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**CONTENT**

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# ADAPTATION OF CEREAL SEEDLINGS TO OXIDATIVE STRESS INDUCED BY HYPERTHERMIA

## SUMMARY

The response of etiolated seedlings of winter rye (*Secale cereale*), bread (*Triticum aestivum*) and durum (*T. durum*) wheats, as well as triticale *(× Triticosecale*) to the action of hyperthermia in relation to their resistance to oxidative stress was studied. Exposure of seedlings to 45°C for 4 hours led to a significant inhibition of the growth of *T. aestivum*, while the growth of *T. durum* and *× Triticosecale* seedlings was less sensitive to hyperthermia, and *S. cereale* seedlings showed the greatest resistance to heat stress. In bread wheat seedlings after heating, intensive development of oxidative stress. In durum wheat and triticale, such effects were less pronounced, and in rye, they were almost absent. In rye, triticale, and durum wheat seedlings, peroxidase activity increased under hyperthermia conditions, while in bread wheat, on the contrary, it decreased. In all four studied cereals, in response to the action of high temperature, the content of multifunctional stress metabolite proline increased, however, in rye, its absolute content significantly exceeded that in other species. The content of sugars during hyperthermia increased in *S. cereale* and *T. durum*, but did not change in the other two cereals. Triticale and especially rye have a high base content of anthocyanins and its increase in response to high temperature. A conclusion was made about the relationship between the ability of cereal seedlings to maintain growth under the action of hyperthermia and their resistance to oxidative stress, which is mainly due to the accumulation of metabolites with antioxidant activity.

**Keywords**: *Secale cereale, Triticum aestivum, T. durum, × Triticosecale, hyperthermia, oxidative stress*

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## INTRODUCTION

Probability of extreme conditions with abnormally high temperatures, lack or excess of precipitation in recent decades has been constantly increasing (Konapala *et al.*, 2020). Climate change is already having major implications for stability of food security crop industry (El Bilali *et al.*, 2020). High temperatures are among the factors that have a particularly strong and dramatic effect on plants, which is not limited to membrane barriers (Wahid *et al*., 2007).

Despite the relevance of the problem of cereals heat tolerance for crop production and breeding, researchers still state the lack of effective markers for assessing this property of plants (Li *et al.*, 2019; Pržulj *et al*., 2020). One of the main reasons for the damaging effect of high temperatures is fluidization of the lipid base of cell membranes, including chloroplast and mitochondrial membranes (Yoshioka, 2016; Choudhury *et al*., 2017). This effect, in turn, causes generation of reactive oxygen species (ROS), which can lead to oxidative damage to biomacromolecules and membrane lipids (Farooq *et al.*, 2011). In this regard, attempts are being made to use markers of development of oxidative stress and state of antioxidant system to screen for heat-resistant cereal genotypes (Gupta *et al.*, 2013). At the same time, strategies for plant adaptation to oxidative stress depend on their taxonomic affiliation (Kolupaev *et al.*, 2015; 2016; 2020). In this regard, a comparison of the functioning of stress-protective systems in cereals belonging to different genera and species and having different resistance is of interest both for a deeper understanding of specific characteristics of adaptation and for developing methods for assessing resistance of varieties and lines that can be used in breeding.

Studies dealing with role of antioxidant system in heat resistance of winter cereals is carried out mainly on adult green plants (Sairam *et al.*, 2000; Hameed *et al.*, 2012). However, in many regions, in particular, in the South and East of Ukraine, hyperthermia and drought have a harmful effect on plants not only in summer, but also in early autumn. Thus, over the past two decades, in the Steppe part of Ukraine, the average temperature in August and September increased by 2.8 and 1.9°C, respectively (Romanenko *et al.*, 2018). It has been shown that the heat tolerance of etiolated wheat seedlings correlates with the field resistance of plants (Oboznyi *et al.*, 2013). In this regard, information about the features of the functioning of stress-protective systems of cereal seedlings can partly be extrapolated to characterize their resistance at later developmental stages.

Despite the simplicity of using seedlings as a model object for screening heat resistance, comparative studies of a species characteristics of stress-protective systems at the early stage of cereal grains development have not yet been carried out durum wheat and triticale are especially poorly studied in this regard (Ahmadizadeh *et al.*, 2001; Blum, 2014; Rampino *et al.*, 2018).

The aim of this work was a comparative study of the response of seedlings of winter rye, bread and durum wheat and triticale to the action of hyperthermia in relation to their resistance to oxidative stress.

## MATERIAL AND METHODS

*Plant material and treatments*. Seedlings of winter rye (*Secale cereale*, cv. Pamyat Khudoyerko), bread (*Triticum aestivum*, cv. Doskonala) and durum (*T. durum*, cv. Priazovskaya) wheats, as well as triticale (× *Triticosecale*, cv. Raritet) were used for research. The experiments were carried out at the Laboratory of Plant Biochemistry of the Yuriev Plant Production Institute. Seed samples were provided by the National Center for Genetic Resources of Plants of Ukraine. Seeds of 2020 and 2021 years of reproduction were used for the research.

Grains were disinfected by immersion in 1% sodium hypochlorite solution for 15 min, after they were thoroughly washed with distilled water and germinated at 24°C for 3 days in Petri dishes on two layers of filter paper moistened with distilled water. Heat resistance of cereal seedlings was assessed by growth response to high temperature using the method proposed by Zhuk and Grygoryuk (Pat. 45879 UA, 2002) with our modifications. Three-day-old seedlings of experimental variants were placed in open Petri dishes in a thermostat with a temperature of 45±1°C and air humidity of 40-45% (exposure 4 hours). To prevent drying of the roots, the filter paper in the cups was moistened every hour with the same amount of distilled water. After the end of the exposure, one part of the seedlings was used for biochemical analyzes, and the other part was placed in a thermostat at a temperature of 24°C to assess the growth response. The seedlings of the control variants were kept in a thermostat at a temperature of 24°C throughout the experiment. 24 hours after heat stress, the inhibition of seedling growth was evaluated using the formula:

$$I = \frac{(C_2 - C_1) - (E_2 - E_1)}{C_2 - C_1} \cdot 100\%,$$

where $I$ is growth inhibition (%); $C_1$ and $C_2$, $E_1$ and $E_2$, respectively, the initial and final values of the fresh weight of seedlings (without grains) in the control and experimental (heat stress) variants.

The water content in the plant material was determined by the gravimetric method by drying at 103°C to constant weight.

*Evaluation of LPO products content*. Analysis of the amount of lipid peroxidation (LPO) products reacting with 2-thiobarbituric acid (mainly malonic dialdehyde, MDA) in the shoots was carried out according to the protocol described earlier (Kolupaev *et al.*, 2015).

*Generation of superoxide anion radicals* by the shoots was estimated by reduction of nitroblue tetrazolium (NBT). Ten shoots of the same size were placed in bottles for 1 h with 5 ml of 0.1 M K, Na-phosphate buffer (pH 7.6) containing 0.05% NBT, 10 μM EDTA, and 0.1% Triton X-100 (Kolupaev *et al.*, 2013). At the end of the exposure, the shoots were carefully removed from the incubation solution and optical density of the incubation solution was measured at a wavelength of 530 nm. The increase in generation of superoxide anion radical under heat stress was determined by the ratio (%) of optical density in the experimental and control variants.

*Evaluation of hydrogen peroxide content*. To determine $H_2O_2$ content, the shoots were homogenized in cold with 5% trichloroacetic acid. The samples were centrifuged at 8000 g for 10 min at 2–4°C; concentration of hydrogen peroxide was determined in supernatant using the ferrothiocyanate method (Sagisaka, 1976).

*Measurement of antioxidant enzymes activity*. To assess the activity of antioxidant enzymes, shoots (200 mg) were homogenized in 10 ml of 0.15 M K, Na-phosphate buffer (pH 7.6) with the addition of EDTA (0.1 mM) and dithiothreitol (1 mM) on ice (Kolupaev *et al.*, 2016). Activity of catalase (EC 1.11.1.6) was analyzed at pH 7.0 of the reaction mixture, estimating the amount of hydrogen peroxide that decomposed per unit time. Peroxidase activity (EC 1.11.1.7) was analyzed with guaiacol used as a hydrogen donor and hydrogen peroxide as a substrate. The pH of the mixture was adjusted to 6.2 by K, Na-phosphate buffer.

*Content of low molecular protective compounds.* Proline content in the shoots was determined according to the modified method of Bates *et al.* (1973).

Total sugar content of the plant material was determined by the modified Morris-Roe method based on anthrone reagent (Zhao *et al.*, 2003).

For determination of anthocyanins, samples of plant material were homogenized in 1% HCl solution in 80% methanol. After centrifugation of the homogenate at 8000 g for 15 min, optical density of the supernatant was determined at a wavelength of 530 nm (Nogues and Baker, 2000).

*Replication of experiments and statistical processing of results*. The experiments had 3 biological replicates. Data for each parameter were statistically analyzed using analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test. Different letters denote values, the differences of which are significant at $P \leq 0.05$.

## RESULTS

*Growth reaction of seedlings and their water content under heat stress*. After a 4-hour exposure to 45°C, inhibition of the growth of rye seedlings was insignificant (Figure 1). The high-temperature effect inhibited the growth of triticale and durum wheat more significantly and approximately to the same extent (by ≈ 36%). The strongest growth inhibitory effect of heat stress was observed in bread wheat seedlings (more than 60%).

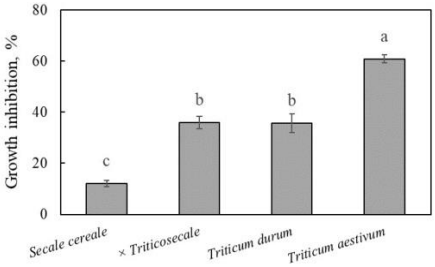


**Figure 1.** Growth inhibition (%) of cereal seedlings after heat stress (45°C, 4 h)

Water content in the roots of seedlings of all four species was 89-90% and did not change significantly after heat stress (results not shown), since the filter paper and the root system were moistened several times with distilled water during the heat treatment period (see the Material and Methods section). At the same time, water content in the shoots decreased in all species, except for rye (Figure 2). The most significant decrease in the water content in the shoots was in bread wheat (almost 4%). For durum wheat and triticale, this effect was less pronounced.

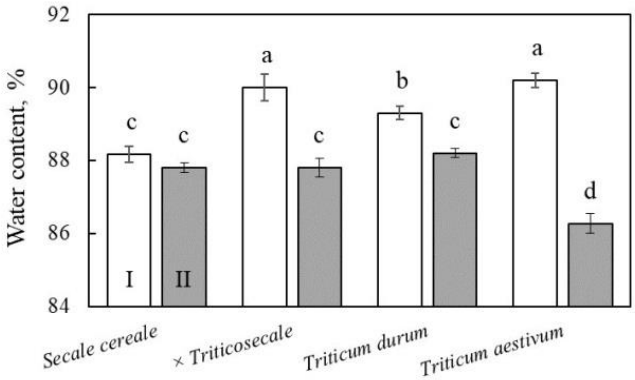


**Figure 2.** Water content (%) in cereal seedlings

*Manifestation of the effect of oxidative stress in seedlings after high temperature exposure.* In seedlings of all studied cereal species, after heat stress, an increase in the content of LPO product MDA was observed (Figure 3A). It was most noticeable in bread wheat, and least significant in rye. In rye seedlings, no significant increase in the generation of superoxide anion radical was registered after exposure to high temperature (Figure 3B). At the same time, in triticale and durum wheat, the $O_2^{\bullet-}$ formation increased by 27-28%. The most significant (2.2-fold) increase in the generation of this ROS under heat stress occurred in the shoots of bread wheat seedlings (Figure 3B).

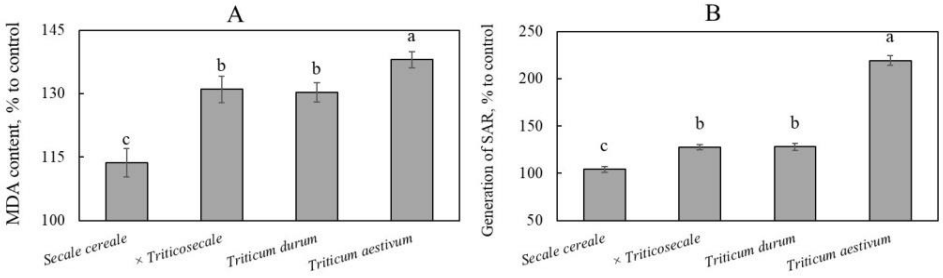


**Figure 3.** MDA content (A, % to control) and generation of superoxide anion radical (SAR) (B, % to control) by cereal seedlings after heat stress (45°C, 4 h)

Changes in the content of hydrogen peroxide under stress conditions in the shoots of cereal seedlings were comparable with the values observed for $O_2^{\bullet-}$.

Rye seedlings had a lower base content of hydrogen peroxide compared to other cereals, while heat stress did not affect its amount (Figure 4). In triticale, the constitutive content of hydrogen peroxide was slightly higher than in rye and increased by 22% after heating. Durum wheat seedlings were characterized by a higher content of hydrogen peroxide compared to other cereals, and heating caused an increase in its amount by 23%. In bread wheat, with a relatively low basic content of hydrogen peroxide, its more than twofold increase was noted after heat stress (Figure 4).

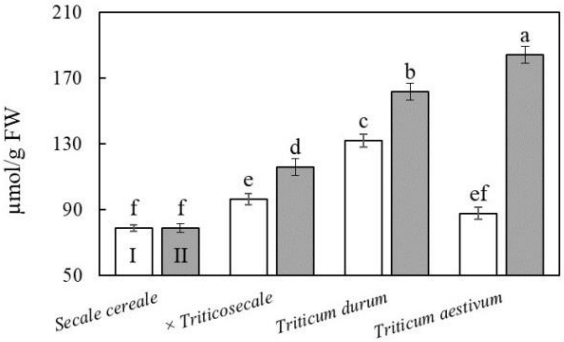


**Figure 4.** The content of hydrogen peroxide (µmol/g FW) in cereal seedlings. I – control; II – stress (45°C, 4 h)

*Activity of antioxidant enzymes in cereal seedlings under heat stress*. The basal level of catalase activity in rye seedlings was somewhat lower than in triticale and two wheat species (Figure 5A). Heat stress caused some decrease in the enzyme activity in rye and triticale. In durum and bread wheat, the activity of the enzyme practically did not change.

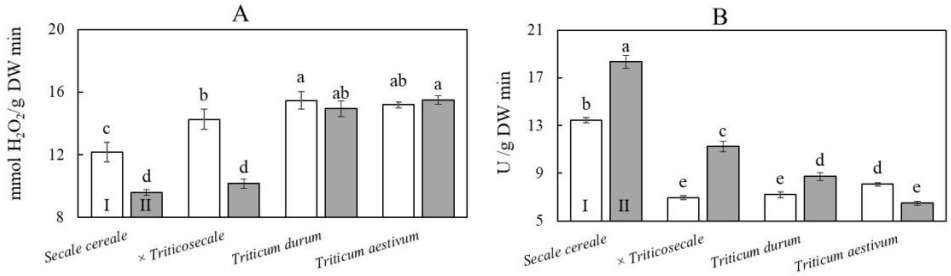


**Figure 5.** Activity of catalase (A, mmol $H_2O_2$/g DW min) and guaiacol peroxidase (B, U/g DW min) in wheat seedlings. I – control; II – stress (45°C, 4 h)

The constitutive activity of peroxidase in rye significantly exceeded that in other species (Figure 5B). In bread wheat, the enzyme activity was slightly higher than in durum wheat and triticale. Under the influence of heating, there was a significant increase in activity of the enzyme in rye and triticale and a slight increase in durum wheat. At the same time, a decrease in peroxidase activity was observed in bread wheat after heat stress (Figure 5B).

*Content of multifunctional stress metabolites in cereal seedlings*. The highest basic content of proline was observed in rye and durum wheat seedlings (Figure 6A). In bread wheat seedlings and especially triticale, it was significantly lower. Effected by heat stress, the content of proline in cereals of all types increased proportionally (approximately 2.5 times compared with the initial values). Where in the absolute values of rye exceeded those of other cereals. At the same time, the content of proline in triticale after stress exposure was noticeably lower than in other cereals (Figure 6A).

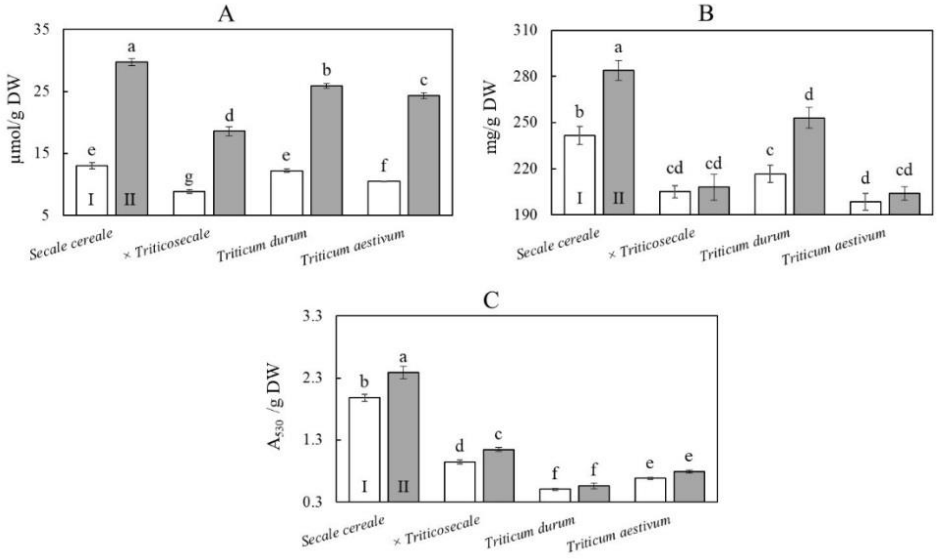


**Figure 6.** Content of proline (A, µmol/g DW) and sugars (B, mg/g DW) and anthocyanins (C, $A_{530}$ /g DW) in cereal seedlings. I - control; II – stress (45°C, 4 h)

The constitutive sugar content in rye and durum wheat was higher than that in the other two cereals (Figure 6B). In those species it also increased under heat stress. At the same time, the content of sugars in triticale and bread wheat seedlings did not change significantly.

A high base content of anthocyanins was characteristic of rye. In triticale, it was two times lower than in rye, and in two types of wheat, anthocyanin content was very low (Figure 6B). In response to heat stress, the content of anthocyanins in rye seedlings and triticale further increased. In the two types of wheat, a significant increase in the content of anthocyanins was not observed.

## DISCUSSION

The results obtained allow us to speak about the dependence of the growth intensity after exposure to hyperthermia on the ability of seedlings to maintain pro-/antioxidant balance. Thus, rye seedlings were characterized by a lower basic content of hydrogen peroxide and the absence of a significant increase in its amount, as well as the $O_2^{\bullet-}$ generation after heat stress (Figures 3,4). They also

showed less accumulation of lipid peroxidation products compared to other cereals after heating (Figure 3A). In durum wheat and triticale seedlings, the manifestation of such oxidative stress signs was more significant, but moderate. Finally, in bread wheat, which growth of seedlings was significantly slowed down, the generation of superoxide anion radical and the accumulation of hydrogen peroxide increased very strongly, in addition, the LPO products content increased more significantly than in other species (Figures 3, 4).

The higher resistance of rye to hyperthermia compared to other types of cereals may be due to high resistance to oxidative stress. Thus, it has been shown that the basic resistance of rye seedlings to direct agents of oxidative stress (hydrogen peroxide and iron(II) sulfate) was significantly higher than that of bread wheat (Kolupaev *et al.*, 2016). It is well known that rye is more frost resistant than most other cereals (Fu *et al.*, 1998; Kolupaev *et al.*, 2015). Fu *et al.* (1998) associated rye resistance to both low and high temperatures with a significant accumulation of various carbohydrates, including fructans. Our work showed that rye seedlings differed from other cereals in their higher total soluble carbohydrate content and its increase after heat stress (Figure 6B), which indicates their possible role in protection against hyperthermia. There is reason to believe that soluble carbohydrates are directly involved in antioxidant defense of cells. Moreover, their effects can be due to both direct interaction with radical ROS (Morelli *et al.*, 2003) and involvement in complex regulatory processes (Khanna *et al.*, 2022).

At the same time, rye seedlings were also distinguished by a high constitutive content of other stress metabolites, in particular, proline and anthocyanins (Figure 6). Proline, along with the osmoprotective and membrane-protective functions, can play the role of antioxidant (Liang *et al.*, 2013). Flavonoid compounds, in particular anthocyanins, have a very powerful antioxidant effect (Gould and Lister, 2006); their basic content in rye was several times higher than in other cereals (Figure 6C).

It is possible that accumulation of various low-molecular-weight compounds contributes to general colligative properties of intracellular solution of rye seedlings. Livingston (2006) stated that rye had a significantly higher amount of sugars than oats and noted their contribution to resistance to dehydration caused by various influences. It is noteworthy that, under the conditions of our experiments, it was rye seedlings that were distinguished by the ability to retain the amount of water in tissues under conditions of heat stress, which is characteristic of physiologically normal conditions (Figure 2). This property may be due to the high content of various osmotically active compounds, such as sugars, proline, anthocyanins (Figure 6) and, probably, many other compounds, including secondary metabolites (Kolupaev *et al.*, 2016). In addition to low-molecular-weight compounds, defense processes in rye seem to involve antioxidant enzymes, in particular, peroxidase (Figure 5B). Its activity in rye was significantly higher than in other cereals under normal conditions and increased after heat stress.

In durum wheat seedlings under the conditions of our experiments, the content of proline and sugars was found to be significantly higher than in bread wheat, especially under stress, which may be one of the reasons for the higher heat resistance of this species (Figure 6). Rascio *et al*. (1994) noted a relationship between proline content and drought tolerance in durum wheat varieties. The maintenance of redox homeostasis in sufficiently heat-resistant durum wheat probably also occurred with the participation of enzymatic antioxidants. Thus, in this type of cereals under heat stress, a slight increase in peroxidase activity was noted, while maintaining the basic level of catalase activity (Figure 5). At the same time, less resistant to heat stress, bread wheat demonstrated decrease in peroxidase activity after heat stress in absence of change of activity of catalase.

Thus, the highest resistance of rye to heat-induced oxidative stress among the studied cereals is probably due to the high content of multifunctional low-molecular-weight protective compounds – proline, sugars, and anthocyanins, as well as the high activity of peroxidase (Figures 5, 6). The moderately high resistance of durum wheat seedlings can also be associated with a rather high content of proline and sugars at relatively low values of other studied parameters of stress-protective systems.

It is more difficult to talk about the adaptive strategy of triticale. The triticale cv. Raritet seedlings used in our experiments did not have high basic content of proline and sugars, although the amount of proline significantly increased after heat stress (Figure 6). It is possible that the relative resistance of triticale to oxidative stress is due to the increased base content of anthocyanins compared to the two types of wheat, which further increased after stress exposure (Fig. 6C). Another feature of the reaction of the triticale antioxidant system may be a significant increase in peroxidase activity after heating (Figure 5B). It is possible that this was the reason for the relatively low content of hydrogen peroxide in triticale seedlings under heat stress (Figure 4).

