 days) and EAB (up to 18.38 • 10 microbes L^{-1}- 16-18 days, 19.78 • 10 microbes L^{-1}- 30-32 days), (p <0.01). Accordingly, the protein-polysaccharide preparation, along with the enhancement of the specific immunity factor, increases the factors of non-specific cell protection.

CONCLUSION

The microbial protein-polysaccharide immunostimulator for subcutaneous administration of infant piglets in a dose of 0.12 ml kg^{-1} body weight, twice in an interval of 7 days, prevents the development of an immunodeficient condition and exhibits a clearly expressed stimulatory effect on the parameters of cellular and humoral immune protection and cell factors natural resistance.

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