As already noted, durum wheat varieties are generally considered to have some advantage in terms of heat tolerance compared to bread wheat varieties (Kavita *et al.*, 2016). The variety of bread wheat used in our experiments was created in conditions of the Forest-Steppe and does not differ in high heat and drought resistance. Along with a strong growth inhibition (Figure 1), bread wheat seedlings showed a significant loss of water during hyperthermia (Figure 2) and a very large increase in ROS generation (Figures 3, 4). At the same time, there was practically no increase in the functioning of the studied stress-protective systems in bread wheat seedlings. The only significant reaction was an increase in proline content in response to heat stress (Figure 6).

Of course, not all known components of the antioxidant system have been studied in this work. In particular, functioning of ascorbate-glutathione system, whose contribution to plant resistance to drought and hyperthermia can be quite high, has not been studied (Lou *et al.*, 2018). Nevertheless, nature of changes in the integral indicators of oxidative stress (generation of superoxide anion radical, content of hydrogen peroxide and MDA) allows us to state the existence of a

relationship between the ability of seedlings of various types of cereals to grow after exposure to hyperthermia and their resistance to oxidative stress.

It is quite natural that resistance of cereal species to hyperthermia may also depend on their varietal characteristics. At the same time, it is possible that in some indicators varietal differences may exceed species differences. However, further study of species characteristics will make it possible to compile a general picture of the strategies of adaptive reactions that are characteristic of certain taxonomic groups of cereals. Knowledge of such strategies is important not only for a fundamental understanding of adaptation mechanisms of plants of different taxonomic affiliations, but also for the selection of a set of heat resistance markers that is optimal for screening this property in each specific cereal species.

## CONCLUSIONS

At the stage of etiolated seedlings, significant differences were found in the heat resistance of the four studied types of cereals. According to this indicator, they were arranged in a row: *Secale cereale* > *Triticum durum* ≥ × *Triticosecale* > *T. aestivum*. After exposure to 45°C, the manifestation of the oxidative stress effect was most pronounced in *T. aestivum*, and the least pronounced in *S. cereale*. The obtained results indicate the relationship between the heat resistance of seedlings of various types of cereals and their resistance to oxidative stress. At the same time, strategies for protection against the oxidative stress development depend on taxonomic affiliation. The most resistant rye is characterized by a high content of proline, sugars, anthocyanins, as well as high peroxidase activity under heat stress conditions. *T. durum* is characterized by a relatively high content of sugars and proline, while × *Triticosecale* is characterized by increased content of anthocyanins and increase in peroxidase activity under stress conditions. In *T. aestivum*, the studied indicators of the functioning of defense systems had low values, and all (except for the content of proline) decreased or did not change after exposure to stress.

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# USE OF GREEN SOLVENTS IN SHAKING AND ULTRASOUND ASSISTED EXTRACTION OF MOBILE AND MOBILIZABLE FRACTIONS OF POTASSIUM FROM SOIL

## SUMMARY

Extraction of potassium from agricultural soil often involves strong acids (HCl, $HNO_3$, $H_2SO_4$, $HClO_4$, HF), whose decomposition results in formation of toxic gases. Green solvents are environmentally friendly and are used in place of conventional solvents that are hazardous to both human and the environment. Also, potassium in agricultural soils is often incorporated in minerals that are highly insoluble, and thus highly unavailable to plants. Usage of strong acids may result in much higher concentrations of potassium, and a wrong insight into potassium bioavailability. This erroneous result for the potassium content could lead to improper usage of fertilizers. The most important fractions of potassium for plant growth are mobile an easily mobilizable fractions. Those fractions are extracted with water and weak acids. Technique for sample preparation that is often used is shaking and ultrasound assisted extraction. In this research, two green extraction solutions with two different extraction mechanisms and two different soil types were used. All results were compared to pseudo-total amounts of metals. pH, EC, moisture content and Al - solution extracted potassium (available to plants), as basic quality parameters were measured. Potassium content was measured by means of flame atomic emission spectroscopy (FAES). Mobile and easily mobilizable fractions correlate to a high extent ($r < 0.800$), but they are negatively correlated to pseudo-total amounts. Pseudo-total amount gives a wrong insight into the content of potassium available to plants. The ultrasound filed could be used in case of soils that are low in a clay content.

**Keywords**: mobile, potassium, pseudo-total, shaking, ultrasound

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## INTRODUCTION

Solvents are often used in chemical industry and chemical analysis. The chemical industry of today uses solvents in large quantities. Therefore, solvents define a major part of the environmental performance of a process and have impact on cost, safety and health issues. The extraction of metals from soil samples is a step without which it is impossible to measure metals by most analytical methods. Strong acids are mainly used for extraction of metals from soil. Hydrochloric, hydrofluoric, nitric, and sulfuric, are some of the strong acids. These acids and their products ($Cl_2$, $F_2$, NO, $NO_2$, $SO_2$) are very corrosive and toxic for people, animals and plants. Mixtures of strong acids (*aqua regia*) are frequently used. The idea of ‘‘green’’ solvents expresses the goal to minimize the environmental impact resulting from the use of solvents in chemical production (Capello *et al.*, 2007). The use of green solvents in chemical industry could lead to a healthier future. Healthier future involves reduced use of strong acids as reagents and solvents. Soils are dynamic systems in which a very delicate equilibrium exists between the pseudo-total content (inactive and inert), the mobile fraction (effective soluble, very active, bioavailable) and the mobilizable fraction (potentially bioavailable, leachable and partly active). However, between these three fractions there is an equilibrium attained as a result of complex physic-chemical and biochemical processes (Gupta *et al*., 1996). The mechanisms of metals affecting their solubility can have crucial impacts on ecosystems. Most metals are essential nutrients for normal growth of plants, hence their practical relevance for productivity of agricultural and forest ecosystems. High concentrations of some metals may give rise to ecotoxicological concerns (McBride, 1994).

Potassium is a macro element (the average value of total potassium in agricultural soils ranges from 10-20 g/kg), and is highly important for plant growth. Average soil potassium content is relatively large, but it is mostly unavailable for plant intake. This is why it is important to introduce soluble potassium salts as fertilizers. Current soil analysis methods for K are insufficient for some common soils posing the risk of imbalanced fertilization (Zörb *et al.*, 2014). Adequate content measurement is thus highly important in order to face the problems of potassium presence in agricultural soil.

*Aqua regia,* which is a mixture of hydrochloric and nitric acid in 1:3 ratio, is used today for the assessment of metal concentration in soil. The *aqua regia* extraction gives the “pseudo-total” (meaning almost total) concentrations of metals. Since *aqua regia* is a strong extraction solution, it gives higher results of potassium content in agricultural soil - the amounts that are not available for plant growth. This may result in false information about potassium content in soils and wrong amounts of fertilizer used. The most important fractions in the nutritive and ecotoxicological terms are mobile and easily mobilizable fractions of metals. Mobile and easily mobilizable fractions are those fractions which are more soluble than others. Distilled water is often used to extract mobile fraction of metals. Easily mobilizable fraction, also known as acid-extractable one, is gained

by hydrochloric acid extraction method. Water is a green solvent, but hydrochloric acid is not. This is why acetic acid as a green solvent (Byrne *et al.*, 2016), will be used. Acetic acid is used as solvent of extraction of mobile fraction of macronutrients (Neugschwandtner *et al.*, 2022). The shaking procedure is very often used for extraction of metals from soil with water, weak acids or complexing agents (DTPA, EDTA) (Néel *et al.*, 2006; Soumaré *et al.*, 2007). Instead of shaking, it is also possible to extract metals by use of ultrasounds, which is a green method (Yebra, 2012) (Câmara *et al.*, 2022). Extraction of metals with acetic acid and ultrasounds was addressed in earlier research (Krasnodębska-Ostręga and Kowalska, 2003). Due to the fact that *aqua regia* is not green method and gives much higher amounts of potassium, the aim of this study was developed: to determine the possibilities of green solvents and green preparation techniques to extract readily mobile and easily mobilizable amounts of potassium.

## MATERIAL AND METHODS

### Samples and sampling

The sampling was conducted with the aid of a plastic shovel, until the depth of 20 cm was reached. Once taken, the samples were transferred in plastic bags, labelled, and transferred to the laboratory. Ten soil samples from Butmir an agricultural soil area of the Faculty of Agriculture and Food Science in Sarajevo (label B) and ten samples from a family-owned agricultural soil in Visoko (label V) were used in this research. In total, twenty samples were investigated in two repetitions. The soil samples from Butmir are taken from a sandy loam type of soil. Sandy loam soils are characterized by portions of sand, clay and slit. The soil samples from Visoko are of a clay loam type, which is characterized by a large portion of clays (Arnold, 2004).

The samples were air dried for few days in the laboratory, homogenized with mortar and pastille, and sieved through a 2-mm sieve.

### Methods

#### *Methods for determination of basic quality parameters*

*pH value*

In soil samples used in this research, the pH value was measured using a potentiometric method, on a Mettler Toledo MP 220. The instrument was calibrated with buffers having pH values of 4.21 and 7.00. The soil extraction solution ratio was 1:5 (10 g soil:50 mL extraction solution), prepared as described in literature (ISO 10390, 1994). The measurement was made using two different extraction solutions: ultra-pure water and calcium chloride (0.01 mol/L).

*Electrical conductivity (EC)*

Electrical conductivity was measured in soil: ultrapure water (1: 5) ratio, on a Mettler Toledo MC 126 instrument. The instrument was calibrated with

standards of a known electrical conductivity (12.88 mS/cm and 1413 μS/cm), applying the EPA 9050 method procedure.

*Moisture content*

Determination of moisture content was conducted in a heating oven, Memmert UN 30. 4-6 ±0.0001 g soil was heated on 105 °C, until the constant mass was reached.

*Extraction of mobile forms of potassium*

Extraction of mobile forms of potassium was done with Egner-Riehm-Domingo method. The AL-method is based on the extraction of potassium from the soil with a buffer solution of ammonium lactate with a pH of 3.75.

After extraction of mobile forms of potassium with Al-solution, the content was determined by means of flame photometer (Systonic, S 935).

*Extraction procedures for metal analysis*

Three different metal extraction procedures were used for the purpose of this research:

-extraction with deionized $H_2O$

-extraction in 4% $CH_3COOH$

-extraction in *aqua regia* (HCl + $HNO_3$) in 3:1 ratio – reference method.

Metal extractions in deionized water and acetic acid were carried out by using the shaking and ultrasounds (550 W) procedures. The *aqua regia* (reference method) was carried out in accordance with the ISO method (BS ISO 11646, 2006).

*Extraction with deionized water*

Water extraction was prepared as described in the literature (Séguin *et al.*, 2004). 10 ±0.0001 g soil was placed into a polyethylene bottle with a cap and an under cap. 50 mL deionized water (1:5 ratio) was added on the soil. Two different extraction procedures were done: shaking (two hours of shaking on 180 rpm) and ultrasounds (two hours under ultrasounds). After the two-hour long 180 rpm shaking, or ultrasounds, the solution was decanted and placed in a centrifuge at 4500 rpm for 10 minutes. After centrifugation, the solution was filtered through a slow filtering paper (pore size: 2.5 μm). The filtrate was conserved with 0.5 mL concentrated $HNO_3$. The solution volume was brought up with deionized water, in a 100 mL volumetric flask. A 10 mL aliquot was transferred into a 50 mL volumetric flask, and 5 mL CsCl was added to make concentration of 2000 mg/L. Deionized water was conserved with 0.5 mL concentrated nitric acid in a 50 mL volumetric flask, which was used as a blank solution. After that, 5 mL acidified deionized water was placed in a 50 mL volumetric flask, into which 5 mL CsCl[2] solution was pipetted.

[2] CsCl was added to reduce ionization interferences in acetilene/air flame.

*Extraction with $CH_3COOH$ (4%)*

10 ±0.0001 g soil was placed into a polyethylene bottle with a cap and an under cap. 50 mL of 4% acetic acid was added on the soil (1:5 ratio). After a two-hour long shaking at 180 rpm, or ultrasounds, the solution volume was brought up with deionized water, until 100 mL was reached. A 10 mL aliquot was transferred into a 50 mL volumetric flask, and 5 mL CsCl was added to make concentration of 2000 mg/L. A blank solution was made by transferring 50 mL of 4% $CH_3COOH$ into a volumetric flask. After that, 5 mL acid was transferred into a volumetric flask into which 5 mL CsCl solution was pipetted. The method was modified as described in literature (Cappuyns, 2012).

*Extraction with aqua regia*

The extraction with *aqua regia* (ISO 11464) was used in this research as a comparison (reference) method. 3 ± 0.0001 g soil was transferred in a round-bottom bottle. This was followed by adding 7 mL concentrated nitric and 21 mL concentrated hydrochloric acid. The solution was mixed and left to rest for 16 h in a digestor hood. After 10 hours the solution was placed onto a heating plate under reflux, and heated at 108 °C for two hours. Solution was filtered through a slow filtering paper and brought up to a volume to 50 mL with 0.5 % nitric acid. 1 mL solution was transferred into a volumetric flask, and CsCl solution was added to make concentration of 2000 mg/L. A blank solution was prepared in the same manner, just without the sample, by mixing 7 mL nitric and 21 mL hydrochloric acid and leaving to rest for 16 hours. The solution was then heated under reflux for two hours, filtered into a 100 mL volumetric flask and volume was brought up with 5% nitric acid. 1 mL solution was transferred into a volumetric flask, where CsCl solution was added to make a concentration of 2000 mg/L.

***Determination of potassium content***

Following the extraction procedures for metals analysis, the potassium content was measured with a FAES method, on a Varian Spectra AA240FS spectrophotometer. The measurement was carried out at 766.5 nm wavelength. The "external standards" calibration method of known potassium concentrations was used. The standards concentrations were from 0.0 to 2.0 mg/kg. The calibration curve equation was $y = 0.357x + 0.049$ ($R^2 = 0.9983$).

***Statistical evaluation***

Statistical evaluation of the results was conducted by means of average value, standard deviation, t-test and correlation coefficients. Correlation coefficients indicating the strength of correlation between the two variables are: 0.0– 0.19 very weak correlation, 0.2 – 0.39 weak correlation, 0.40 – 0.59 moderate correlation, 0.60 – 0.79 strong correlation, 0.80 – 1.0 very strong correlation. Correlation can be uphill (the coefficient of correlation is positive), or

downhill (the coefficient of correlation is negative). The correlations were calculated in MS Office Excel.

## RESULTS AND DISCUSSION

### *Results of the basic quality parameters*

Basic quality parameters were measured in order to gain an insight into the basic characteristics of the soil samples.

The results of basic quality parameters: pH value (Water and $CaCl_2$), EC, moisture and AL-$K_2O$ content, are shown in Table 1.

**Table 1.** Basic quality parameters

| | pH (H2O) | pH (CaCl2) | EC µS/ cm | Moisture (%) | $K_2O$ mg/ 100g | | pH (H2O) | pH (CaCl2) | EC µS/cm | Moisture (%) | $K_2O$ mg/ 100g |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $B_1$ | 6.02 | 5.08 | 231 | 11.34 | 25.2 | $V_1$ | 6.71 | 6.10 | 1068 | 11.95 | 53.3 |
| $B_2$ | 6.10 | 5.11 | 263 | 16.28 | 28.0 | $V_2$ | 6.46 | 6.60 | 626 | 12.67 | 53.1 |
| $B_3$ | 6.13 | 5.34 | 270 | 17.47 | 17.6 | $V_3$ | 6.75 | 6.09 | 598 | 12.98 | 39.2 |
| $B_4$ | 7.61 | 6.86 | 492 | 15.03 | 23.1 | $V_4$ | 5.89 | 4.90 | 187 | 10.17 | 10.6 |
| $B_5$ | 6.29 | 5.30 | 300 | 17.22 | 25.8 | $V_5$ | 5.85 | 4.96 | 289 | 10.24 | 31.1 |
| $B_6$ | 6.85 | 6.14 | 617 | 14.24 | 73.3 | $V_6$ | 6.99 | 6.25 | 600 | 14.17 | 61.6 |
| $B_7$ | 6.06 | 6.03 | 452 | 15.83 | 34.6 | $V_7$ | 6.95 | 6.21 | 466 | 11.65 | 49.2 |
| $B_8$ | 6.60 | 6.02 | 362 | 17.91 | 34.5 | $V_8$ | 6.60 | 5.94 | 501 | 12.98 | 25.8 |
| $B_9$ | 7.00 | 6.25 | 333 | 17.63 | 31.4 | $V_9$ | 6.96 | 6.23 | 658 | 13.11 | 46.8 |
| $B_{10}$ | 6.51 | 5.63 | 323 | 10.40 | 25.0 | $V_{10}$ | 6.97 | 6.11 | 685 | 14.13 | 35.0 |
| SD | 0.51 | 0.57 | 120.9 | 0.47 | 15.3 | SD | 0.43 | 0.56 | 239.2 | 1.09 | 15.4 |
| Aw. | 6.53 | 5.78 | 364.3 | 10.87 | 40.5 | Aw. | 6.61 | 5.94 | 567.8 | 13.04 | 31.8 |

All results of pH measurement indicate a slight acidity of the medium. The maximum value of pH was found in $B_9$ sample and the minimum in $V_5$ sample. The average value of pH measurement for B samples was 6.53 and for V samples 6.61. These pH values define soils under the investigation as neutral soils (Sirsat *et al*., 2017). The pH measurement in calcium chloride is, in each case, lower than pH in water. This result is expected, because calcium removes some of the hydrogen ions from soil matter, thus causing higher amount of $H^+$ in solution and lower pH (Minasny *et al.*, 2017).

The average value of electrical conductivity was higher in V labelled samples (567.8 µS/cm). The average value for electrical conductivity in B samples was 364.3 µS/cm. The maximum value was found in sample $V_1$, and minimum in sample $V_4$. Since electrical conductivity is the measure of the concentration of ions that are extractable by water, it could be concluded that V samples (clay loam) contain more ions, which is expected, since clays have a highly active surface attracting ions from solution (Yuehmin *et al.*, 2016). Moisture content was quite uniform. The higher average value of water content was found in V samples (13.06 %). The Al-solution extraction of potassium resulted in higher concentration in V samples, than in B samples.

The correlations between basic quality parameters are shown in Table 2.

**Table 2.** Correlations between the measured values

| | pH | pH ($CaCl_2$) | EC | Moisture | $K_2O$ |
|---|---|---|---|---|---|
| pH | 1 | | | | |
| pH ($CaCl_2$) | 0.856 | 1 | | | |
| EC | 0.649 | 0.648 | 1 | | |
| Moisture | 0.171 | 0.196 | - 0.135 | 1 | |
| $K_2O$ | 0.423 | 0.550 | 0.690 | -0.062 | 1 |

The expected very strong correlation was observed between the results of pH in water and pH in calcium chloride. Those two parameters are very similar because they represent the amount of $H^+$ ion. The moderate correlation between pH and EC is the result of the fact that a higher EC is derived from a higher ions content. The less acidic pH value is derived from the $OH^-$ ion presence in soil, which could give higher EC.

Potassium amounts available to plants ($K_2O$) showed strong correlation with EC. pH value showed moderate correlation with $K_2O$ value. (Kassa, 2021) had similar finding. Available potassium, same as the $H^+$ ion contributes to electrical conductivity.

### *Extraction of potassium by means of different solvents and different intensification techniques*

The results obtained for the extraction of potassium from soil samples using demineralized water and acetic acid and shaking or ultrasounds as intensification technique are presented in Figures 1-3.

Water, as extractant, was used for the extraction of mobile fractions of potassium. The extractions were conducted by applying the ultrasounds or the shaking technique.

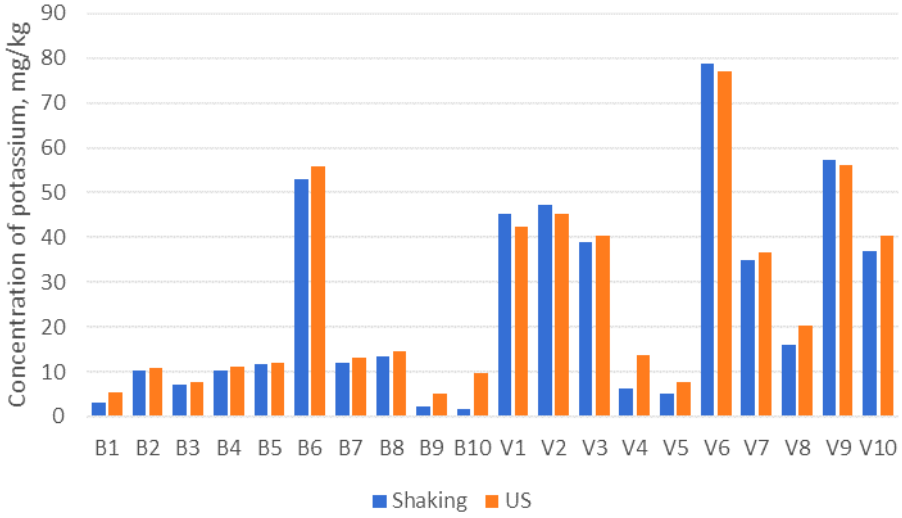


**Figure 1**. Shaking and US extraction with water

The extraction with water resulted with lowest amount of potassium. The average concentration of potassium using the ultrasounds field extraction was 26.29 mg/kg, and 24.59 mg/kg using the shaking method. The application of ultrasounds resulted in slightly higher average concentration. The higher concentrations of mobile amounts of potassium were found in samples from Visoko, where the average value was 36.69 mg/kg. The V samples showed the higher amount of bioavailable potassium content (AL-$K_2O$). The average value of mobile fraction of potassium in B samples was 12.49 mg/kg. The higher concentration of potassium in V samples was expected due to the higher electrical conductivity of these samples. Recent research (Lu, 2021) resulted in similar potassium content. Diluted acetic acid was the second extraction solution used for measuring the mobilizable fractions of potassium. Potassium concentrations gained with acetic acid extraction with two different extraction techniques, shaking and ultrasounds are presented in Figure 2.

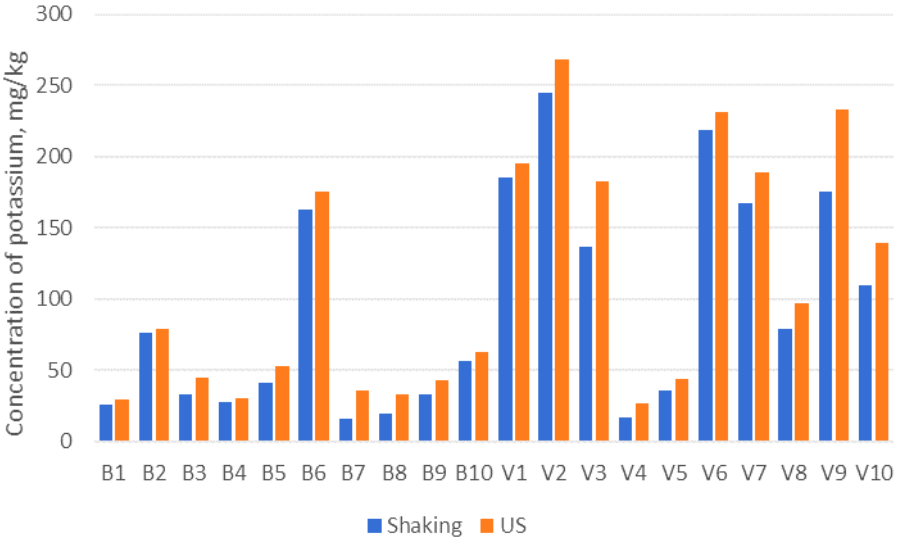


**Figure 2.** Shaking and US extraction with acetic acid

The ultrasounds field extraction with acetic acid resulted in the average value of 109.65 mg/kg, while the shaking technique recorded the average value of 93.01 mg/kg. Some recent studies show similar results (Linquist *et al.*, 2022). The average value in case of acetic acid shaking-assisted extraction for B samples was 49.04 mg/kg, and 58.71 mg/kg in case of ultrasounds-assisted extraction. V samples analysis resulted in 136.99 mg/kg of potassium with shaking, and 160.59 mg/kg with ultrasounds. The results gained with acetic acid were higher than in case of water. The reason lies in the fact that acetic acid is more aggressive than water. The higher amount of potassium recorded with the use of the ultrasound's technique is probably due to the fact that ultrasounds produce more energy than shaking. Ultrasound-assisted extraction of metals is based on the cavitation

phenomenon. The cavitation process comprehends formation, expansion, and collapse of microbubbles. Ultrasounds waves pass through the medium, inducing cycles of expansion and compression in the particles resulting in microbubbles that, upon reaching a critical size, collapse violently and release large amounts of energy (Rutkowska *et al.*, 2017).

Determining the amount of pseudo-total potassium content was conducted with *aqua regia* extraction. The results of potassium content extracted with *aqua regia* are shown in Figure 3.

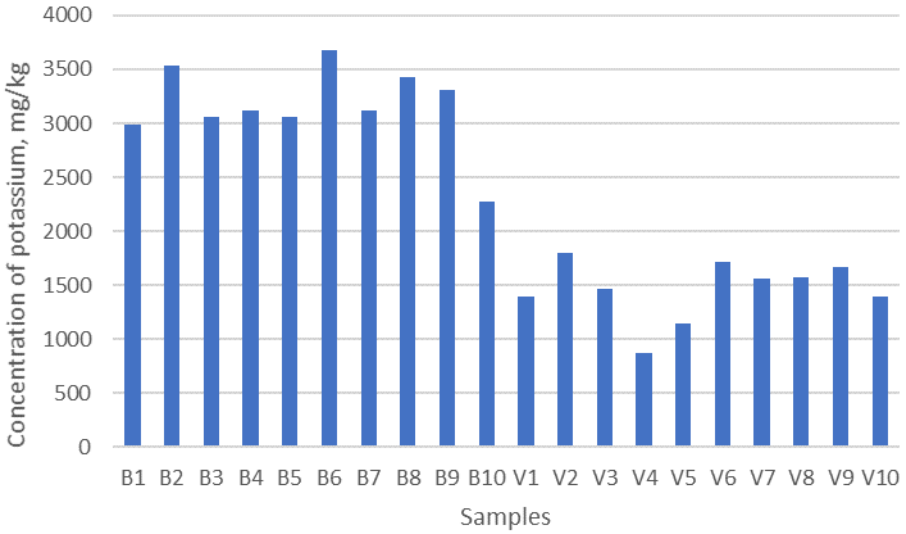


**Figure 3.** Extraction with *aqua regia*

The potassium concentrations were quite uneven because of the fact that samples under the investigation were very heterogenous. The results of water and acetic acid extractions were compared with the results obtained by the *aqua regia* extraction. Amounts of potassium extracted with *aqua regia* were much higher than those extracted with water or acetic acid. The average value for B samples was 3.16 g/kg, while the average amounts for V samples were 1.46 g/kg. Although the electrical conductivity was higher in the V samples, the aqua regia extraction showed much smaller amounts of potassium (two times smaller). This means that there are other ions, e.g., calcium, magnesium, hydrogen etc., present in the "V" samples contributing to electrical conductivity. If the results of metal extraction from B labelled soils were compared to each other, it is possible to extract only 0.62% of potassium with distilled water, and only 3.4 % with acetic acid. In the case of V labelled samples, it is possible to extract only 5.1 % of potassium with distilled water, and only 10.21 % with acetic acid. The samples from Visoko showed higher amounts of mobile and easily mobilizable potassium.Very large difference between pseudo-total and mobile or easily mobilizable fractions of metals was observed. Potassium is mostly present in

forms that are not available to plants. It is believed that it is structurally incorporated in very stable minerals, e.g., silicates. Silicate minerals that contain potassium in soils are mainly muscovite, biotite and feldspars. K-feldspars can directly release potassium into soil, but K in micas is tightly bonded (Singh and Goulding, 1997). Extraction of potassium from soil with ISO method could lead to an inadequate insight into the potassium presence in the soil.

The results obtained by three different extraction methods were also subjected to correlation calculation. Correlations between the extraction methods are shown Table 3.

**Table 3.** Correlations between the extraction methods (shaking and ISO)

| | Water | Acetic acid | ISO |
|---|---|---|---|
| Water | 1 | | |
| Acetic acid | 0.904 | 1 | |
| ISO | -0.515 | -0.605 | 1 |

A very strong correlation between water and acetic acid extraction was found. It means that mobile and mobilizable amounts of potassium have very similar solubility characteristics. They differ slightly only in amount. Another thing that can be noted is that there is a moderate correlation between ISO method and water/acetic acid extraction – a negative one. The negative correlation actually proves that potassium is mainly present in highly insoluble compounds in soil. On the one hand, similarity between results obtained with shaking and ultrasounds as intensification techniques, could be seen in their high correlation ($r = 0.998$). On the other hand, the t-test (paired, uneven variance) showed that shaking and ultrasound field were statistically significantly different (t-test = -3,487; $p = 0.001$).

When extractions with shaking and ultrasounds from Butmir and Visoko are separately compared, the results from Butmir do not show significant difference between methods (t-test = -0.468; $p=0.645$), but samples from Visoko do (t-test= -5.230; $p=0.000$). The samples from Butmir are characterized as sandy loam. Samples from Visoko are clay loam. From the results of the t- test, we can conclude that sandy loam is more suitable for extraction with ultrasounds, than clay loam. This can be explained by the fact that clays are mostly aluminum – potassium silicates, which contain large amounts of potassium, which could be partially released by ultrasound. Ultrasounds are high in energy. Because of that, ultrasounds field extraction gives a larger amount of potassium. Average difference in potassium content when shaking and ultrasounds are compared in case of Butmir samples is 1.6 mg/kg, Samples from Visoko resulted in 16.06 mg/kg difference (ten times higher - clay loam).

## CONCLUSIONS

It is possible to extract mobile and easily mobilizable fractions of potassium from soil with the use of green solvents and with application of shaking or ultrasounds. There is a very large difference in the amounts of mobile

and easily mobilizable fractions of potassium when compared to the pseudo-total amounts. The aqua regia extraction could lead to an inadequate insight into the potassium status in soil.

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# ASSESSMENT OF SOIL FERTILITY AND LEAF NUTRIENTS IN OLIVE ORCHARDS

## SUMMARY

The status and relationship between soil characteristics and nutrient contents in olive leaves of autochthonous Montenegrin cultivar 'Žutica' and Italian 'Leccino' were investigated. The sampling was done in the municipality of Tivat.

The content of nutrient elements in olive leaves depend on cultivars, physiological phases of sampling, edaphoclimatic, pedological characteristics and agronomic techniques.

Regarding the results of soil and leaf analysis, it is generally recommended to decrease fertilization with K, but in some orchards to increase with N. Since the content of Fe, and in the most cases of Mg, was below optimal, the foliar fertilizers should be applied. In saline and calcareous soils, the application of organic fertilizers could improve nutrient uptake, transport and availability to the plant.

The correlation between Ca and N in olive leaves was significantly negative ($p=0.005$). The negative relationships (very close to significance level 0.05) of soil clay component with leaf Cu, and between silt component and P, but positive relationship of humus component with leaf Zn were found.

**Keywords**: Žutica, Leccino, olive leaves, soil, nutrients, fertilizer

## INTRODUCTION

The olives (*Olea europaea* L.) are cultivated worldwide on nearly 11 million ha, with more than 90% of that area in the Mediterranean Basin, characterized by cold and wet winters, but hot and dry summers (Zipori *et al*., 2020). The factors which effect on quality and quantity of olive yield are season climatic conditions, irrigation, soil characteristics and agronomic practices (Knežević *et al*., 2017; Markoč, 2019; Mhanna *et al*., 2021).

About 70% of the olive orchards are traditional and marginal with a medium to very low productivity due to the lack of appropriate orchard

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management (in a significant degree). In these rainfed orchards, water is very often the limiting factor for nutrient availability and uptake. Insufficient rainfall may lead to significant decrease of yield, because of lack of nutrients in the root zone. Excessive rainfall has the same effect on yield due to significant losses of nitrogen from the rhizosphere, thus autumn fertilization is not recommended. About 30% of the olive orchards are usually drip-irrigated, fertirrigated and planted with quick-growing young olive plantings, which have suitable productivity but usually associated with higher environmental impacts (Gargouri *et al*., 2006; Gencer *et al*., 2019; Zipori *et al*., 2020). The farmers often manage their orchards regardless the soil fertility. Unnecessary applications of fertilizers i.e. over- or under-application cause also economic losses, environmental pollution and unsatisfactory yields. It may be prevented by taking into account the analysis of soil and plant (Miranović, 2006).

Although olives prefer deep loam texture soil, they can grow well on almost any well-drained and aerated soil with moderately fine texture (ranging from sandy to silty clay, loamy soils). The most suitable soil is 6.5–8.5 (from the neutral, slightly alkaline values to alkaline ones). It is known that olive trees are tolerant to mild saline conditions (Gargouri *et al*., 2006; Toscano *et al*., 2015).

The olive trees are very tolerant towards soil carbonates. Namely, excellent yield and vegetative growth were observed, in both soils with low content of carbonates and where this parameter being 50%, with a limit of 76% (Gargouri *et al*., 2006).

Organic matter enhances the olive tree productivity through positive effects on soil structure, water retention capacity and availability of some nutrients (limiting erosion, nitrogen leaching, phosphorus precipitation and iron inactivation). Soyergin *et al*. (2002) considered that the content of soil organic matter more than 1% being suitably for well growth. Thus, preserving soil richness in organic matter is very important in the sustainability of olive farming.

The available fraction of P depends on adsorption by various minerals, mostly by carbonates and may be influenced by several factors like pH and soil organic matter (Topalović *et al*., 2006).

Potassium as a relatively mobile nutrient can be rapidly leached in sandy ones. The available K content in the soil is correlated to clay content. The olive trees can compensate, partially, the lack of soil P and K due to uptake of these nutrients from huge soil volume (Gargouri *et al*., 2006). Tubeileh *et al*. (2014) found that available soil K and soil depth explained together 77% of the yield variability, which increased to 83% by incorporating leaf B and Fe concentrations.

Microelements such as Fe, Zn, Mn, and Cu are required in small or very small amounts. Due to fact that most olive orchards are grown on calcareous soils, their availability may be limited.

Besides the functions of nutrients in plant metabolism (Pasković *et al.*, 2020), the plant tolerance or resistance to biotic or abiotic stresses can be affected by their status (Fernández-Escobar, 2019).

This research was carried out to determine status and relationship between soil characteristics and nutrient levels in olive leaves of autochthonous Montenegrin cultivar 'Žutica' and Italian 'Leccino' (as the most common and widespread allochthonous olive cultivar).

## MATERIAL AND METHODS

The soil samples were taken from 0–30 cm and 30–60 cm depth from olive orchards in Tivat Municipality (Grbalj, Gornji Grbalj, Pelinovo, Dumidran, Radovići). The soil sampling was done on 12$^{th}$ March, and olive leaves on 1$^{st}$ July from the middle of current season non-fruit-bearing shoots.

The soil parameters were determined by methods described by Džamić *et al*. (1996, cited by Lekić, 2016). The concentrations are expressed on air-dried basis.

Plant material was dried at 65ºC and ground in laboratory mill. After acid digestion with $HNO_3$ and $HClO_4$ (2:1), the total elemental concentrations – Ca, Mg, Fe, Mn, Zn and Cu were determined by FAAS (Shimadzu, AA – 6800). Total nitrogen was determined by Kjeldahl method. The concentrations are expressed on dry matter.

Due to fact there are no meteorological data for Tivat, the average values of temperature and precipitation were given for three coastal towns in Montenegro (Herceg Novi, Bar and Ulcinj) in period 1981–2010 (http://www.meteo.co.me/).

The data statistical analysis was performed by SPSS 23.0. The descriptive statistics, bivariate correlation analysis and Principal component analysis (PCA) with Varimax rotation were done.

## RESULTS AND DISCUSSION

The optimal conditions for olive growth, development and fruit yield are average annual temperatures between 15°C and 25°C. It can withstand maximum temperatures of up to 50 °C, but minimal temperature -7 °C (no longer than 8–10 days). The annual precipitation demand lies between 700–850 mm (Ozturk *et al*., 2021).

The average annual temperature for three coastal towns was around 16°C, and annual sum of precipitation in the range 1189–1771 mm. The changes of parameters per months are given in Fig. 1.

The results of the soil analysis (Table 1 and 2) show great variability in soil properties. These soils belong to the category of fertile ones, with weakly acidic to alkaline reaction, from carbonate-free to calcareous conditions, and from optimal to very high humus content.

The available concentrations of nutrients ranged from optimal, through high to very high in the samples of topsoil layer, and in the most soil samples of the underlying layer. These results indicate the high effect of fertilization on soil properties.

In the upper layer, the soils mainly belong to the textural class of clay loam, and in the underlying layer to light clay. Only at one location, there is a sandy loam soil.

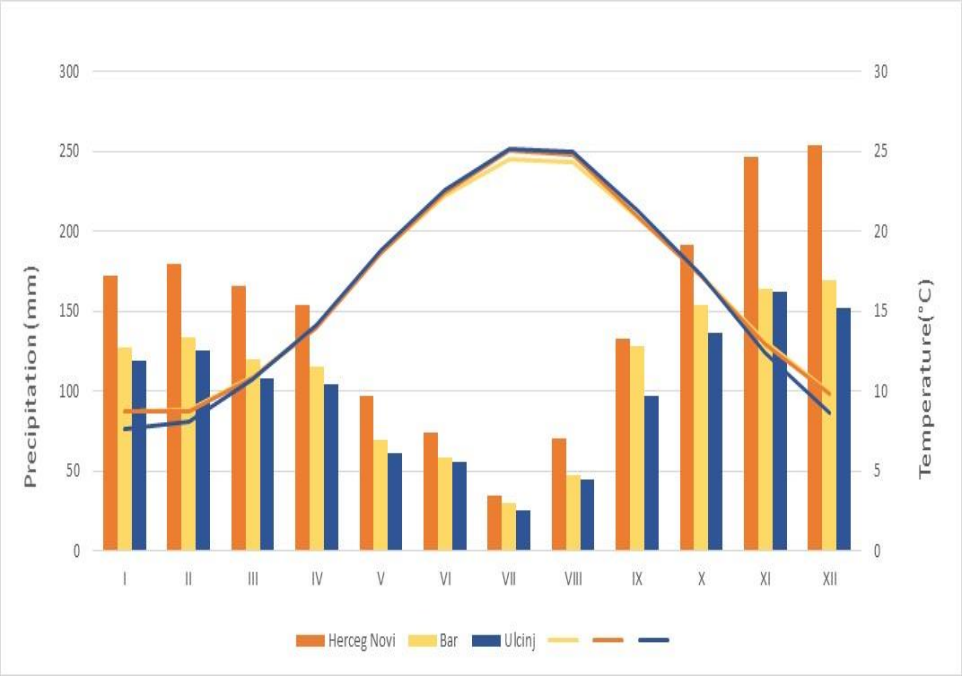


**Figure 1.** Climatic parameters in three coastal towns in Montenegro

The nutrient contents in leaves of Žutica' and 'Leccino' are presented in Fig. 2 and 3. In some orchards of both cultivars the N deficiency was recorded. The Fe content was below optimal in both cultivars, while Mg content was mainly below or at the lower limit of optimal. Also, Ca in 'Žutica' leaves was at the lower limit of optimal content, in average. The potassium content was above the optimal value in both cultivars, as well as Cu in 'Leccino'. The supply of olives with other nutrients was generally optimal (Connell & Vossen, 2007).

In the leaves of five olive cultivars (two Croatian – 'Istarska bjelica' and 'Lastovka', two Italian – 'Pendolino', 'Leccino', and one Spanish cultivar – 'Hojiblanca') sampled as in our study, Pasković *et al.* (2013) found lower concentrations for P (0.08−0.12%) and K (0.77−1.24%), mainly similar or higher for Mg (0.08−0.15%) and Ca (1.14−2.04%), respectively.

The concentration of microelements in our samples were close to maximum for Fe (38−54.5 ppm), close to minimum for Zn (17.8−30.17 ppm) and Mn (23.8−33.4 ppm), but higher for Cu (5.8−7.17 ppm) than ones found in the mentioned 5 cultivars grown in Croatia (Pasković *et al.*, 2013).

**Table 1.** Descriptive statistics for soil parameters (topsoil samples from olive orchards in the municipality of Tivat)

| Soil layer (0-30 cm) in olive orchard | Leccino | | | Žutica | | |
|---|---|---|---|---|---|---|
| | Min | Max | Mean±sd | Min | Max | Mean±sd |
| pH($H_2O$) | 6.27 | 7.60 | 6.92 ± 0.67 | 6.39 | 7.58 | 7.02 ± 0.60 |
| pH(KCl) | 5.69 | 7.13 | 6.38 ± 0.72 | 5.76 | 7.00 | 6.41 ± 0.62 |
| Total carbonates (%$CaCO_3$) | 0.00 | 53.70 | 17.90 ± 31.00 | 0.00 | 25.20 | 9.83 ± 13.48 |
| Humus (%) | 5.67 | 6.61 | 6.11 ± 0.47 | 5.39 | 7.46 | 6.28 ± 1.07 |
| Available P (mg $P_2O_5$/100 g) | 7.10 | 57.70 | 40.00 ± 28.52 | 8.40 | 18.00 | 14.23 ± 5.12 |
| Available K (mg $K_2O$/100 g) | 17.20 | 115.70 | 56.10 ± 52.41 | 20.50 | 111.00 | 60.37 ± 46.20 |
| Electrical conductivity (microS/cm) | 86.70 | 154.80 | 114.10 ± 35.95 | 86.50 | 161.90 | 116.53 ± 39.97 |
| Exchangable Ca (mg/100 g) | 238.00 | 2212.00 | 905.00 ± 1131.98 | 292.00 | 2104.00 | 923.00 ± 1023.58 |
| Exchangable Mg (mg/100 g) | 9.60 | 27.60 | 18.23 ± 9.02 | 18.50 | 33.60 | 24.67 ± 7.92 |
| Available Fe (mg/kg) | 10.90 | 33.30 | 25.57 ± 12.71 | 15.40 | 22.40 | 19.83 ± 3.86 |
| Available Mn (mg/kg) | 13.20 | 103.30 | 54.53 ± 45.51 | 17.00 | 93.10 | 48.47 ± 39.72 |
| Available Cu (mg/kg) | 12.69 | 31.00 | 19.62 ± 9.93 | 4.61 | 27.72 | 12.80 ± 12.94 |
| Available Zn (mg/kg) | 2.01 | 8.55 | 5.60 ± 3.32 | 1.85 | 10.62 | 4.87 ± 4.98 |
| Coarse sand (%) | 2.62 | 24.10 | 14.28 ± 10.86 | 1.33 | 20.21 | 7.84 ± 10.72 |
| Fine sand (%) | 36.87 | 45.61 | 41.84 ± 4.49 | 23.72 | 46.92 | 37.37 ± 12.13 |
| Silt (%) | 22.15 | 31.00 | 27.01 ± 4.49 | 24.23 | 39.72 | 32.89 ± 7.91 |
| Clay (%) | 10.70 | 23.90 | 16.88 ± 6.64 | 16.83 | 27.52 | 21.90 ± 5.37 |
| Total sand (%) | 48.22 | 67.15 | 56.12 ± 9.85 | 43.45 | 48.25 | 45.21 ± 2.64 |
| Total clay (%) | 32.85 | 51.78 | 43.88 ± 9.85 | 51.75 | 56.55 | 54.79 ± 2.64 |

In olive leaves cv. Picual, Cornicabra and Manzanilla sampled in harvest (November) and usual pruning (March) in the southwest region of Spain, the concentration of nutrients were P: 0.09−0.15%, K: 0.66−0.98%, Mg: 0.07−0.18%, Ca: 1.73−2.70%, Fe: 76−117 ppm, Mn: 36−64 ppm, Zn: 14−19 ppm and Cu: 24−111 ppm (Martínez-Navarro *et al*., 2021). The content of Mg, Zn, P and K was similar to higher, but lower for Fe, Mn and Cu in tested samples of 'Žutica' and 'Leccino' in Montenegro.

In the study of 21 olive cultivars (17 Turkish), the concentration of nutrients in leaves taken at the beginning of December were N: 1.89−2.03%, P: 0.12−0.2%, K: 0.84−1.02%, Ca: 1.72−2.12%, Mg: 0.15−0.27%, Fe: 53.6−78.8 ppm, Mn: 23.1−32.92 ppm, Zn: 15.1−26.8 ppm and Cu: 11−25 ppm (Toplu *et al*., 2009). Nevertheless the different cultivars, physiological phases of sampling, edaphoclimatic and pedological characteristics as well as the agronomic techniques, our results are higher for K, lower for Ca and Mg, but similar for Fe,

Zn and Cu in comparison to ones monitored in cultivars grown in Türkiye. Besides, Toplu *et al*. (2009) also found significantly negative correlation between Ca and N in leaves, and positive between Zn and Mn. These correlation coefficents are -0.940 ($p$=0.005) and 0.772 ($p$=0.072) for altogether leaf samples of 'Žutica' and 'Leccino'.

**Table 2.** Descriptive statistics for soil parameters (samples of underlying soil layer from olive orchards in the municipality of Tivat)

| Soil layer (30-60 cm) in olive orchard | Leccino | | | Žutica | | |
|---|---|---|---|---|---|---|
| | Min | Max | Mean±sd | Min | Max | Mean±sd |
| pH($H_2O$) | 7.04 | 7.85 | 7.41 ± 0.41 | 6.28 | 7.71 | 7.03 ± 0.72 |
| pH(KCl) | 6.40 | 7.31 | 6.81 ± 0.46 | 5.67 | 7.13 | 6.47 ± 0.74 |
| Total carbonates (%$CaCO_3$) | 0.00 | 59.90 | 22.03 ± 32.94 | 3.80 | 26.60 | 11.40 ± 13.16 |
| Humus (%) | 3.01 | 4.60 | 3.91 ± 0.81 | 3.99 | 6.94 | 5.11 ± 1.60 |
| Available P (mg $P_2O_5$/100 g) | 5.50 | 14.70 | 9.33 ± 4.79 | 0.20 | 17.30 | 6.40 ± 9.47 |
| Available K (mg $K_2O$/100 g) | 8.00 | 42.50 | 23.13 ± 17.64 | 18.90 | 25.30 | 21.27 ± 3.51 |
| Electrical conductivity (microS/cm) | 79.80 | 206.10 | 137.77 ± 63.78 | 62.10 | 163.50 | 106.63 ± 51.81 |
| Exchangable Ca (mg/100 g) | 238.00 | 2185.00 | 968.00 ± 1060.93 | 157.00 | 2077.00 | 869.00 ± 1051.72 |
| Exchangable Mg (mg/100 g) | 6.00 | 29.90 | 17.37 ± 11.99 | 16.20 | 37.00 | 24.70 ± 10.91 |
| Available Fe (mg/kg) | 7.30 | 24.10 | 15.57 ± 8.40 | 10.20 | 20.40 | 16.80 ± 5.72 |
| Available Mn (mg/kg) | 7.20 | 42.50 | 29.93 ± 19.72 | 18.30 | 37.30 | 29.53 ± 9.96 |
| Available Cu (mg/kg) | 2.97 | 29.35 | 12.85 ± 14.38 | 2.40 | 9.63 | 6.31 ± 3.65 |
| Available Zn (mg/kg) | 0.32 | 2.98 | 1.99 ± 1.45 | 1.05 | 3.13 | 2.30 ± 1.10 |
| Coarse sand (%) | 7.61 | 24.19 | 18.62 ± 9.54 | 0.97 | 19.13 | 7.14 ± 10.39 |
| Fine sand (%) | 30.99 | 40.89 | 35.41 ± 5.03 | 22.77 | 51.28 | 34.45 ± 14.94 |
| Silt (%) | 20.70 | 26.13 | 24.03 ± 2.92 | 21.50 | 36.90 | 30.71 ± 8.13 |
| Clay (%) | 14.22 | 35.27 | 21.93 ± 11.60 | 21.20 | 36.00 | 27.70 ± 7.56 |
| Total sand (%) | 38.60 | 65.08 | 54.04 ± 13.78 | 30.28 | 52.60 | 41.59 ± 11.16 |
| Total clay (%) | 34.92 | 61.40 | 45.96 ± 13.78 | 47.40 | 69.73 | 58.41 ± 11.17 |

According to the highest loadings of topsoil parameters (with the greatest olive root densities) after Varimax rotation, component 1 could be assigned as carbonate component, component 2 as clay, component 3 as silt (negative) and component 4 as humus component (Fig. 4).

The bivariate correlation analysis between the scores of soil parameters and the content of leaf elements shows negative relationships of clay component with Cu ($p$=0.055), and between silt component and P ($p$=0.053); but positive relationship of humus component with Zn ($p$=0.055). The clay particles could decrease the plant uptake of some nutrients, because of their adsorption affinity

for Cu and P. On the other hand, humus has beneficial effects on nutrient uptake, transport and availability to the plant in saline and calcareous soil conditions.

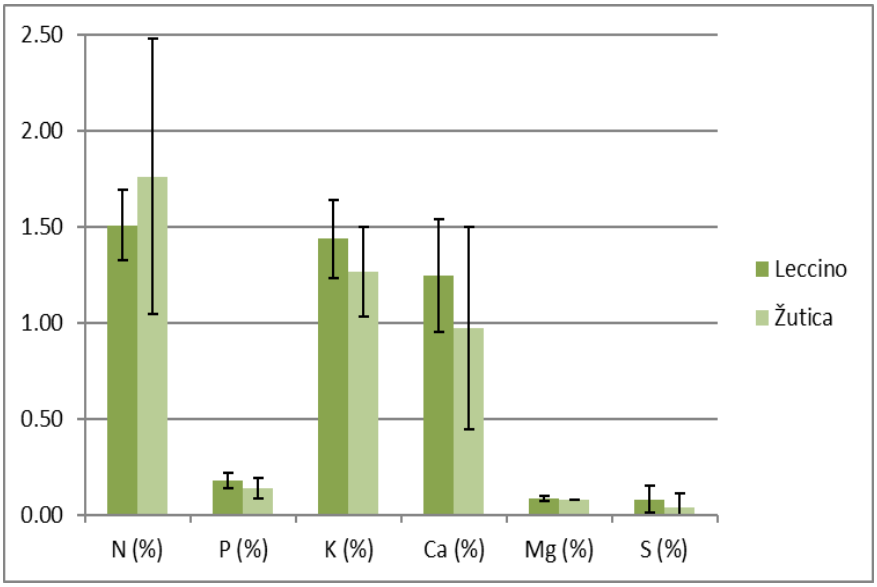


**Figure 2.** Macro- and mesoelements in olive leaves

In our study, there were no concrete data on fertilization. Farmers applied fertilizers according to their usual practice without insight into soil fertility and olive nutritional status.

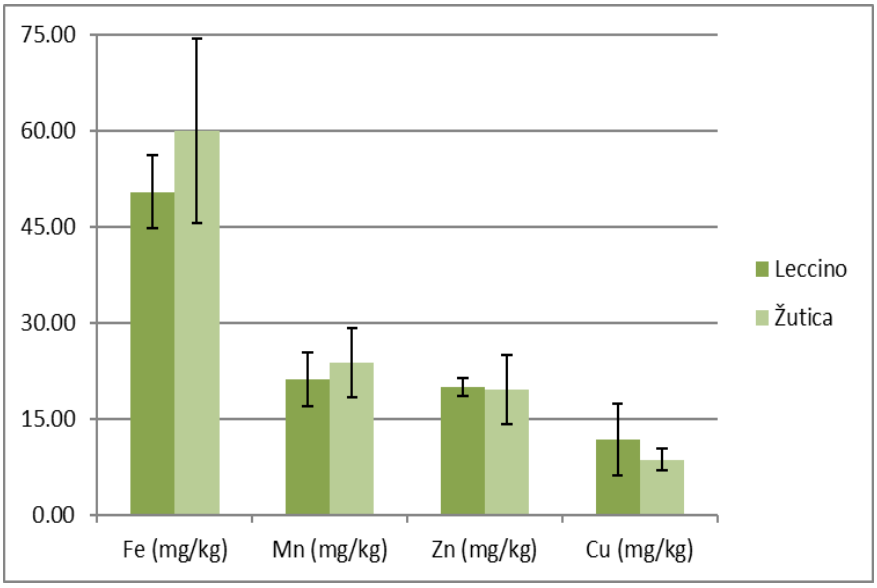


**Figure 3.** Microelements in olive leaves

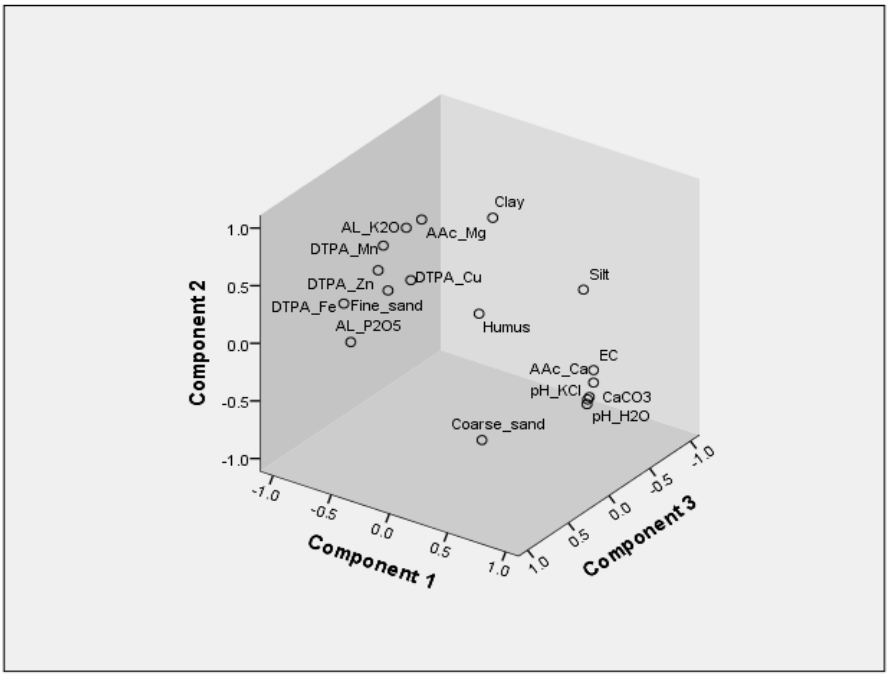


**Figure 4.** Component Plot in rotated space (PCA with Varimax rotation)

For drip-irrigated olive orchard, Cayuela *et al*. (2004) recommended that the doses for N, $P_2O_5$ and $K_2O$ – 150, 30, and 250 kg/ha, respectively, could be achieved by combined application of compost (dose 40 kg per tree i.e. 14.3 t/ha) and mineral fertilizers.

The results of the olive tree fertilization trials in Marocco showed that the annual dose for N and $K_2O$ of 0.5 kg per tree allowed a good yield, while phosphorus did not have a significant impact on the olive tree (Bouhafa, 2022). In Spain, Garcia (2009) proposed a balanced formula between $N:P_2O_5:K_2O$ of 20:8:14 for the fertilization of olive trees.

## CONCLUSIONS

Olive trees mostly uptake N, K and Ca as macro- and mesoelements, as well as Fe as microelement from soils. Amount of the required P is less than N and K. Fertilizers mostly used in olive production are the ones with N, K, and P. Fertilization program for the olive trees established without soil and/or leaf analysis results is at the head of these problems.

From aspect of soil fertility and olive supply with nutrients, it is generally recommended to decrease fertilization with K, but in some orchards to increase with N. Since the content of Fe and mostly Mg was below optimal, these elements should be applied through foliar fertilizers.

In saline and calcareous soils, the application of organic fertilizers is preferable.

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# SEED HEALTH TREATMENTS IN ORGANIC SEED PRODUCTION

## SUMMARY

The basic principles for the development of organic agriculture has been prescribed by the International Federation of Organic Agriculture Movements - IFOAM and the European Union (Commission Regulation No 209/91), on whose standards EU regulations are founded. The field of organic production at the international level and issues of seeds and planting material are regulated by the IFOAM Basic Standards 2002, which stipulates that seed and planting material used in organic agriculture has to be produced in line with the regulations applicable to organic crops. Unlike the conventional seed production, in organic seed production, there is a higher risk of contamination with pathogens, i.e. seed-borne diseases. The aim of this study was to point out the existing methods of seed treatments in the organic production system in order to obtain healthy seeds. Seed-borne pathogens, including bacteria, fungi, viruses and viroids, are responsible for disease recurrence in subsequent cycles of seed multiplication and spread of diseases in new geographic regions. According to various authors, there are several classifications of treatments including physical treatments, application of natural compounds, such as plant extracts and oils, use of inorganic natural products and biological control (use antagonistic microorganisms). In order to overcome various pathogens different biocontrol strategies should be developed. Microorganisms can be used in diverse crop protection practices, i.e. several seed treatments can facilitate high levels of both disease control and production yield.

**Keywords**: seed borne diseases, microorganisms, plant extract, plant oils

## INTRODUCTION

Organic farming is characterised by the production of organic, high-quality food, while preserving plant health and complete biodiversity, soil fertility, the environment and the entire ecosystem (Popović *et al*., 2016).

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The share of the land used for organic farming has been steadily increasing worldwide for many years, as well as, the organic products market (organic-world.net, 2022; Golijan *et al*., 2017; Golijan and Dimitrijević, 2018). Organic products are more beneficial and environmentally safe, similarly or more alimental and contain lower amounts of pesticide residues or do not contain any residues compared to conventionally produced food (Golijan *et al.*, 2021; Golijan & Sečanski, 2021a). The aim of organic farming can be achieved by the application of various practices including polycrop rotation, diverse combination of crops and farm animals, pulses, organic manure and biological pest control (Vaško and Kovačević, 2020; Golijan *et al*., 2021). Organic farming is frequently recommended as an option to farmers who cannot be lucrative in conventional farming. To achieve the optimal cost-benefit ratio, farmers take into consideration various combinations of production factors and their use. They very often face the dilemma whether to use a conventional or organic farming system. Organic farmers have not only moral commitment but also corporate social responsibility (Vaško and Kovačević, 2020).

The International Federation of Organic Agriculture Movements - IFOAM and the European Union (Regulation 209/91) prescribed the basic principles for the development of organic agriculture. Seed production is one of the most important segments of plant production. The field of organic production at the international level and issues of seeds and planting material are regulated by the IFOAM Basic Standards 2002. The 1991 EC Council Regulation (EEC) No 2092/91 is one of the first regulations at the level of the European Economic Community regulating the field of seeds and planting material in organic production. The field of seed production in organic plant production is regulated by the EU Directives 834/2007 and 889/2008. The Regulation (EC) No. 834/2007 imposes the need for each Member State to create a computerised database that is set up to contain varieties of which organic-produced seed is available in its territory (Golijan, 2020; Golijan and Sečanski, 2021b). The offer of organic varieties and organic seeds is at odds with the expansion of organic agriculture, which slows down the development of organic production (Ugrenović *et al*., 2010). The production and treatments of seeds, bulbs and tubers according to the principles of organic production represent an additional challenge for development of high-quality plant material for further propagation. Early harvest is one of the possible measures to improve seed health and there are various forms of seed treatments. High-quality seeds, i.e. seed production in harmony with principles of organic production, is of exceptional importance for organic farming (Kolašinac *et al*., 2017; Golijan *et al*., 2018). There are significant technical challenges in organic production of healthy seeds with germination of a high percentage. The aim of current regulations in many countries is to produce all seeds and planting material, used for organic farming, according to prescribed organic methods. A similar consideration applies to materials used for treatments, film coating and pelleting of seeds (Döring *et al*., 2012). The European Commission Regulation (EC) No. 1452/2003 states that only organic seeds can

be used in organic agriculture. Deviations from this rule are allowed if organic seeds are not available on the market, and conventional seeds are not chemically treated. In case that a user wants to use organic seed of a certain variety and if seed is not listed in a database of all commercially obtainable organic seeds, the user is allowed to use conventional seeds (Spadaro *et al*., 2017). Furthermore, methods that can be applied in seed treatments used against pathogens have become scarce (Howard, 2009), i.e. as the application of chemicals is limited, risk of contamination with weed seeds and seed-borne pathogens are much more expressed in the organic seed production (Roschewitz *et al*., 2005).

The use of chemical fungicides is common in conventional seed production. This treatment reduces losses in seeds and seedlings caused by seed- and soil-borne diseases. The use of the majority of seed protectants in organic production is not allowed. Nevertheless, some seed treatments, such as priming, pelleting and the use of hot water could be an option for organic growers to improve seed traits (Gatch: https://eorganic.org/node/749). Organic production of seed is more subjected to risk of contamination with weed seeds and seed-borne pathogens than conventional seed production. Moreover, seed-borne pathogens can be accumulated and can become severe problem after several cycles of seed multiplication. According to Emily Gatch, Washington State University (https://eorganic.org/node/749):

*„The purpose of any seed treatment is to improve seed performance in one or more of the following ways: 1) eradicate seed-borne pathogens or protect from soil-borne pathogens, 2) optimize ease of handling and accuracy of planting (reduce gaps in stand or the need for thinning of seedlings, particularly when mechanical planters are used), and 3) improve germination rates."*

## SEED HEALTH TREATMENTS

These treatments are used to improve health of seeds and seedlings by killing seed-borne pathogens or by protecting germinating seeds from infestation of soil-borne pathogens. Many authors refer to different categories of seed treatments in organic production. For instance, Spadaro *et al*. (2017) classified treatments as follows: physical treatments, use of natural compounds, use of inorganic natural products, antagonistic microorganisms (biological control), and induced resistance. According to Kumari *et al*. (2013), the organic seed is produced under the organic system in which seeds are typically treated with materials from organic sources (Table 1).

**Table 1.** Seed treatments with materials from organic sources

| Botanicals | Biofertilizers | Cow's product | Biocontrol agent | Other |
|---|---|---|---|---|
| Neem leaf extract | *Rhizobium* | Panchagavya | *Pseudomonas* spp. | Coconut milk |
| Mint leaf extract | *Azatobactor* | Cow milk | *Trichoderma* spp. | Tender coconut |
| Sarani leaf extract | *Azospirillum* | Curd | | Vermicompost |
| Prosopis leaf extract | *Phosphobacteria* | Cow urine | | Vermiwash |
| Arappu leaf extract | | Cow dung | | |

Source: Kumari *et al.* (2013)

Since it is usually impossible to produce healthy, disease-free seed and since the application of conventional treatments with chemical is not allowed, many studies on alternative seed treatments have been done and are still in progress. According to Kumari *et al.* (2013), different treatments that have been tested can be grouped into the following categories:

1. **Thermal treatment**: Hot water seed treatments are quite efficient when applied in some crops, but they should be carefully applied to prevent seed destruction. The limiting factor is that seed have to be dried quickly after the treatment and it is difficult to achieve it in the process of industrial production. To elude this problem, the aerated steam method has been suggested. Due to the fact that the seed is not immersed in water but is exposed to hot humid air, drying is not t a problem anymore. The selection of the temperature and its control is essential.

2. **Use of antagonists**: A number of antagonists have been tested, and some results are as follows;

- *Trichoderma* spp. are used against the collar rot disease of groundnut caused by *Aspergillus niger*
- *Pseudomonas chlororaphis, Bacillus subtilis, Fusarium oxyporum, Streptomyces* spp. are used against *Alternaria* spp. on *Brassica* seeds.
- *Bacillus subtilis* is used against *Tilletia caries* on wheat.
- *Trichoderma viride* is used against *Fusarium* spp. and *Bipolaris sorokiniana* on wheat and barley.
- Several antagonists are used against *Rhizoctnia solani*.

3. **Natural compounds**: Essential oils, occasionally with chelator and natural detergent have been tested. Thyme and oregano oils are successful against *Botritis aclada, Alternaria dauci, Clavibacter michiganensis* pv. *michiganensis* and *Xanthomonas campestris* pv. *campestris*. The mustard powder Tellecur expresses good results against different pathogens, especially T*elletia caries* in wheat. On the other hand, Chitosan gives excellent results against *Fusarium* spp. and *Bipolaris sorokiniana* on wheat and barley. A complex product, Biokal (57% of medicinal herb extracts, 38% bio-humus extracts, 5% volatile oil and metal and trace elements) has proven to give some good results against *Ascochyta pisi* on pea seeds.

4. **Other products**: Tests of organic acids (acetic, ascorbic, citric, lactic and propionic) and antiseptic products such as potassium permanganate and copper sulphate are in progress.

## PHYSICAL TREATMENTS

Thermotherapy is one of the oldest methods of heat treatments of seeds, but due to many practical limitations, it has never been widely used in conventional agriculture for control of seed-borne fungi. On the other hand, the progress in organic agriculture aroused interest in methods of physical seed treatments, such as thermotherapy, including the use of hot water, hot humid air, and microwave radiation (Szopińska and Dorna, 2021). According to Spadaro *et*

*al.* (2017) physical treatments encompass mechanical treatments (sorting progress of organic farming and brushing), heat treatments (warm water, aerated steam or hot air), ultrasonic treatments and radiations (with microwaves resulting in higher temperatures), UV-C light and redox treatments as with cold plasma and electrons.

**Hot water treatment.** The hot water treatment destroys the majority of bacterial organisms that cause diseases on or within seeds, Miller and Lewis Ivey (2021). This treatment is recommended for seeds of eggplant, pepper, tomato, carrot, spinach, lettuce, celery, cabbage, turnip, radish, and other crucifers. Hot water can damage seeds of cucurbits, such as watermelons, pumpkins, squash, gourds, etc., and therefore this treatment should not be used for these seeds. Since the use of this treatment reduces seed vigour over time, seeds treated with hot water should be kept no longer than a season. The period between destruction of the pathogen and the seed injury is usually short and therefore a precise control of the intensity and the length of the treatment is needed. A successful thermotherapy without seed damage is difficult, particularly for large seeds, such as for legumes. Seed lots, even those of the same variety, can greatly differ in sensitivity for the hot water treatment. The sensitivity can depend on the seed maturity, water content, or the period of seed storage (Forsberg, 2004).

Miller and Ivey (2021) state that the seed treatment is performed according to the following steps:

„*Step 1: Wrap seeds loosely in a woven cotton bag (such as cheesecloth) or nylon bag.*

*Step 2: Pre-warm seed for 10 minutes in 100°F (37°C) water.*

*Step 3: Place pre-warmed seed in a water bath that will constantly hold the water at the recommended temperature (see table that follows). Length of treatment and temperature of water must be exactly as prescribed.*

*Step 4: After treatment, place bags in cold tap water for 5 minutes to stop heating action.*

*Step 5: Spread seed in a single, uniform layer on screen to dry. Do not dry seed in area where fungicides, pesticides, or other chemicals are located.*

*Step 6: Dust seed with Thiram 75WP (1 tsp/1 lb seed) once the seed is completely dry.*“

Table 2 presents a list of crop seeds and the temperatures and times recommended for the hot water treatment.

Groot *et al.* (2006) observed effects of seed maturity on the susceptibility to hot water, aerated steam and electron treatments. Two seed lots each of *Brassica oleracea* L. and *Daucus carota* L. commercially produced were chosen as they contained relatively great amounts of insufficiently mature seeds. Less mature *B. oleracea* seeds were more susceptible to hot water and aerated steam treatments, while *D. carota* seeds were more susceptible to the hot water treatment. On the other hand, seed maturity did not affect susceptibility to the

applied electron seed treatments. Although seed lots were not selected for infections caused by pathogens carried on the seeds, it was observed that less mature seeds were more frequently infected. Thus, seeds should be harvested at full maturity and less mature seeds should be removed during seed processing. Categorisation of seeds by their level of chlorophyll fluorescence provides an effective method of sorting seed lots of *B. oleracea* and *D. carota*.

**Table 2.** List of crop seeds and the temperatures and times recommended for the hot water treatment.

| Seed | Water temperature | | Minutes |
|---|---|---|---|
| | °F | °C | |
| Brussels sprouts, eggplant, spinach, cabbage, tomato | 122 | 50 | 25 |
| Broccoli, cauliflower, carrot, collard, kale, kohlrabi, rutabaga, turnip | 122 | 50 | 20 |
| Mustard, cress, radish | 122 | 50 | 15 |
| Pepper | 125 | 51 | 30 |
| Lettuce, celery, celeriac | 118 | 47 | 30 |

Source: Miller and Ivey (2021)

According to Nega *et al.* (2003) the hot water treatment at 50ºC for 20 to 30 min, or at 53ºC for 10 to 30 min controlled *Alternaria dauci*, A. *radicina*, *A. alternata*, and *A. brassicicola* on seeds of carrot, cabbage, celery, parsley, and lamb's lettuce. Pryor *et al.* (1994) applied the treatment of water or 1.0% NaOCl heated to 50ºC for 20 min on carrot seeds. This teatement led to eradication of *A. radicina* with a minimum reduction in germination. Du Toit and Hernandez-Perez (2005) performed spinach seed treatments in 1.2% NaOCl for 10 to 60 min, or hot water (40, 45, 50, 55, and 60°C) for 10 to 40 min, in order to evaluate eradication of *Cladosporium variabile, Stemphylium botryosum,* and *Verticillium dahliae* from seeds. A significant reduction in germination was recorded in the hot water treatment at 50°C for ≥30 min or 55 or 60°C for ≥10 min. Eradication of *C. variabile* was observed in seeds treated in 40°C water for 10 min. *V. dahliae* was eradicated from seeds treated at 55°C for ≥30 min or 60 °C for ≥10 min. Furthermore, eradication of *S. botryosum* was possible from seeds in a lightly infected seed lot (5% incidence) by hot water treatment at 55 or 60 °C for ≥10 min, but eradication was not possible from two heavily infected lots (>65% incidence), even at 60°C for 40 min. According to Hermansen *et al.* (2000) the treatment of carrot seeds with 54ºC water for 20 min eradicated *A. dauci*, but germination, emergence, or yield were not adversely affected.

**Other physical treatments.** The possibility of microwaves to raise temperatures in seeds has also beenstudied. The routine application of hot humid air (ThermoSeed technology) to control seed-borne pathogens in cereals has been common in Sweden and Norway for many years (Forsberg *et al.*, 2002; Forsberg *et al.*, 2005). There are quite a few advantages of microwave technology, including safety, high efficiency, and environmental protection. Microwave

radiation, causing microbial inhibition, is based on the internal heating of the seeds that results from molecular movements in the pulsing electromagnetic field. As a result, the denaturation of proteins, enzymes, and nucleic acids occurs. Heating affects proteins and damages them directly because the bonds that hold them together are destroyed. This also implies the risk of loosing enzymatic activities, which are essential for carrying out metabolic processes (Schmidt *et al*., 2018; Wang *et al.*, 2019). According to Knox *et al*. (2013), fungal pathogens in wheat could not be significantly eliminated by microwave treatments without seed being damaged. The higher seed moisture content increases efficacy of microwave radiation against seed-borne fungi, Mangwende *et al*. (2020). As water molecules are polar, they rotate when exposed to microwaves. This rotation of water molecules produces heat. There are no effects on dried samples because of the lack of polar molecules, while those in the presence of water can reach lethal temperatures (Gartshore *et al*., 2021). Szopińska and Dorna (2021) recorded the highest seed germination (81%, 85% and 77%) in carrot cultivar Amsterdam when the microwave wet treatment at power output levels of 500 W, 650 W and 750 W was applied for 75 s 45 s 60 s, respectively. On the other hand, corresponding values of 46% and 43% were recorded in carrot seeds of cultivar Berlikumer when treated for 60 s at 500 and 650 W, respectively. Seeds of both samples soaked in water and treated with microwaves for over 30 s, regardless of the power output, were significantly less infested with *Alternaria* spp. Tylkowska *et al*. (2010) reported that the microwave treatment of dry seeds (9.5% m.c.) of common bean in a microwave oven with a power output of 650 W and frequency of 2450 MHz for 15-120 s did not affect *A. alternata* and *Fusarium* spp., but reduced the presence of *Penicillium* spp. Microwave radiation less affects dark, multi-celled, and thick-walled spores, as well as dark mycelium (e.g., *Alternaria* spp. or *Bipolaris* sp.) than hyaline and one-celled spores (e.g., *Aspergillus* spp. or *Penicillium* spp.). Schmidt *et al*. (2018) performed the study on *A. parasiticus* and established that the severity of DNA damage increased with higher temperatures.

Electroporation is one of the non-thermal effects that might be caused by microwave irradiation. Microwaves at sub-lethal temperatures stimulate the pore formation in a cellular membrane as a result of their interaction with polar molecules. These pores allow the content of cells, including DNA, to leak outside (Gartshore *et al*., 2021).

Another way to reduce the inoculum load of mainly fungal pathogens on seed is to apply ultrasound. Frequencies ranging from 20 to100 kHz are typically used to generate a powerful cavitation that can destroy and detach microorganisms from surfaces. According to Sagong *et al*. (2011) the combination of the ultrasound treatment with organic acids effectively increased the pathogen reduction in comparison with individual treatments without significantly affecting quality. These authors demonstrated the potential of this novel method in increasing microbial safety on organic fresh lettuce.

A low energy electron treatment of seeds was developed to control cereal seed-borne pathogens (Burth *et al*., 1991). The electron penetration depth is limited to the seed surface and external parts of the seed coat (0.025-0.5 mm), and therefore pathogens in the endosperm and embryo remain unaffected. Waskow *et al.* (2021) observed and compared seed decontamination by the cold atmospheric-pressure plasma and the low-energy electron beam regarding their effects on quality of seeds and seedlings. Results showed that both technologies provided large potential for inactivation of microorganisms on seeds. Cold plasma yielded a higher efficiency with 5 log units than a maximum of 3 log units after the electron beam treatment. Regardless of the applied technique, the short plasma treatment (< 120 s), or all applied doses of the electron beam treatment (8–60 kGy)), seed germination was accelerated, defined by the percentage of hypocotyl and leaf emergence at 3 days. Nonetheless, even the lowest dose of the electron beam treatment (8 kGy) caused root abnormalities in seedlings, implying a detrimental effect on the seed tissue. The cold plasma treatment eroded the seed coat and increased seed wettability compared to electron beam treated seeds. A good effect was also achieved against *Xanthomonas hortorum* pv. *carotae* on carrot seeds (Jahn and Puls, 1998).

Selcuk *et al*. (2008) employed the low-pressure cold plasma system using air gases to inactive *Aspergillus* spp. and *Penicillium* spp. on seed surfaces. The fungal attachment to seeds was reduced below 1% of the initial load depending on the initial contamination level by applying the plasma treatment, while preserving quality of seed germination. A significant decrease of 3-log for both species was achieved in the course of 15 min of the SF6 plasma treatment time. The non-thermal plasma treatment of rice seeds for 76 s resulted in a 90% level of control against *Gibberella fujikuroi*, a fungal plant pathogen that causes bakanae disease (Jo *et al*., 2014).

Seed-borne pathogens can be eliminated by seed treatments with UV-C, UV light with a low wavelength (100-280 nm). UV-C has powerful germicidal properties and can cause the photochemical damage of the DNA of viruses and microorganisms. According to results gained with water-based solutions and with surfaces, pulsed UV light is more effective than continuous UV light. Practically three orders of magnitude of increased inactivation have been accomplished with the photosensitised UV process on surfaces (McDonald *et al*., 2000).

According to Brown *et al.* (2001), the optimum UV-C dose of 3.6 kJ m was effective in reducing black rot and the population density of *Xanthomonas campestris* pv. *campestris* in infected cabbage leaves. Plants grown from seeds treated with UV-C at 3.6 kJ m had the most desirable colour, greatest weight, largest head diameter and delayed maturity. The impact of the storage period at room temperature on the disease occurrence of black rot of cabbage grown from seeds treated with a low hormetic UV-C dose of 3.6 kJ m was 90% of black rot in plants from UV-C treated seeds stored for 2 days, 40% stored for a day, 60% stored for 5 days and 60% stored for 8 months, 8 weeks after transplanting cabbage plants.

**Chlorine Treatment.** The chlorine treatment successfully removes bacterial pathogens from the seed surface. Unlike the hot water treatment, it does not eliminate pathogens in the seed. This treatment is recommended for both large- and small-seeded vegetables if no other treatments were applied to the seeds and if the possibility of pathogens being carried inside the seeds is not a concern (Miller and Ivey, 2021).

According to Miller and Ivey (2021), the procedure of seed chlorine treatment should be performed in the following steps:

„*Step 1: Agitate seed in a solution of 25 oz Clorox plus 100 oz water with one teaspoon surfactant for 1 minute. Use 1 gallon of disinfectant solution per pound of seed (conversions provided below) and prepare a fresh solution for each batch.*
*Step 2: Rinse seed thoroughly in cold running tap water for 5 minutes.*
*Step 3: Spread seed in a single, uniform layer on screen to dry. Do not dry seed in area where fungicides, pesticides, or other chemicals are located.*
*Step 4: Dust seed with Thiram 75WP (1 tsp/1 lb seed) once the seed is completely dry*."

It is very important to test seed germination after a hot water and chlorine treatments. According to Miller and Ivey (2021) germination is tested in the following way:

"*1. Mix seeds in each seed lot and count out 100 seeds per seed lot.*
*2. Treat 50 of the seeds exactly as described in the fact sheet.*
*3. After treated seeds have dried, plant the two groups of seeds separately in flats containing planting mix according to standard practice. Label each group as "treated" or "untreated."*
*4. Allow the seeds to germinate and grow until the first true leaf appears (to allow for differences in germination rates to be observed).*
*5. Count seedlings in each group separately.*
*6. Determine the % germination in each group:*

$$\frac{\#\ \text{seedlings emerged}}{\#\ \text{seeds planted}} \times 100$$

*7. Compare % germination in each group: they should be within 5% of each other."*

Conversions:
8 oz = 1 cup
16 oz = 1 pint
32 oz = 1 quart
128 oz = 1 gallon

Du Toit and Derie (2003), observing the occurrence of *Stemphylium botryosum* in a spinach seed lot, determined that it reduced from 54.8 to 23.3% when the seed was soaked in 1.2% NaOCl for 10 min. This reduction was less

than 20% for the seed soaked in chlorine for 20, 30, or 40 min. On the contrary, the reduction of the occurrence of *Corynebacterium. variabile* ranged from 49.0 to 0.3% after chlorine treatment for 10 min, and was not detected in seeds treated with chlorine for over 10 minutes. Moreover, Du Toit and Hernandez-Perez (2005) treated spinach seeds with 1.2% NaOCl for 10 to 60 min, and found out that *C. variabile* and *Verticillium dahliae* were largely eliminated by the chlorine treatment lasting 10 or more minutes. Although the chlorine treatment reduced the occurrence of *S. botryosum*, this fungus was not eliminated after 60 min in chlorine. Even after the 60-min chlorine treatment seed germination was not negatively affected.

## USE OF NATURAL COMPOUNDS – PLANT EXTRACTS AND OILS

A number of plant oils including oils produced from garlic, savory, clove, oregano, thyme, lemongrass, and cinnamon express some potential in suppressing damping-off (Golijan and Sečanski, 2022). Thyme oil is used in Europe as a seed treatment. The majority of the studies carried out on seed disinfection with natural compounds have been aimed on cereal seed-borne pathogens. It has been determined that pure soya bean or mineral oils had reduced storage moulds not only of soya bean but also of maize. In order to establish feasibility of seed treatment protocols based on essential oils it is necessary to continue with research on the disease suppressive potential of these oils (https://eorganic.org/node/749).

Antifungal activities of essential oils against *Fusarium* spp. have been reported in many studies previously performed for different laboratory media and plant materials (Kumar *et al*., 2016; Matusinsky *et al*., 2015; Ferreira *et al*., 2018). According to Schmitt *et al.* (2009), thyme oil (1%) was effective against *Phoma valerianellae* on seeds of lamb's lettuce. Different essential oils, organic acids and plant extracts were tested by Van Der Wolf *et al.* (2008) with the intention to use them to disinfect vegetable seeds. Thirty-minute treatments with certain essential oils eliminated 99% of the bacteria on cabbage seeds. Furthermore, it reduced fungi in blotter tests. High concentrations of organic acids (>2.5%) reduced bacteria on seeds. However, a concentration higher than 1% of certain products such as propionic acid, cinnamon oil and Biosept, negatively affected seed germination, whereas thyme oil had the best efficiency. Perczak *et al.* (2019) reported that essential oils have great potential for the inhibition of the growth of *Fusarium* fungi on maize seeds.

Shukla *et al.* (2002) stated that the toxicity of the ajowain (*Trachyspermum ammi*) oil was fungicidal at 0.1%, which inhibited heavy doses of inocula (25 fungal discs, each of 5-mm diameter) and killed the test pathogen in no more than 2-3 s. This oil also exhibited a wide antifungal activity against *Aspergillus flavus*, *A. parasiticus*, *Curvularia lunata*, *Cladosporium cladosporioides*, *Alternaria alternate*, *Colletotrichum capsici*, *Colletotrichum falcatum*, *Helminthosporium maydis*, *Helminthosporium oryzae*, *Penicillium implicatum*, *P. italicum* and *P. minio-Iuteum*, and was more active than some commercial synthetic pesticides

(benlate, captan, mancozeb, and thiram). Mohamed *et al.* (2020), determined antifungal activities of *Origanum majorana* essential oils against four seed-borne fungi in rice: *Fusarium verticilliodies*, *F. graminearum*, *Bipolaris oryzae*, and *Curvularia lunata*. Chen *et al*. (2014) demonstrated the effectiveness of a citronella (*Cymbopogon nardus*) essential oil on *A. alternate* in in vitro and in vivo assays. Moumni *et al.* (2021) tested in vivo seven essential oils for disinfection of squash seeds (*Cucurbita maxima*) that had been naturally contaminated with *Stagonosporopsis cucurbitacearum, Alternaria alternata, Fusarium fujikuro, Fusarium solani, Paramyrothecium roridum, Albifimbria verrucaria, Curvularia spicifera*, and *Rhizopus stolonifer*. The seeds were treated with essential oils produced from *Cymbopogon citratus, Lavandula dentata, Lavandula hybrida, Melaleuca alternifolia, Laurus nobilis*, and *Origanum majorana* (#1 and #2). The occurrence of *S. cucurbitacearum* was decreased, ranging from 67.0% in *L. nobilis* to 84.4% in *O. majorana* #2. Seed germination was not affected by treatments at 0.5 mg/mL essential oils, even though radicles were shorter than control ones, except for *C. citrates* and *O. majorana* #1 essential oils. The occurrence of *S. cucurbitacearum* was reduced by approximately 40% in plantlets developed from seeds treated with *C. citratus* essential oil. Xu *et al.* (2014) demonstrated that 0.5 mg/mL of their *L. nobilis* essential oil protected cherry tomatoes from infection with *A. alternata*. Moreover, Xu *et al.* (2017) showed an exceptional antifungal activity of essential oil made from bay tree in tests against *A. alternata*. Eke *et al.* (2020) reported that essential oil made from lemon grass protected young plants of French bean from diseases caused by *F. solani*, under conditions both of the laboratory and the greenhouse.

One of difficulties of using plant extracts are high amounts of water that are needed and a drying step that is necessary afterwards. In order to obtain a stable emulsion of essential oils in water, a sonication procedure was developed. The activity may be possibly enhanced if chelating divalent cations are added. They stabilise the anionic lipopolysaccharide layer in the outer membrane of Gram-negative bacteria (Skandamis *et al*., 2001).

## BIOLOGICAL SEED TREATMENTS (USE OF ANTAGONISTS)

Other approaches to seed treatments encompass in-field applications to plants for the reduction of disease-causing fungi and bacteria that can develop on the seed. Biological seed treatments control seed pests by parasitising the pest organisms, which defeat them in the competition for food on the root system, or which produce toxic compounds that inhibit pathogen growth. Various organisms such as filamentous fungi, bacteria and yeast have been used as biocontrol agents (BCAs) against seed-borne pathogens. In order to be more successful against plant pathogens, a BCA have to be able to colonise strongly the plant rhizosphere, to prevent the attack of pathogens and to compete with other microorganisms in the plant rhizosphere. Disease control can be effective if BCAs survive and

develop in the spermosphere before sowing and in the rhizosphere after seed germination (Spadaro *et al*., 2017).

Comprehending the mechanism of action is vital for developing optimal commercial formulations and application procedures with the intention to maximise the efficacy of BCAs (Spadaro and Gullino, 2005). Microbial antagonists use diverse mechanisms to control plant pathogens, such as food competition, hyperparasitism, production of lytic enzymes, secretion of antibiotics, and interference with quorum sensing. Moreover, microorganisms can also elicit localised and systemic host defences (Mukerji and Chincholkar, 2007).

In the study with commercially formulated micro-organisms, Tinivella *et al.* (2009) established that two out of seven products tested were effective. When specific strains of microorganisms are combined, numerous traits that antagonise the pathogen can also be combined and this may result in more efficient protection. De Boer *et al.* (2003), controlled *Fusarium* wilt of radish by combining *P. putida* strain WCS358, which competed for iron through the production of its pseudobactin siderophore, with *P. putida* strain RE8, which induced systemic resistance against *F. oxysporum* f.sp. *raphani*. Studies of Bennett *et al.* (2009) and Wharton *et al.* (2012), showed that *Trichoderma* spp. effectively controlled soil- and seed-borne pathogens such *as Pythium, Phytophthora, Rhizoctonia* and *Fusarium* spp. in different crops. Raupach and Kloepper (1998) applied a mixture of three different plant growth-promoting rhizobacteria (PGRP), as a seed treatment. PGRPs intensively promoted the pant growth and reduced numerous cucumber diseases. Many strains of bacteria or fungi used in biocontrol produce antibiotics that inhibit the growth of other fungi. The introduction of the gene encoding the housekeeping sigma factor into a strain of *P. fluorescens* increased the production of pyoluteorin and 2,4-diacetylphluoroglucinol (DAPG) (Schnider *et al*., 1995).

*Pseudomonas* and *Bacillus* species as PGPRs have attracted much attention for their role in mitigating and reduction of plant diseases. When applied as seed treatments, PGPR resulted in significant reduction of *Phytophthora* blight disease of squash (Zhang *et al*., 2010). The development of mixtures containing strains communicating with each other to maximise antibiotic production and disease control could be another approach in the improvement of control of soil-borne diseases (Becker *et al*., 1997; Davelos *et al*., 2004). According to De Sousa *et al.* (2021), inoculation with *Trichoderma* increased the length of the radicle and hypocotyl and showed no fungi in the seeds. Some treatments increased the height and the plant root dry mass in seedlings.

*Pseudomonas chlororaphis* has a suppressive ability against the pathogens as it produces antifungal metabolites with broad activities. This bacterium efficiently controls many seed-borne diseases present on seeds or near seed coats but it does not control soil-borne diseases and pathogens located deeper in seeds. Although active on the seed in the soil, this bacterium does not provide sufficient effect later (Kilany *et al.,* 2015; Shah *et al.,* 2017, BioAgri, 2019).

According to Emily Gatch, Washington State University (https://eorganic.org/node/749), Kodiak (*Bacillus subtilis*), Mycostop (*Streptomyces grieseoviridis*), SoilGard (*Gliocladium virens*), T-22 Planter Box (*Trichoderma harzianum*), Actinovate (*Streptomyces lydicus*) are products used for biological treatments of seeds that can be purchased on the world market.

## CONCLUSIONS

Organic seed production in comparison to conventional seed production is more prone to risk of contamination with weed seeds and seed-borne pathogens that can accumulate and become a problem after several cycles of seed multiplication. It is currently very difficult to achieve the desired seed quality standards for a large number of crops. Weed, disease and pest control is a particularly sensitive segment as many difficulties occur due to the almost complete absence of chemical measures. Various simple and complex methods regarding seed treatments have been developed and tested for the past several decades. There are numerous effective and sustainable seed treatments used to control seed-borne diseases that are successfully applied world-wide: 1) physical seed treatments, including mechanical treatments, thermal treatments, radiations, and redox treatments, 2) the use of natural compounds, in which organic compounds comprise plant extracts, essential oils, as well as purified microorganism compounds, and 3) biological control, based on the use of antagonistic microorganisms. Since the use of pesticides is not allowed in organic agricultural production, seed production in compliance with the prescribed principles for such a production mostly faces difficulties within the filed of plant protection and therefore further studies are necessary in order to coin measures for successful control of pathogens, especially seed-borne diseases.

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*Šahinović, E., Alkić Subašić, M., Hrkić, M., Murtić, S. (2022). Antimicrobial and antioxidant properties of Gentiana lutea L. roots. Agriculture and Forestry, 68 (4): 59-68. doi: 10.17707/AgricultForest.68.4.05*

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# ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF *GENTIANA LUTEA* L. ROOTS

## SUMMARY

In this study, the antimicrobial and antioxidant properties of yellow gentian roots (*Gentiana lutea* L.) were investigated. Root samples were collected at the Vranica Mountain, situated in the central part of Bosnia and Herzegovina. The yellow gentian roots were evaluated for antimicrobial activities against four bacteria, namely, Gram-positive bacteria: *Staphylococcus epidermidis*, *Enterococcus faecalis* and Gram-negative bacteria: *Escherichia coli* and *Klebsiella pneumoniae*. Total antioxidant capacity of root extracts was evaluated using ferric reducing antioxidant power (FRAP) assay. Total phenolic and total flavonoid contents were also evaluated. The antimicrobial assays indicated that the yellow gentian roots were more effective against *Staphylococcus epidermidis* and *Escherichia coli* than against *Enterococcus faecalis* and *Klebsiella pneumoniae*. The results also showed that the extracts of yellow gentian roots contained relatively high amounts of phenolic compounds and high antioxidant activity rates. The antioxidant activity of the root extracts was found to be positively associated with the total phenolic and flavonoid contents.

**Keywords**: bacteria, flavonoids, health, mountain habitats, phenolic compounds

## INTRODUCTION

*Gentiana lutea* L., commonly known as yellow gentian, is distributed in the mountain areas of central and Southern Europe, including the Balkan Peninsula. It mostly occurs on grassy mountain meadows and grows at an elevation of 600-1700 m, and even more. The plant blooms from June until August.

Yellow gentian is a perennial herbaceous plant, rising to 1-2 m tall, with round, strong and vertical flower stem. Leaves are opposite, lanceolate to elliptic,

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10-30 cm long and 4-12 cm wide. Most of them belong to a basal rosette until flowering; however, basal leaves are slightly bigger than others, 30 cm long and 15 cm wide. The flowers, placed on the upper half of the stem are yellow, with the corolla distributed nearly to the base into 5-7 narrow petals. Fruit is a capsule, approximately 6 cm long, with plenty of seeds (Prakash *et al.*, 2017).

Yellow gentian root is well developed and branchy. It occurs as single or branched sub-cylindrical parts of various lengths and usually 10 - 40 mm in thickness. Dried fragments of gentian roots are identified by disagreeable smell and bitter taste. They contain some important bitter glycosides (gentiopicrin, gentianin) and alkaloids (gentiomarin) that have a wide range of pharmaceutical and medicinal utilities, primarily in treatments of various human digestive disorders (Olennikov *et al.*, 2015). Yellow gentian roots are also used as a bitter flavoring for alcoholic drinks, and in traditional medicine to stimulate the appetite and improve digestion (Catorci *et al*., 2014; Karalija *et al*., 2021).

Generally, the pharmacological activities of yellow gentian roots have been scientifically validated; however, the knowledge about their antioxidant and antimicrobial properties is still not well explored, particularly in the contexts of yellow gentian from natural habitats in Bosnia and Herzegovina. Therefore, the main aim of the present study was to evaluate the antioxidant and antibacterial properties of yellow gentian roots. This study focuses on the wild populations of yellow gentian at Mountain Vranica (Bosnia and Herzegovina).

## MATERIAL AND METHODS

*Study area*

Mountain Vranica is part of the Dinaric mountain range, situated in central part of Bosnia and Herzegovina between the town Gornji Vakuf in the west and the town Fojnica in the east. The highest peak is Nadkrstac at 2112 m altitude, followed by Krstac (2069 m) and Rosinj (2059 m). Approximately 20 km east of the Nadkrstac, at 1660 m altitude, glacial lake ‘Prokoško Jezero’ is located (Figure 1).


**Figure 1.** Study area location

Lake Prokoško jezero and the surrounding area received a protected status due to the exceptional richness of flora and fauna, including some endemic plants such as *Edraianthus niveus* G. Beck, *Euphorbia gregersenii* K. Maly and *Alchemilla vranicensis* Pawlowsky.

Climatic patterns in this area are primarily mountainous with cold snowy winters and cool summers, however in lower zones (below 1300 m), especially on the southern and western slopes, the climate is moderately continental due to the influence of the sub-Mediterranean climate.

The vegetation of the Vranica Mountain area is a unique mosaic of mountain meadows, pastures, and high-altitude forests (fir, spruce, beech, and green alder). Vranica Mountain is also known for its abundance of blueberries and cranberries. Furthermore, a considerable number of Balkan endemic plants occur in habitats of Vranica Mountain such as *Ranunculus crenatus* W. et K., *Crepis aurea* (L.) Cass., *Festuca panciciana* (Hack) Richt. and *Scabiosa leucopylla* Borbas.

*Gentiana lutea* L. (yellow gentian) is also occurring in habitats of this area, but, unfortunately, very rarely and only in small populations. Although yellow gentian is an endangered plant species, its population on the Vranica mountain is in constant decline, primarily because unregulated and inappropriate harvesting. Therefore, in this study, very small root fragments from three individuals of yellow gentian were sampled.

*Collection and processing of root samples*

Root samples of three individuals of yellow gentian were collected from the same locality at mountain Vranica (mountain meadow bellow Ločika peak, 1950 m above sea level, latitude: 44° 56' 20" N, longitude: 17° 44' 27" E) in September 2021. The sampled individuals of yellow gentian were not particularly close to each other, but they were scattered within the study area. Since the yellow gentian is an endangered plant, fragments from older roots were cut with a knife very carefully. The collected fresh roots were oven-dried at 40 °C (3 days) to avoid degradation of their chemical compounds. Thereafter, dried root samples were ground to a fine powder using an electric blender and stored at 4 °C until further use.

*Preparation of root extracts*

1 g of dried root powder was extracted with 30 ml of 60% aqueous ethanol solution (room temperature for 24 h). Subsequently, the extract was filtered through coarse filter paper into the volumetric flask and diluted to the 50 ml mark with extracting solution. The extract thus obtained was used for the determination of total phenolic content, total flavonoid content and total antioxidant capacity.

*Total phenolic content*

Total phenolic content of different root extracts was determined by the Folin-Ciocalteu method (Ough & Amerine, 1988). Briefly, 0.1 ml of prepared

extract was mixed with 6 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent (diluted in distilled water 1:2, v/v before use). After 10 min, 1.5 ml of saturated $Na_2CO_3$ (20% w/v) solution was added. The mixture was made up to 10 ml with distilled water, heated in a water bath at 40 °C for 30 min, and then cooled in an ice-bath. The absorbance of this prepared sample solution was read at 765 nm. A standard solution of gallic acid was used to prepare a calibration curve (0-500 mg $l^{-1}$, R2 =0.999). Results were expressed as mg gallic acid equivalents (GAE) $g^{-1}$ dry weight.

*Total flavonoid content*

Total flavonoid content of different root extracts was determined by the aluminum chloride colorimetric assay (Zhishen *et al*., 1999). Briefly, 1 ml of extract solution was diluted with 4 ml of distilled water. To this solution, 0.3 ml of 5% $NaNO_2$ was added. After 5 min, 0.3 ml of 10% $AlCl_3$ and 2 ml of 1 M NaOH was added. Then, the mixture was made up to 10 ml with distilled water and incubated at room temperature for 1 h, after which the absorbance was read at 510 nm. A standard solution of catechin was used to prepare a calibration curve (0-100 mg $l^{-1}$, R2 = 0.997). Results were expressed as mg catechin equivalents (C) $g^{-1}$ dry weight.

*Ferric reducing antioxidant power (FRAP) assay*

Total antioxidant capacity of different root extracts was determined using the ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996). Briefly, 80 µl extract was diluted with 240 µl distilled and then 2080 µl of fresh FRAP reagent was added. The FRAP reagent was prepared immediately before use by mixing acetate buffer (300 mM, pH=3.6), 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in 40 mM HCl and 20 mM $FeCl_3$ in a volume ratio of 10:1:1. Thereafter, the mixture was heated at 37 °C for 15 min in a water bath, after which the absorbance was read at 595 nm. A standard solution of $FeSO_4 \times 7\ H_2O$ was used to prepare a calibration curve (0-2000 µmol $l^{-1}$, R2 = 0.996). Results were expressed as µmol $Fe^{2+}$ $g^{-1}$ dry weight. Amersham ultrospec 2100 spectrophotometer (Biochrom, USA) was used for all spectrophotometric measures.

*Bacterial strains*

Two Gram positive bacterial strains *Enterococcus faecalis* and *Staphylococcus epidermidis*, as well as two Gram negative bacterial strains *Escherichia coli* and *Klebsiella pneumoniae* have been isolated from food samples and food environment. After isolation of bacterial strains on nutrient agar plates, strains were identified and characterized by microscopic analysis and biochemical tests; Gram-straining, catalase, coagulase, citrate, oxidase, urease, indole, etc. All bacterial strains, used in the study, were cultured on nutrient agar (Liofilchem, Italy) and incubated at 37 °C for 18-24 h.

*Preparation of bacterial inoculum*

Direct colony suspension method was used in preparing the inoculum from colonies grown during 18 to 24 h. Briefly, three to five morphologically similar colonies from fresh overnight grown cultures were transferred with a loop into 5 ml of physiological solution (0.85% NaCl, w/v) in a capped test tube, and mixed thoroughly using a vortex for 1 min. The suspension was then adjusted to give a turbidity equivalent to that of a 0.5 McFarland standard (CLSI, 2012).

*Antimicrobial activity evaluation*

Evaluation of the antimicrobial activity of gentian roots was conducted according to the agar dilution method (CLSI, 2012). The aim of agar dilution methods is to determine the lowest concentration of the assayed antimicrobial agent (minimum inhibitory concentration, MIC) that, under defined test conditions, inhibits the visible growth of the bacterium being investigated. This procedure involved the incorporation of different concentrations of the antimicrobial agent into a nutrient agar medium (ranged from 3 μg $ml^{-1}$ to 24 μg $ml^{-1}$), followed by the inoculation of a defined microbial inoculum on the surface of the agar plate. The plates were then incubated at 37 °C for 48 h.

Classification of the bacterial strains into susceptible (S), intermediate resistant (IR) and resistant (R) was based on specific ability of each strain to grow on the whole surface area of Petri plates. Cut-off values to differentiate among resistant and susceptible groups were defined on the basis of the growth distribution of the population after incubation at 37 °C. The determination of biofilm forming categories was done per standardized formulas for manual calculation of biofilm forming categories (weak, moderate, strong) according to the CLSI (2012).

*Statistical analysis*

All assays were performed in triplicates, and measurement data were expressed as the mean ± standard deviation. The Pearson correlation coefficients were calculated in order to identify the relationship between the phenolic/flavonoids and total antioxidant capacity. Microsoft Excel 2010 package for Windows (Office 2010, Redmond, WA, USA) was the software used for the statistical analysis.

## RESULTS AND DISCUSSION

Plants with bioactive properties play an important role in health promotion and disease prevention. Antioxidant capacity is without a doubt one of the basic bioactive properties of plants due to the presence of different types of phenolic compounds and other antioxidants (Pinto *et al*., 2021). Even though yellow gentian roots have long been traditionally used, their antioxidant capacity is still insufficiently explored, particularly in the contexts of yellow gentian native to Bosnia and Herzegovina.

In the present study we therefore evaluated the antioxidant capacity of yellow gentian roots from natural habitats of Vranica Mountain. Total phenolic and flavonoid contents were also evaluated. The evaluation of both phenolic and flavonoid contents is relevant because the antioxidant capacity of plant has been mainly attributed to their presence in plants (Yu *et al*., 2021).

The quantification of total phenolic contents (TPC), total flavonoid contents (TFC) and total antioxidant capacity (TAC) in root samples of yellow gentian roots are shown in Table 1.

**Table 1.** Total phenolics (TPC), total flavonoids (TFC), and total antioxidant capacity (TAC) of yellow gentian roots

| Individuals | TPC (mg $g^{-1}$ DW) | TFC (mg $g^{-1}$ DW) | TAC (µmol $Fe^{2+}$ $g^{-1}$ DW) | TFC/TPC ratio |
|---|---|---|---|---|
| No. 1 | 8.90 ± 0.67 | 1.37 ± 0.11 | 25.54 ± 4.62 | 0.15 ± 0.03 |
| No. 2 | 9.05 ± 1.25 | 1.41 ± 0.27 | 24.84 ± 4.45 | 0.16 ± 0.02 |
| No. 3 | 9.35 ± 1.01 | 1.55 ± 0.32 | 27.88 ± 3.01 | 0.17 ± 0.03 |

The obtained experimental values for TPC and TFC of yellow gentian root samples are in good agreement with those findings reported in literature. The values found in the literature for the TPC and TFC in dried yellow gentian roots were in the range of 0.96 to 17.46 mg $g^{-1}$ and 0.48 to 2.66 mg $g^{-1}$, respectively (Nastasijević *et al*., 2012; Azman *et al*., 2014; Drobyk *et al*., 2015; Mudrić *et al*. 2020). Interestingly, the TFC/TPC ratios in the studied root samples of yellow gentian were in the range of 0.15 to 0.17, indicating low percentage rate of TFC vs. TPC. Huang *et al*. (2013) also reported the low TFC/TPC ratios in six different *Gentiana* spp. root samples.

In this study, there were no significant differences in TPC, TFC and TAC in the root samples among the three individuals of yellow gentian studied. These results were expected since all individuals of yellow gentian studied were sampled from the same locality.

The correlation of TPC/TFC with TAC in yellow gentian roots samples is shown in Figure 2.

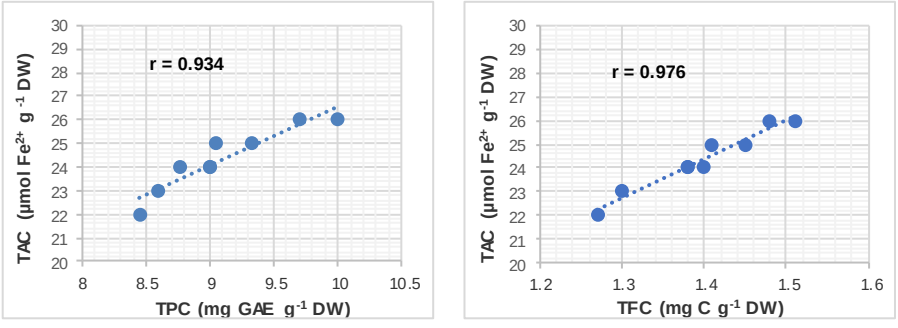


**Figure 2.** Correlation between TPC/TFC and TAC of the samples

A strong positive linear relationship was found between TPC/TFC and TAC of the samples, indicating that phenolic compounds, especially flavonoids, are highly responsible for the antioxidant activity of yellow gentian root extracts. Numerous studies have also revealed that phenolic compounds play an important role in the antioxidant activity of plants (Stagos, 2019; Khan *et al*., 2020; Kiani *et al*., 2021). Mucha *et al*. (2021) reported that phenolic compounds have high antioxidant activity mainly because of their ability to donate a hydrogen anion, i.e., an unpaired electron to free radicals, which interrupts the cycle of their new creation. The antioxidant capacity of phenolic compounds is also attributed to their ability to chelate metal ions responsible for the generation of free radicals (Tungmunnithum *et al*., 2018).

The antimicrobial activities of yellow gentian root powder were also evaluated in this study (Table 2). This activity was tested against two Gram positive bacterial strains: *Enterococcus faecalis* and *Staphylococcus epidermidis*, and two Gram negative bacterial strains: *Escherichia coli* and *Klebsiella pneumoniae*. Gram-positive and Gram-negative bacteria tested in this study were categorized under opportunistic pathogens which can cause severe nosocomial infections. Hence, they are difficult to treat because of multiple drug resistance to the various classes of antibiotics (Cepas *et al*., 2019; Senobar Tahaei *et al*., 2021).

**Table 2.** Antimicrobial and antibiofilm activity of yellow gentiana root powder against tested bacteria

| MIC (minimum inhibitory concentration) | *Staphylococcus epidermidis* | | *Enterococcus faecalis* | | *Escherichia coli* | | *Klebsiella pneumoniae* | |
|---|---|---|---|---|---|---|---|---|
| | growth | biofilm | growth | biofilm | growth | biofilm | growth | biofilm |
| 3μg $ml^{-1}$ | IR* | weak | IR | moderate | IR | moderate | R | strong |
| 6μg $ml^{-1}$ | IR | weak | IR | moderate | IR | moderate | R | strong |
| 9μg $ml^{-1}$ | IR | weak | R | strong | S | weak | R | strong |
| 12μg $ml^{-1}$ | IR | weak | R | strong | S | weak | R | strong |
| *R - resistant, IR - intermediate resistant, S - susceptible | | | | | | | | |

The strain *Staphylococcus epidermidis* was intermediate resistant (IR) to all minimum inhibitory concentrations (MICs) of gentian root powder used in this study (ranged from 3 to 24 μg $ml^{-1}$) and all MICs inhibited biofilm formation capability of tested *Staphylococcus epidermidis*.

The strain of *Enterococcus faecalis* showed intermediate resistance to MIC>3μg $ml^{-1}$ and MIC>6μg $ml^{-1}$ and high-level resistance to MIC>12μg $ml^{-1}$ and MIC>24μg $ml^{-1}$ of gentian root powder, due to strong biofilm formation. Numerous studies have revealed that the natural products with or without antimicrobial activity can stimulate bacterial biofilm formation (Singh *et al*. 2002; Bleich *et al*. 2015; Yu *et al*. 2017), and the results obtained in this study support this hypothesis. The bacteria present inside a biofilm are protected against the action of the antimicrobial drugs, thus permitting their survival (Pinheiro *et al*., 2014). In study of Hassan *et al*. (2011) several relationships were

found between the ability to form biofilm and antimicrobial resistance, being different for each species.

In the present study, the strain *Escherichia coli* showed moderate biofilm production capability at MIC>3 μg $ml^{-1}$ and MIC>6 μg $ml^{-1}$ of gentian root powder in agar media, while MIC>12 μl $ml^{-1}$ and MIC>24 μl $ml^{-1}$ inhibited the growth of *Escherichia coli* strain as well as biofilm formation.

Gentian root did not show any antibacterial effect to the strain *Klebsiella pneumoniae*, indicating the high-level resistance of studied *Klebsiella pneumoniae* strain to all MICs of yellow gentian root used in this study.

## CONCLUSIONS

In sum, the results of this study revealed that the extracts of gentian roots contained relatively high amounts of phenolic compounds and high antioxidant activity rates. The antioxidant activity of the root extracts was found to be positively associated with the total phenolic and flavonoid contents.

The antimicrobial assays indicated that the yellow gentian roots were more effective against *Staphylococcus epidermidis* and *Escherichia coli* than against *Enterococcus faecalis* and *Klebsiella pneumonia*.

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# SOIL MANAGEMENT IN SUSTAINABLE AGRICULTURE: ANALYTICAL APPROACH FOR THE AMMONIA REMOVAL FROM THE DIARY MANURE

## SUMMARY

This study analyses the viability of converting the organic nitrogen possessed by dairy manure from Sri Lankan cows into ammonia using hydrolysis. Furthermore, ammonia removal from anaerobically digested dairy manure was evaluated with the experimental analysis. Hydrolysis was conducted to determine the impact of total solids, retention time, and temperature on the ammonia recovery. Experimental studies have shown that 85% of organic nitrogen in dairy manure was recovered into ammonia at 35 $^{O}$C within 7 days a 12.1 – 13.8 Total Solid (TS) content. Furthermore, it was also inferred that acidification occurs along with ammonium in the diary manure. Ammonia removal from anaerobically digested manure was investigated using Head Space Flushing (HSF), where it was identified that 73% of influent ammonia was removed at 35$^{O}$C after 7 days. This result can be effectively used as an appropriate method for converting and removing ammonia from dairy manure in countries with large cattle herds.

**Keywords**: Organic Nitrogen; Hydrolysis; Ammonification; Acidification; Total Solid content; Head Space Flushing

## INTRODUCTION

To minimise greenhouse gas (GHG) emissions, organic wastes are highly urged to be utilised as renewable energy sources (Daniel-Gromke *et al.*, 2015; Kader *et al.*, 2021). Generally, the animal production farms are constructed and maintained in the locations far from the population intense areas to mitigate odour issues (Hellstedt, 2020). Due to its high biodegradable calorie concentration, cattle manure (i.e., cow dung) is one of the significant organic materials being used in the manufacture of biogas (Shaibur *et al.*, 2021). The main constituents of cattle manure are grains and digested grass that possess 24 types of minerals such as

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potassium, iron, magnesium, manganese, and cobalt (Gupta *et al.*, 2016; Huang *et al*., 2017).

However, due to the existence of ammonia which is a turbulent and smelly compounds (Ndegwa *et al.*, 2011), utilizing cattle excrement as fertiliser without purification may cause major environmental problems (Kumar *et al.*, 2013), GHG emissions, water body eutrophication, air pollution ,soil acidification and nitrate seeping are all examples (Chukwu *et al*., 2022). The production of biogas is very significant in Sri Lanka, and it is highly recommended to incorporate a sustainable framework to recycle and reuse the cattle manure (Shuraik & Lizny, 2022). In 2021, Sri Lanka possessed 1,131,080 cattle (Kader, 2022; Statistics, 2022) where the majority of the waste manures are not handled adequately.

If cattle dung is to be utilised as a nutrient for microbial development, it must be substantially diluted due to its high nitrogen concentration (Markoska & Spalevic, 2020), which hinders anaerobic metabolism within the anaerobic digesters (Chen *et al*., 2008; Gerardi, 2003). Chemical parameters like temperature, moisture content and pH (Calli *et al.*, 2005) greatly influence nitrogen hydrolysis (Chen *et al.*, 2008). The phenomena of organic nitrogen hydrolysis in cattle manure induce ammonia ($NH_3$) production during ammonification (Abendroth et al, 2015). Conventional methods like denitrification are mainly focused on biological treatments to remove nitrogen by converting it into nitrogen gas (Kader et al, 2022). Since this method is not applicable to raw cattle manures, modern methods like Struvite precipitation (Uludag-Demirer *et al.*, 2005), Gas-Permeable membrane (Filho *et al.*, 2018; Vanotti & Szogi, 2010), Alternative Stripping Reactors (Filho *et al.*, 2018), chemical amendments (Kavanagh *et al.*, 2019), by using chemical acidifier, commercial products and agricultural waste (Kavanagh *et al.*, 2021) and biofilter (Shang, *et al.*, 2021) were successfully used in past research to recover ammonia from the extracted solid manures and slurries.

In the Indian subcontinent, the most common method for removing ammonia from animal manures is Ammonia stripping (Srinivasan *et al.*, 2008), where the cattle slurry is treated for ammonia removal using steam or biogas (Dos Santos *et al.*, 2020; Ferraz *et al.*, 2013; Huang & Shang, 2006). The efficiency of air stripping is high in liquid manure (Bonmatıi & Flotats, 2003) and it heavily depends on the rate of air supply, temperature, and pH (Buonomenna, 2013; Lodhi & Lal, 2017). However, it was observed that the major crisis in using air stripping on an industrial scale for ammonia recovery is the formation of air bubbles or foams at unprecedented levels (Ndegwa *et al.*, 2009; van Niekerk *et al.*, 1987). By having an overall analysis on past studies, our research was scoped to implement hydrolysis on raw cattle manure to study the effects of total solid content, retention time and temperature and then to use Head Space Flushing to remove ammonia from absorbed cattle manure by lowering the pressure gradient of its toxic components.

## MATERIAL AND METHODS

***Outsourcing of specimens.*** The raw diary manure was taken from the poultry farms of Pottuvil (Eastern Province), Polonnaruwa (North Central Province), Kalutara (Western Province), Hambantota (Southern Province) and Jaffna (Northern Province) of Sri Lanka, and the specimens for hydrolysis were prepared from these extractions. Digestates from the hydrolysis experiments were used for HSF. Table 1 describes the characteristics of solid cattle manure from past experimental outcomes (Matulaitis et al., 2015) carried out in Lithuania.

Table 1: Characteristics of Solid cattle manure (Matulaitis *et al.*, 2015)

| **Solid manure Specimen** | **Total solids (%)** | **Total Kjeldahl nitrogen (%)** | **pH** | **Volatile solids (%)** | **Total Ammonium nitrogen (%)** |
|---|---|---|---|---|---|
| Dairy cattle | 13.75 ± 2.72 | 0.37 ± 0.05 | 7.35 ± 0.33 | 10.67 ± 2.60 | 0.15 ± 0.04 |
| Non-dairy cattle | 12.09 ± 4.55 | 0.41 ± 0.05 | 8.13 ± 0.07 | 8.27 ± 4.89 | 0.14 ± 0.02 |

**Experimental setup**

***Hydrolysis.*** There were two batch hydrolysis studies were carried out in temperature monitored environment, using 200 ml volumetric flask with 100 w/w dairy manure with raw species and replicates under ASTM E1426 standards. First batch experiment was undergone at three different temperatures of 25$^{o}$C, 35$^{o}$C, 45$^{o}$C and the second batch experiment was undergone at a constant temperature of 35$^{o}$C. Following the introduction of diary manure, gaseous nitrogen was used in anaerobic environment to ventilate the volumetric flask.

Different magnitudes of temperature, retention times and total solid loads (31%, 28% and 23%) were exerted on raw diary manure during the batch hydrolysis for 14 days to observe the behavioural changes. The efficiency of hydrolysis was determined using the "NH4-N/Total Kjeldahl Nitrogen (TKN)" ratio. At the second phase of batch hydrolysis, Total Solid loadings from 31% - 5% were exerted at 35$^{o}$C for the course of 7 days. The experimental outcomes of those two hydrolysis experiments were described by Table 2.

Table 2: Characteristics of the diary manure based on batch hydrolysis

| **First batch** | | | **Second batch** | | |
|---|---|---|---|---|---|
| Temperature ($^{o}$C) | $NH_4$-N influent (mg/L) | TS content (%) | Temperature ($^{o}$C) | $NH_4$-N influent (mg/L) | TS content (%) |
| 25 | 5 378 | 31 | 35 | 5 378 | 31 |
| 25 | 4 474 | 28 | 35 | 4 474 | 28 |
| 25 | 3 856 | 23 | 35 | 3 856 | 23 |
| 35 | 5 378 | 31 | 35 | 3 117 | 20 |
| 35 | 4 474 | 28 | 35 | 2 639 | 17 |
| 35 | 3 856 | 23 | 35 | 2 201 | 13 |
| 45 | 5 378 | 31 | 35 | 1 654 | 9 |
| 45 | 4 474 | 28 | 35 | 970 | 5 |
| 45 | 3 856 | 23 | 35 | NA | NA |

***Head Space Flushing (HSF).*** To eliminate ammonia from digested dairy manure, an HSF experiment was carried out. HSF studies were conducted in 250 ml volumetric containing 200 ml of decomposed Cow dung for 5 days. To achieve smooth blending without suspensions, these volumetric flasks were shaken using a rotary sieve shaker at 450 25 rpm. At 165 ± 5 rpm, the acquired solution in the second volumetric flask was mixed. The digested dairy manure's headspace was constantly aerated with an Eheim air pump 100 at a 5 L/min airflow rate. All trials were carried out at a constant temperature of 35ºC. A 200 ml solution of 2N Sulphuric acid ($H_2SO_{4(aq)}$) was used to scour $NH_{3(g)}$ in the second volumetric flask from the head space of the previous volumetric flask.

Using a DR-5000 spectrophotometer and nesslerization, total ammoniac nitrogen was determined. Furthermore, in this analytical work, nesslerization was employed to measure the Total Kjeldahl Nitrogen by monitoring the Kjeldahl digestion and distillation. Gas chromatography was used to determine the composition of biogas effluents.

## RESULTS AND DISCUSSION

***Hydrolysis.***

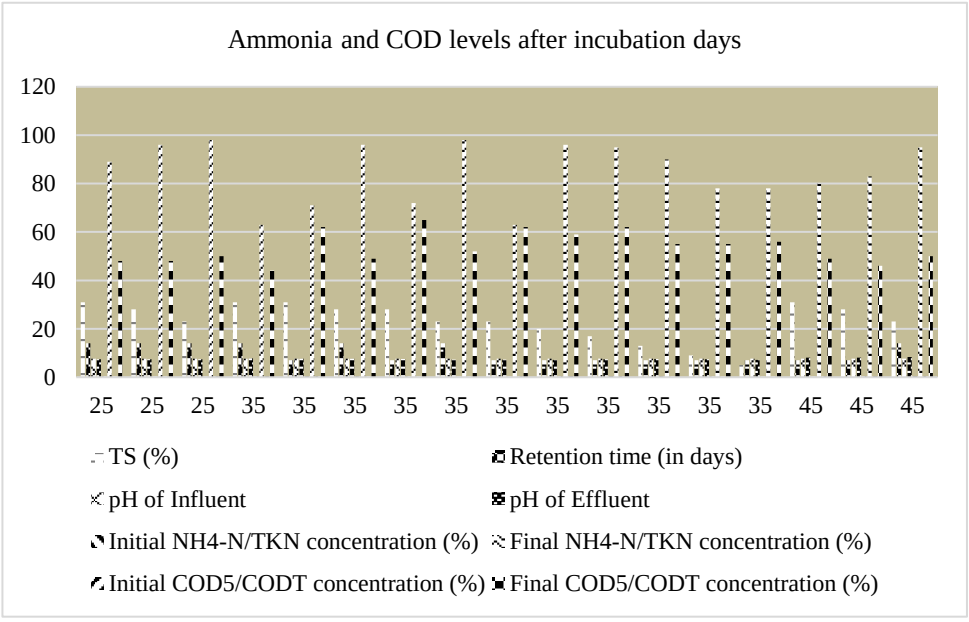


Figure 1: Ammonia level after the first and second phase hydrolysis tests

By the end of the first batch hydrolysis testing after 14 days, 33% of the initial NH4-N:TKN proportion had reached 89%. Hence, $COD_5/COD_T$ ratio was also increased from 21% to 48% and the average loss of $COD_T$ computed to be 7.21%. This observation proves the substantial occurrences of acidification during the hydrolysis of manure. Figure 1 shows that at all temperatures, the maximum

ammonification was found with a TS concentration of 23%. This experimental outcome validates the past studies (Alino *et al.*, 2022; Chen *et al.*, 2008; Surmeli *et al.*, 2017) those conclude that the TS concentration is inversely related to nitrogen hydrolysis. Temperature has a modest effect on the outcome of this hydrolysis experiment when compared to the effects of TS concentration.

According to the chromatographic results, $CO_2$ (85%) and 3.4% $H_2$ are formed due to organic matter degradation in dairy manure, whereas no indications of methane ($CH_4$) generation were observed during both the first and second stages of hydrolysis. Our findings support (Abendroth *et al.*, 2015; Bayrakdar *et al.*, 2017; Niu *et al.*, 2014; Qi *et al.*, 2021; Zejak *et al.*, 2022) by demonstrating that methanogenic processes are hindered during manure hydrolysis and that the quantity of COD5 excreted may be insignificant. Our findings also demonstrate that an acceptable quantity of organic ammonia conversion from nitrogenous substances takes three to seven days. The results were corroborated by Figure 1, which revealed that at 20% TS content at 35°C, 96% of NH4-N:TKN was attained.

***Head Space Flushing (HSF)***

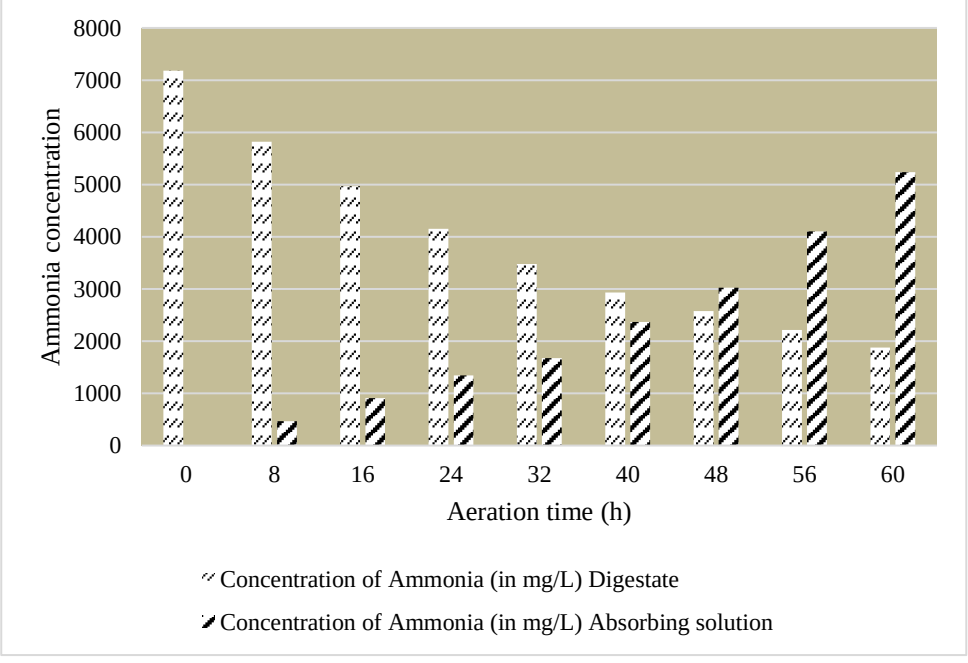


Figure 2: Ammonia removal and recovery results of HSF test on diary manure

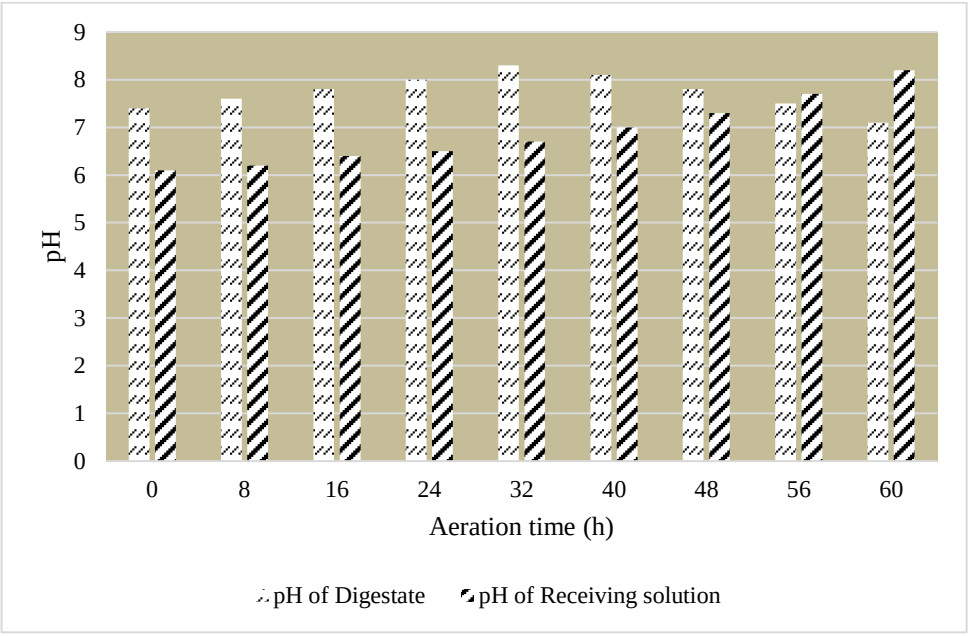


Figure 3: pH variation during the HSF test on diary manure

$$\text{Rate of ammonia recovery} = \frac{\text{Ammonia concentration of absorbing solution}}{\text{Ammonia concentration of diary manure digestate}}$$

$$= \frac{5236\text{mg/L}}{7183\text{mg/L}} \text{x } 100 = 72.89\% \approx 73\%$$

Because the pH range of digestate in the recruited diary manure samples is alkaline (i.e., 7.4-8.3), HSF tests on the anaerobically digested diary manure were performed using a laboratory digester. Because of its strong foaming potential due to its alkaline pH (Moeller *et al.*, 2015; Moeller *et al.*, 2015; Serna-Maza *et al.*,), the hydrolyzed dairy manure could not be properly mixed. The main cause of this increased pH is $CO_2$ stripping, which could be explained as follows:

$HCO_3^-{}_{(aq)} + \text{air} \longrightarrow OH^-_{(aq)} + CO_{2(g)}$ (García-González, Vanotti, & Szogi, 2015) (1)

$OH^-_{(aq)} + NH_4^+{}_{(aq)} \longrightarrow NH_{3(g)} + H_2O_{(l)}$ (2)

Figure 2 demonstrates the change in ammonia removal and recovery during HSF tests over a 5-day period (i.e., 60 hours). The ammonia content in the digestate

was reduced to 1879 mg/L from 7183 mg/L at the start. The ultimate concentration of the absorbing solution, on the other hand, was 5236 mg/L (i.e., ≠ 7183 mg/L - 1879 mg/L), indicating the presence of non-recoverable ammonia losses with a minor amplitude. The rate of ammonia removal was much greater than the rate of recovery in the first 32 hours. According to Figure 3, this could be explained by the high pH of the influent digestate. However, the increase in recovery rate was steadily overcome from 32 to 60 hours due to a pH decrease far below 8. Overall, the rate of ammonia removal and ammonia recovery was approximately 73%.

## CONCLUSION

The potential of extracting and recovering organic nitrogen in Sri Lankan dairy manure for sustainability issues was effectively explored utilising a batch hydrolysis and the HSF experimental framework. The hydrolysis of nitrogen in untreated dairy manure and ammonia with digestate from the HSF effluent was examined. Hydrolysis studies were carried out at 25, 35, and 45 degrees Celsius, as well as at 31, 20, and 15% TS concentration. Under anaerobic conditions at 35$^{o}$C and 23% TS content, almost 85% of the total nitrogen in dairy manure was transformed as ammonia in 7 days. Although significant ammonium efficiencies were reached, methanogenesis used relatively little organic materials.

HSF was used to eliminate ammonia from digested CM. The dissolved $CO_2$ in the digestate was eliminated, and the ammonia removal was improvised during HSF by raising the pH of the absorbing solution. Secondly, it is established that pH, TS concentration, and airflow rate all have substantial impacts on hydrolysis rate. The outcomes of this analytical research demonstrate that biogas is a feasible alternative to air that may be utilised to reduce the syntrophic methanogenic components and to mitigate the aerobic degradation during the HSF in the degraded leachate from the dairy manure outsourced from Sri Lankan cattle herds.

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# SOME ASPECTS OF BEESWAX HYDROLYSIS

## SUMMARY

Beeswax is the secretion of a bee's abdominal glands. According to its chemical composition, beeswax belongs to the group of lipids and it is formed a very complex composition of about 300 organic substances. It mostly contains different classes of esters, of which the majority are monoesters. Alkaline hydrolysis of esters (saponification) is a significant chemical reaction in the chemical industry. The saponification value together with the melting point, the acid value and the ester value represent the basic physico-chemical parameters for determining the quality, authenticity and potential faking of beeswax. Saponification has not been fully researched, nor is it an efficient process. Many researchers examine the reaction of ester hydrolysis in order to obtain a process with as little energy consumption as possible, a higher yield and a shorter duration of the hydrolysis process. The main factors that affect the wax saponification process are the size and structure of the alcohol and acid that make up the ester, the wax/alkali mass (molar) ratio, the duration of the process and the temperature of the reaction. With an increase in the mass of the wax sample, i.e. an increase in the wax/alkali mass ratio, the saponification value, i.e. the degree of hydrolysis, decreases. With an increase in the duration of hydrolysis and temperature, the degree of hydrolysis of wax esters, increases. The highest saponification value (SV=79.3; degree of hydrolysis 76.5%) was achieved by the hydrolysis of 0.5 g of wax at a temperature of 105°C for 150 minutes.

**Keywords**: beeswax, saponification, esters, hydrolysis, saponification value, acid value

## INTRODUCTION

Beeswax, produced by honeybees as an excretion from the abdominal glands, is a complex mixture of organic compounds. According to its chemical composition and properties, it belongs to the group of lipids.

The uses and significance of beeswax are very diverse. It is used in various fields of medicine and pharmacy, and in the production of cosmetics, but also in art, during religious ceremonies, as an indicator of environmental pollution, for the production of foodstuffs, in construction, in various branches of the

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engineering industry, and in various other fields. (Ebrahim, 2015, Svečnjak *et al.*, 2019). One characteristic property of wax is that it can preserve its properties unchanged for thousands of years.

Waxes are, for the most part, monoesters of higher fatty acids with higher monohydroxyl alcohols. Fatty acids and higher alcohols usually have an even number of C-atoms and feature a straight chain. In addition to esters, waxes also contain a significant proportion of impurities (often more than 50%) consisting of free fatty acids, oxy-carboxylic acids, free alcohols, hydrocarbons, resinous substances and various others (Ebrahim, 2015, Svečnjak *et al.*, 2019). The properties of natural waxes are not determined by the properties of the esters that make them up, but rather by the properties of the impurities, most notably in terms of their share.

According to its chemical structure, beeswax is a complex mixture that includes over three hundred different substances. The most abundant constituent is myricyl-palmitate, $C_{15}H_{31}CO\text{-}O\text{-}C_{30}H_{61}$. However, there is considerable variation in the chemical composition of beeswax. This is to be expected considering that the chemical composition of the wax depends on the prevailing geographical and climatic conditions, the species and genetics of the bees, the age of the wax and other similar factors. Based on the analyzes of Tulloch (1980), Hepburn (1986), Aichholz and Lorbeer (1999), Negri *et al.* (2000), Garnier *et al.* (2002), Kuzene (2013), and Ebrahim (2015), beeswax contains, in general terms, 35-45% esters, 5.6-14% diesters, 3% triesters, 4-9.5 hydroxymonoesters, about 8% hydroxy-polyesters, 1% acid esters, 2% acid polyesters, about 12% free polyesters, 12-23.5% hydrocarbons, 3.6-18% free fatty acids, 0.6-4.3% free higher alcohols, and about 6% other substances.

In recent times, more than 15 different natural and industrial waxes have been created on the basis of the adulteration of beeswax around the world (Bogdanov, 2004, Svečnjak *et al.*, 2015). Among them, paraffin waxes represent the most significant problem, due to their wide availability and low cost. In addition, paraffin is a white or colorless substance, and is also odorless and chemically inert, which makes it very suitable for adulteration. Occasionally, other unwanted substances, such as stearic acid, stearin, tallow, microcrystalline wax, and others, are also noticed in beeswax.

Determining the physico-chemical properties of beeswax is necessary to determine both its quality and authenticity. The recommendation of the International Honey Commission (IHC) is that the basic physicochemical parameters for quality assessment and the detection of possible adulteration are the melting point, the acid value, the saponification value, the ester value and the ester/acid ratio.

Determining the physicochemical properties of beeswax, in order to determine its authenticity, has both advantages and disadvantages. The advantages include the fact that it can be done using inexpensive equipment, accessories and reagents. It is also easy and quick in terms of its application and no special training is required for the chemical analysis. Furthermore, there is an

obvious and clear set of reference values for pure beeswax, which makes comparison straightforward. The disadvantages include the insufficient reliability of certain characteristics when determining the authenticity of beeswax, and the high limit of detection of unwanted impurities (5-50%), Moreover, the analysis process is often destructive and requires a larger amount of sample, meaning that it is necessary to carry out a larger number of analyses in order to determine the presence and the extent of any adulteration to the beeswax.

## MATERIAL AND METHODS

**The determination of the melting point.** The melting point was determined automatically using an instrument based on a capillary tube (a Melting-Point Meter KSPII). The instrument contains a microprocessor to allow for a controlled increase in temperature. Melted beeswax was introduced (approximately 1 cm in total) into a capillary with a length of 10 cm and an inner diameter of 2 mm. The test tube was kept at room temperature for about 24 hours with the solidified beeswax. Then the sample was slowly heated at a rate of 1°C per minute. The melting temperature was the one at which the beeswax was completely melted, which means that it was completely transparent with no cloudiness.

**The determination of solubility.** In order to test the solubility of the beeswax, at room temperature, 1 ± 0.0001 g of the tested beeswax was introduced into 7 glasses of 250 $cm^3$. 50 ± 0.05 $cm^3$ of each solvent was added in each of the glasses in the following order: petrol ether, a mixture of higher alkanes ($C_9$-$C_{15}$), mixture of xylene, cyclohexane, acetone, chloroform and ethyl ethanoate. The glasses were hermetically sealed and left at room temperature for 24 hours with occasional mixing.

**The determination of the acid value.** The acid value (AV) of the wax is the number of milligrams of KOH required to neutralize the free fatty acids in 1 g of beeswax.

We measured 3 ± 0.0001 g of wax in a 250 $cm^3$ erlenmeyer flask and added 50 $cm^3$ of solvent, composed of equal parts of ethanol and xylene. We heated the solution in a water bath (at about 60°C) until it dissolved, and then cooled the solution to room temperature. After that, we added 2-3 drops of phenolphthalein and titrated with a 0.1 M ± 0.0001 alcoholic solution of KOH. The analysis procedure was repeated three times.

The acid value was calculated according to the equation:

$$AV = \frac{c_{KOH} \times V_{KOH} \times 56.1}{w_{beeswax}}$$

where: $C_{KOH}$ is the concentration of the KOH solution in $moldm^{-3}$, $V_{KOH}$ is the volume of the used KOH solution in $cm^3$ and *w* is the mass of the measured wax in grammes.

**The determination of the saponification value.** The saponification value (SV) is the number of milligrams of KOH required to neutralize the total fatty acids in 1g of wax. The saponification value was determined according to the

method prescribed by the European Pharmacopoeia 7th Edition of 2008. A wax mass of 2.0 g ± 0.0001 g was measured in a 250 $cm^3$ erlenmeyer flask. 30 $cm^3$ of an ethanol: xylene mixture (1:1 V/V) was added to the wax sample. After that, it was heated in a water bath until it dissolved, and 25.0 $cm^3$ of standard alcoholic solution KOH, c = 0.5 ± 0.0001 M, was added from a burette. Hydrolysis was performed with reflux for 3 hours. The hot solution was titrated with 0.5 ± 0.0001 M HCl solution, with phenolphthalein as an indicator. The control sample was prepared and titrated in the same way.

The saponification value was calculated according to the equation:

$$SV = \frac{c_{HCl} \times (B - S) \times 56.1}{w_{beeswax}}$$

where *c* is the molarity of the HCl solution, in $moldm^{-3}$, B is the number of $cm^3$ of HCl solution used for the control sample, S is the number of $cm^3$ of HCl solution used for the sample, and *w* is the weight of the sample in grammes. In our experiments, in order to test the degree of hydrolysis, we measured three different masses of wax: 0.5 g, 1.0 g and 2.0 g ± 0.0001 g. The hydrolysis was carried out with reflux at three different temperatures (85°C, 95°C and 105°C) and over three time intervals: 30 minutes, 90 minutes and 150 minutes.

**The ester value (EV) and the ester/acid ratio.** The ester value is defined as the amount of KOH in milligrams needed to neutralize 1 g of ester linked acids of beeswax, being calculated by subtracting the saponification value from the acid value: EV = SV – AV. This method is intended to give a measure of the esterified fatty acids in beeswax.

The ester/acid ratio is the quotient of the ester and acid values of individual measurements. According to the International Honey Commission (IHC, 2016), the ester/acid ratio should be in the range of 3.3-4.3.

## RESULTS AND DISCUSSION

**The melting point of beeswax.**The determination of the melting point is not a reliable analytical method for determining beeswax adulteration. For example, paraffin wax is often added to beeswax for adulteration, because it has a similar melting point to beeswax. The melting point of beeswax is not constant, because the composition of the wax varies depending on its origin. Moreover, waxes melt without decomposition and harden again without changing. In our work, the melting point of authentic wax was determined from 5 measurements and is in the range of 62.3-65.4°C. The mean value of the measurement and the standard deviation was shown to be 63.8±1.79°C. The measured value of the melting point is in line with the reports from other literature on the expected value for authentic beeswax (Bernal *et al.*, 2005, Serra Bonvehı and Ornantes Bermejo, 2012, Maia and Nunes, 2013). The melting point of beeswax is not constant, because its composition varies depending on its origin. Various pharmacopoeias offer an expected range of 61–66°C or, more commonly, 62–65°C (Serra Bonvehı and Ornantes Bermejo, 2012). However, even values within this range are not a guarantee of wax purity. Small amounts of impurities change

the melting point. If more than 20% of the total mass of the beeswax is in the form of stearic acid, carnauba wax or microcrystalline wax, the melting temperature increases, reaching on average above 65°C. Conversely, when the wax is contaminated with paraffins to a degree greater than 10% of the total mass, the melting point decreases to below 61°C (Svečnjak *et al*., 2015).

**Solubility in organic solvents.** Beeswax is insoluble in water and resistant to many acids, but it is soluble in most organic solvents, including aromatic hydrocarbons, chloroform, ethers, esters and ketones (Breed *et al.*, 1995, Akoh and Min, 2008, Hossain *et al.*, 2009, Endlein and Peleikis, 2011). According to the European Pharmacopoeia (EP, Council of Europe, 2007) beeswax is practically insoluble in water, partially soluble in hot alcohol (90% V/V) and completely soluble in fatty and essential oils. The solubility of waxes is strongly temperature dependent. At room temperature, beeswax is not completely soluble in any of the above-mentioned solvents. However, when heated, after reaching the melting temperature, it is completely and easily dissolved (Endlein and Peleikis, 2011). Hossain *et al.* (2009) investigated the structure of beeswax using scanning electron microscopy, and found that beeswax contains many crystalline and semi-crystalline compounds. In our tests, the solubility of beeswax at room temperature in the tested organic solvents decreases in the following order: chloroform (97.2%) > a mixture of xylene (93.0%) > cyclohexane (90.3%) > petrol ether (75.0%) > a mixture of higher alkanes, $C_9$-$C_{15}$ (66.7%) > ethyl ethanoate (36.3%) > acetone (13.7%).

**The acid value.** The minimum and maximum value of the acid number of the examined beeswax are 15.1 and 18.2, respectively. The mean value ± standard deviation is KN=16.7 ± 1.55. The presence of other substances of an acidic nature can lead to an error in the determination of this value. Slightly higher values of the acid number of authentic beeswax, compared to ours, are shown in the works of certain authors (Bernal *et al.*, 2005, Aguilar *et al.*, 2007, Maia and Nunes, 2013, Svečnjak *et al.*, 2015). Based on a comparative review of the quality parameters of beeswax according to FAO (2005), European legislation (2009/10/EC; EP, 2007) and the International Honey Commission (Bogdanov, 2016), the generally accepted range of acid number values is 17-24(22). Assuming that of the free fatty acids in the wax, only palmitic acid is present, then the content of fatty acids in the examined beeswax is 7.63% w/w. If converted to lignoceric (tetracosanoic) acid, the content of free fatty acids would be 11.0% w/w.

*Apis mellifera* beeswax contains the highest content of free fatty acids (18%) compared to the beeswax of other studied species (Aichholz and Lorbeer, 1999). Of the free fatty acids, saturated monocarboxylic, unbranched acids with a higher number of C-atoms (C-14 to C-36) are the most abundant. Many authors (Tulloch, 1980, Aichholz and Lorbeer, 1999, 2000, Jimenez *et al.*, 2003) state that the most abundant fatty acid in wax is tetracosanoic (lignoceric) acid, while Serra Bonvehi and Ornantes Bermejo (2012) suggest that it is palmitic acid. Of

the unsaturated fatty acids, oleic acid (18:1:9) and linoleic acid (18:2:9,12) are the most abundant.

Fatty acids have a role in bee colony recognition for honeybees in the hive (Breed *et al.*, 1995, Hepburn *et al.*, 2014). Although it is significantly present in the wax, stearic acid does not play a role in the recognition of individuals in the bee hive. Fatty acids play a significant role in the mechanical properties of beeswax, above all by giving it the qualities of resistance and stiffness (Buchwald *et al.*, 2009).

Determining the acid number is a reliable method for determining the contamination of wax by both paraffins and stearic acid. In the case of the falsification of beeswax with the addition of paraffin, the acid value decreases, while the acid value increases with an increase in the content of stearic acid.

**The saponification value.** The saponification value of the tested sample of beeswax was determined from 3 measurements and was SV = 98.5 ± 6.63. The minimum and maximum values were 92.3 and 105.5, respectively. A similar saponification value for authentic beeswax has also been found by other researchers (Bernal *et al.*, 2005, Maia and Nunes, 2013, Svečnjak *et al.*, 2015). Based on a comparative review of beeswax quality parameters according to FAO (2005), European legislation (2009/10/EC; EP, 2007) and the International Honey Commission (Bogdanov, 2016), the range of saponification values should be 87-102(104). In waxes and lipids in general, the saponification value can be a measure of the average molecular weight of the esters present in the sample. The lower the saponification value, the higher the average molecular weight of the wax esters.

The disadvantage of applying the saponification value in beeswax quality assessment is the variations of certain values reported in the literature (Tulloch, 1973, Serra Bonvehı, 1990, Bernal *et al.*, 2005, Maia and Nunes, 2013, Svečnjak *et al.*, 2015). The deviation of the value of the saponification number is due to exposure to high temperatures (121-140°C) of beeswax used for the construction of honeycombs (Tulloch, 1973, Svečnjak *et al.*, 2019). Therefore, differences in saponification number values can be partially explained by heating (>100°C) during beeswax processing (Tulloch, 1973, Svečnjak et al., 2015, Bogdanov, 2016). However, this does not explain the different ranges shown for beeswax samples collected directly from the hives (Bernal *et al.*, 2005, Maia and Nunes, 2013, Svečnjak *et al.*, 2015). Certain variations in the range of saponification values can also be explained by the different geographical origins of the beeswax.

**The saponification of beeswax.** Saponification is an important chemical reaction in the chemical industry due to the widespread practical application of esters. In the alkaline hydrolysis (saponification) reaction, esters give the corresponding salt of carboxylic acid and the corresponding alcohol. In this reaction, the alkali is both a reactant and a catalyst. The reaction is irreversible, slow and has a low yield. Saponification as a process has not yet been fully researched, nor is it especially efficient. Many researchers have examined the reaction of ester hydrolysis in order to obtain a process with the minimum

possible energy consumption, a higher yield and a shorter duration of the hydrolysis process. The main factors that affect the wax saponification process are the size and structure of the alcohol and acid that make up the wax esters, the wax/alkali mass (molar) ratio and the reaction temperature.

Table 1 shows the mean saponification values, under experimental conditions with 3 variables (sample mass, temperature and time).

**Table 1.** Mean of the saponification value of beeswax from 3 measurements, for the given experimental conditions

| time → / sample mass↓ (wax/alkali mass ratio) | 30 min | 90 min | 150 min | temperature |
|---|---|---|---|---|
| 0.5 g (0.7) | 48.1 | 66.3 | 72.8 | 85 $^0$C |
| 1.0 g (1.4) | 44.9 | 64.6 | 71.7 | 85 $^0$C |
| 2.0 g (2.8) | 34.2 | 58.7 | 68.7 | 85 $^0$C |
| 0.5 g (0.7) | 48.7 | 71.7 | 77.2 | 95 $^0$C |
| 1.0 g (1.4) | 45.4 | 63.3 | 75.7 | 95 $^0$C |
| 2.0 g (2.8) | 34.8 | 61.5 | 71.5 | 95 $^0$C |
| 0.5 g (0.7) | 67.0 | 75.9 | 79.3 | 105 $^0$C |
| 1.0 g (1.4) | 58.2 | 71.9 | 78.3 | 105 $^0$C |
| 2.0 g (2.8) | 51.4 | 69.8 | 72.2 | 105 $^0$C |

The obtained experimental saponification values are lower than the actual values. This is to be expected, considering that the hydrolysis was carried out under cooler conditions. With an increase in the mass of the wax sample, i.e. an increase in the wax/alkali mass ratio, the saponification value decreases. Conversely, when there is an increase in the duration of hydrolysis and temperature, the saponification value, i.e. the degree of hydrolysis of wax esters, increases. Increasing the mass of the wax sample from 0.5 g to 1.0 g, (and thus increasing the wax/alkali mass ratio from 0.7 to 1.4) slightly reduces the saponification value. A significantly greater decrease in saponification is observed with a further increase in the mass of the sample, i.e. the mass ratio of wax/alkali. After 30 and 90 minutes of saponification, the influence of increasing the temperature from 95 to 105°C on the degree of hydrolysis is greater. After 150 minutes, the process is reversed, the degree of hydrolysis is higher when the temperature increases from 85 to 95°C. The highest saponification value (SV=79.3) was achieved by the hydrolysis of 0.5 g of wax, at a temperature of 105°C for 150 minutes. The lowest value of the saponification number (34.2) was achieved by hydrolysis of 2.0 g of wax at a temperature of 85°C for 30 minutes.

**The ester value.** The ester value of the tested beeswax is $EV_{total}$=81.8±9.32. In Table 2, the ester values (EV) for different experimental conditions are given. According to European legislation (2009/10/EC; EP, 2007) the range of ester number values should be 70-80, while according to the International Honey Commission (IHC, 2016) the permissible range is 70-90. The result from this work are similar to those found by other researchers (Akoh and Min, 2008, Maia and Nunes, 2013, Svečnjak *et al.*, 2015). Slightly lower results

were reported by Aguilar *et al.* (2007) and Serra Bonvehi and Orantes Bermejo (2012). By contrast, Serra Bonvehi (1990), Puleo and Ritt (1992) and Bernal *et al.* (2005) recorded slightly higher results.

In Table 2, the values of the degree of hydrolysis of wax esters in % for different process conditions are given. The degree of hydrolysis (h) was calculated as: $h=EV/EV_{total}$. For the given experimental conditions, the minimum degree of hydrolysis of esters present in the tested sample of beeswax was 21.4%. The maximum degree of hydrolysis was achieved by the hydrolysis of 0.5 g of wax at a temperature of 105°C for 150 minutes. This produced a result of 76.5%. The ester/acid ratio of the studied beeswax is in the range 4.4-4.8. This is slightly higher than the recommendation of the International Honey Commission (IHC, 2016). A slightly larger range of ester/acid ratio values has also been reported by other researchers (Bernal *et al.*, 2005, Svečnjak *et al.*, 2015). The ester/acid ratio values for the set experimental conditions are given in Table 2.

**Table 2.** Esterification value (EV); degree of hydrolysis (h) and ester/acid ratio (EV/AV)

| time → | 30 min | | | 90 min | | | 150 min | | | Temperature |
|---|---|---|---|---|---|---|---|---|---|---|
| sample mass (wax/alkali mass ratio) | EV | h | EV/AV | EV | h | EV/AV | EV | h | EV/AV | |
| 0.5 g (0.7) | 31.4 | 0.38 | 1.9 | 49.6 | 0.61 | 3.0 | 56.1 | 0.69 | 3.4 | 85 $^0$C |
| 1.0 g (1.4) | 28.2 | 0.34 | 1.7 | 47.9 | 0.59 | 2.9 | 55.0 | 0.67 | 3.3 | 85 $^0$C |
| 2.0 g (2.8) | 17.5 | 0.21 | 1.0 | 42.0 | 0.51 | 2.5 | 52.0 | 0.64 | 3.1 | 85 $^0$C |
| 0.5 g (0.7) | 32.0 | 0.39 | 1.9 | 55.0 | 0.67 | 3.3 | 60.5 | 0.74 | 3.6 | 95 $^0$C |
| 1.0 g (1.4) | 28.7 | 0.35 | 1.7 | 46.6 | 0.57 | 2.8 | 59.0 | 0.72 | 3.5 | 95 $^0$C |
| 2.0 g (2.8) | 18.1 | 0.22 | 1.1 | 44.8 | 0.55 | 2.7 | 54.8 | 0.67 | 3.3 | 95 $^0$C |
| 0.5 g (0.7) | 50.3 | 0.62 | 3.0 | 59.2 | 0.72 | 3.5 | 62.6 | 0.76 | 3.7 | 105 $^0$C |
| 1.0 g (1.4) | 41.5 | 0.51 | 2.5 | 55.2 | 0.68 | 3.3 | 61.6 | 0.75 | 3.7 | 105 $^0$C |
| 2.0 g (2.8) | 34.7 | 0.42 | 2.1 | 53.1 | 0.65 | 3.2 | 55.5 | 0.68 | 3.3 | 105 $^0$C |

## CONCLUSIONS

The solubility of the examined beeswax at room temperature in the tested organic solvents decreases in the following order: chloroform (97.2%) > mixture of xylene (93.0%) > cyclohexane (90.3%) > petrol ether (75.0%) > a mixture of higher alkanes, $C_9$-$C_{15}$ (66.7%) > ethyl ethanoate (36.3%) > acetone (13.7%). These results are to be expected.

The minimum and maximum value of the acid number are 15.1 and 18.2, respectively.The mean value ± standard deviation is AV=16.7±1.55. Assuming that of the free fatty acids in the wax, only palmitic acid is present, then the content of fatty acids in the examined beeswax is 7.63% w/w. When converted to lignoceric (tetracosanoic) acid, the content of free fatty acids would be 11.0% w/w.

The saponification value of the tested beeswax sample was: SV = 98.5 ± 6.63. The minimum and maximum values were 92.3 and 105.5, respectively.

The main factors that affect the wax saponification process include the size and structure of the alcohol and acid that make up the ester, the wax/alkali mass (molar) ratio, the duration of the process and the temperature of the reaction. With an increase in the mass of the wax sample, i.e. an increase in the wax/alkali mass ratio, the saponification value, i.e. the degree of hydrolysis, decreases. On the other hand, when there is an increase in the duration of hydrolysis and temperature, the saponification value, i.e. the degree of hydrolysis of wax esters, increases. The highest saponification value (SV=79.3; degree of hydrolysis 76.5%) was achieved by the hydrolysis of 0.5 g of wax at a temperature of 105°C for 150 minutes. The lowest value of the saponification number (SV=34.2; degree of hydrolysis 21.4%) was achieved by the hydrolysis of 2.0 g of wax at a temperature of 85°C for 30 minutes.

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# WATER–YIELD RELATIONS OF PROCESSING POTATO UNDER SURFACE AND SHALLOW SUBSURFACE DRIP IRRIGATION IN TEMPERATE CLIMATIC ENVIRONMENT

## SUMMARY

Field experiment was conducted to study the effects of surface (SDI) and shallow subsurface drip irrigation (SSDI) on potato (*Solanum tuberosum* L.) tuber yield, evapotranspiration, water use efficiency (WUE), and yield response factor ($K_y$). The experiment was carried out under semiarid climatic conditions in the Vojvodina region in 2020. The trial was established as a block design and adapted to technical specifications of drip irrigation system. In addition, the nonirrigated, control variant was also included in the trial. Irrigation was scheduled on the basis of water balance method. Daily water used on plants evapotranspiration ($ET_d$) was calculated by multiplying reference evapotranspiration ($ET_o$) with crop coefficients ($k_c$). $K_c$ values were 0.5, 0.7, 1.1, 0.9, 0.7 from planting to emergence, early vegetative development, tuber initiation, tuber enlargement and senescence, respectively. The potato processing variety 'Taurus' was used for the experiment. Obtained results indicate a significant effect of irrigation on potato yield compared to the nonirrigated variant (38.33 t $ha^{-1}$**)** but differences in the yield using the SDI (58.06 t $ha^{-1}$) and the SSDI (61.15 t $ha^{-1}$) were not significant. In the study period, seasonal evapotranspiration in irrigation conditions ($ET_m$) and in rainfed control variant ($ET_a$) was 478 mm and 319 mm respectively. IWUE values were 9.39, 10.85 kg $m^{-3}$ and 27.64, 29.09 kg $m^{-3}$ but ETWUE values were 12.40, 14.35 kg $m^{-3}$ and 12.14, 12.79 kg $m^{-3}$ for SDI and SSDI respectively. The seasonal yield response factor ($K_y$) of 1.03 and 1.12 for SDI and SSDI indicates that potato can be grown without irrigation in the temperate climate of Vojvodina.

**Keywords**: potato, drip irrigation, yield**,** evapotranspiration, water productivity

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## INTRODUCTION

Production of potato (*Solanum tuberosum* L.) takes a very important place in world agriculture, with a production potential of about 370 million tonnes harvested and 17.3 million hectares planted area with an average yield of 20.9 t $ha^{-1}$ (FAOSTAT, 2019). It rates fourth among the world's agricultural products in production volume, after wheat, rice, and corn (Fabeiro *et al.*, 2001). Over the last three years, a total of 36,000 hectares were devoted to potato in Serbia with an annual production of 597,000 tons and an average yield of 16.8 t $ha^{-1}$. In Vojvodina, the northern part of the Republic of Serbia potato is grown at about 5.700 hectares, with an annual production of 130,000 with an average yield of 23.0 t $ha^{-1}$ (Statistical Yearbook of the Republic of Serbia, 2021). In the region, the potato is mostly cultivated under rainfed conditions. Irrigation systems (portable sprinklers) cover only 12-15% of the potato growing area (Broćić and Stefanović, 2012). Lower average potato yields in Serbia, compared to those achieved in the leading potato growing countries (USA 49 t $ha^{-1}$, New Zealand 49 t $ha^{-1}$, Denmark 42 t $ha^{-1}$, Holland 42, Australia 40 t $ha^{-1}$, FAOSTAT, 2019), are primarily a consequence of inadequate management practices, insufficient amount and unfavorable distribution of precipitation in the growing season, production mostly under rainfed conditions as well as poor irrigation management.

In the variable climatic conditions of Vojvodina, in which summers are arid (Bošnjak, 2001), high and stable yields of potato can be reliably obtained only by supplementing crop water requirements through irrigation. Only optimum moisture conditions permit the plants to use water according to their needs, i.e., to the level of potential evapotranspiration (ETP). Bošnjak and Pejić (1995) found seasonal ETP of potato in the interval from 460 to 480 mm for the conditions of the Vojvodina region with the seasonal average and maximum daily values of 3.5 mm and 7 mm respectively.

Irrigation in Vojvodina is most commonly used to supplement infrequent or irregular precipitation during drought periods which regularly occur especially in July and August (Dragović *et al.*, 2012). Due to the unpredicted amount and distribution of precipitation in the growing season, irrigation in the Vojvodina region is mainly supplemental (Pejić *et al.*, 2011a, Pejić *et al.*, 2018).

Many irrigation experiments, conducted in a wide range of environments, have confirmed that potato yields increase with well-scheduled irrigation (Yuan *et al.*, 2003; Onder *et al.*, 2005; Milić *et al.*, 2010; Badr *et al.*, 2012; Pejić *et al.*, 2014; Aksić *et al.*, 2014). Rational irrigation, in addition to providing plants with the necessary amounts of water during the growing season, especially in the critical stages of development, implies the correct choice of irrigation methods. Potatoes in the region are most often irrigated by sprinklers, but due to numerous advantages of drip irrigation, both surface (SDI) and shallow subsurface (SSDI) irrigation have recently been applied, especially in the cultivation of vegetables. The SSDI system is the latest method of irrigation. Camp (1998) reported that drip irrigation is superior to sprinkler irrigation due to efficient use of water resources, the possibility of placing water and other chemicals precisely directly

to the root zone (Solomon, 1993; Bartolo, 2005), and significantly larger areas can be watered in one day, preventing crust formation which disturbs soil aeration and rainwater infiltration (Kalfountzos *et al.*, 2007). SSDI offers many advantages over SDI including reduced evaporation (Patel and Rajput, 2009), cut down surface runoff (Camp, 1998), water saving (Ayars *et al.*, 1999; Patel and Rajput, 2007), higher yields (Singh *et al.*, 2006; Pejić *et al.*, 2018), wind drift, vandalism and damage by animals. As well, SSDI has an advantage over SDI when using saline irrigation water in terms of yields and water use efficiency (Tingwu *et al.*, 2003), because SSDI can result in suitable root-zone salinity (Hanson *et al.*, 2009). The question of the depth at which laterals are posed has been the focus of researchers in recent years. Generally, it was suggested to place laterals in shallower layers of soil depending on cultivated plants and the physical properties of the soil (Al-Jamal *et al.* 2001; Patel and Rajput, 2009; Pejić *et al.*, 2018). There is almost no information in the literature regarding subsurface irrigation with laterals placed just below the soil surface (shallow subsurface drip irrigation) which are removed from the plot before harvest and used in the following years. Our knowledge indicates that the biggest advantage of SSDI compared to SDI is the possibility of placing the laterals together with the sowing or planting of plants, because it can be used for the uniform and timely emergence of plants, especially in arid and semi-arid regions. SDI can be placed only after the emergence of plants, at a certain stage of plant growth, i.e. plants must protect the laterals from wind movement (Pejić *et al.*, 2018).

The sensitivity of potato plants to water stress could be determined by using the yield response factor ($K_y$) which relates relative yield decrease to relative ET deficits (Doorenbos and Kassam, 1979). A greater $K_y$ value indicates an increased sensitivity of the cultivated plant to water stress. Doorenbos and Kassam (1979) estimate that the average value of $K_y$ is 0.7 for the potato growing season. The ultimate goal of irrigation is to utilize added water efficiently, i.e. that can give the greatest yield increase from added water (IWUE). If the irrigation regime is not harmonized with the plant water needs and water-physical properties of the soil, the effect of irrigation may be absent. IWUE generally tends to increase with a decline in irrigation but only in case that water deficit does not occur during a single growth period (Howell, 2001). The importance of analyzing evapotranspiration water use efficiency (ETWUE) is illustrated by the efforts of numerous researchers to direct total water use for evapotranspiration (ET) towards transpiration (T) as the productive part of water for plants (Allen *et al.*, 1998; Howell, 2001). Wang *et al.* (1996) pointed out that crop yield depends on the rate of water use, and that all factors increasing yield and decreasing water used for ET favorably affect the ETWUE.

The objectives of this study were to determine the effects of surface and shallow subsurface drip irrigation on potato tuber yield, evapotranspiration, water use efficiency, and yield response factor. The obtained results will be used to provide the professionals with useful information about the practical possibilities of drip irrigation and to give recommendations for rational potato irrigation, which implies high and stable yields.

## MATERIAL AND METHODS

A trial with irrigated potato was conducted on a private farm in Čenej (45°22' N latitude, 19°47' E longitude, and 85 m.a.s.l.) near Novi Sad, the Republic of Serbia, in the Calcic Chernozem soil according to the the IUSS Working Group (WRB) (FAO, 2007), in 2020. In the period 2000-2018, the average seasonal air temperature and precipitation were 19.4°C, and 338 mm, respectively. According to the Hargreaves climate classification system, the study area is classified as arid in the summer period, from Jun to August (Bošnjak, 2001).

The previous crop was the carrot. The soil was ploughed at a depth of 0.3 m in the autumn. Rotary harrowing, fertilizing, planting and ridging were done simutaniously by Brand Grimme mashine („All-in one system"). The potato processing variety 'Taurus' was planted on 23 April. The crop spacing was 0.75 by 0.30 m. All recommended agronomic practices regarding cultivation and plant protection were applied at the experimental plot. The experiment was set up as a block design with three replicates and adapted to the technical specifications of drip irrigation. The trial also included the nonirrigated, control variant. The plants were irrigated with a lateral placed in every row (the distance between laterals was 0.75 m) on surface and subsurface variant (depth 0.1 m in the ridge, Petel and Rajput, 2007 reported that the maximum potato yield was recorded when drip tape was buried at 0.1 m) with drippers spaced every 0.2 m. Drippers had an average flow of 1.1 L $h^{-1}$ under a pressure of 70 kPa. Irrigation was scheduled on the basis of water balance method. Daily water used on plants evapotranspiration ($ET_d$) was calculated by multiplying reference evapotranspiration ($ET_o$) with crop coefficients ($k_c$). $K_c$ values were 0.5, 0.7, 1.1, 0.9, 0.7 from planting to emergence, early vegetative development, tuber initiation, tuber enlargement and senescence respectively (King and Stark, 1997, FAO, 2007, Table 2). $ET_o$ was calculated by Hargreaves equation (Hargreaves and Allen, 2003). Daily $ET_o$ values were taken from the website of the Hydrometeorological Service of the Republic of Serbia, (RHMZS). Irrigation started when readily available water (RAW) in the soil layer of 0.4 m (Wang *et al*. 2006 reported that most of the potato root is located at the depth of 0.4 m) was completely absorbed by plants. The irrigation rate was 30 mm at the beginning of the season and 40 mm in the middle season. The volume of irrigation water and the pressure in the system were controlled by the flow meter and the pressure gauge installed in the hose nozzle used for irrigation. Runoff and capillary rise were assumed negligible, but in the case of heavy precipitation, greater than the capacity of the soil for RAW in a layer of 0.4 m, percolated water into deeper soil layers was calculated. The size of the experimental unit was 10 $m^2$ (13.3 m x 0.75 m). The middle two rows in each plot were harvested by hand at physiological maturity on 31 August. The yield (t $ha^{-1}$) was computed based on the yield measured at the experimental unit. The number of tubers per plant, tuber yield per plant (g), and mean tuber weight (g) were determined from 10 randomly selected plants before harvest. After harvesting, tubers of each plot were graded into three size categories (>40, 35–40,

and <35 mm) and weighed. This classification has also been used in Serbian companies that processed potato. Potato dry matter (%) was determined by the hydrometer (Zeal Manual Hydrometer). YSI 2700 Biochemistry analyzer Marshall Scientific apparatus was used for the determination of sugar concentration in potato tubers.

Yield response factor ($K_y$) during the growing season of potato was determined using Stewart's model (Stewart *et al*., 1977) as follows:

$$\left(1-\frac{Y_a}{Y_m}\right)=K_y\left(1-\frac{ET_a}{ET_m}\right) \quad \textbf{(1)}$$

$Y_a$ = the actual harvested yield (nonirrigated, t ha$^{-1}$),

$Y_m$ = the maximum harvested yield (under irrigation, nonlimiting conditions, t ha$^{-1}$),

$K_y$ = the yield response factor,

$ET_a$ = the actual evapotranspiration (mm), corresponding to $Y_a$

$ET_m$ = the maximum evapotranspiration (mm) corresponding to $Y_m$, and

$(1–ET_a/ET_m)$ = the relative evapotranspiration deficit and $(1–Y_a/Y_m)$ the relative yield decrease.

IWUE's and ETWUE's calculations were done in two ways:

IWUE = $Y_m – Y_a$ /I (Bos, 1985) **(2)**

IWUE = $Y_m$ /I (Viets, 1962) **(3)**

ETWUE = $Y_m – Y_a/ET_m - ET_a$ (Bos, 1985) **(4)**

ETWUE = $Y_a/ET_m$ (Erdem *et al*., 2006) **(5)**

Where I = the total seasonal irrigation water applied (mm)

Data reported for yield and yield components were subjected to analyses of variance (ANOVA). The significant differences for examined traits were calculated using the LSD test at the significance level of $p\leq0.05$.

## RESULTS AND DISCUSSION

In the growing season of the experimental year (April-August), the mean air temperature and total precipitations were 19.3 °C and 319 mm, respectively (Tab. 2). Daily precipitation was measured on the experimental plot by a rain gauge, whereas the air temperature data were obtained from a weather station located at Rimski Šančevi, near the experimental field (Figure 1). As expected, seasonal precipitation of 319 mm (Tab. 1) was not sufficient for potato production, to allow plants to consume water in relation to their needs or evapotranspiration (478 mm, Tab. 2). For this reason, irrigation was needed to get acceptable yields of potato. The amount of water added by irrigation was 210 mm (Figure 1, Table 2). The examined year can be characterized as an average for potato production in comparison with long-term values of precipitation and air temperature (Table 2).

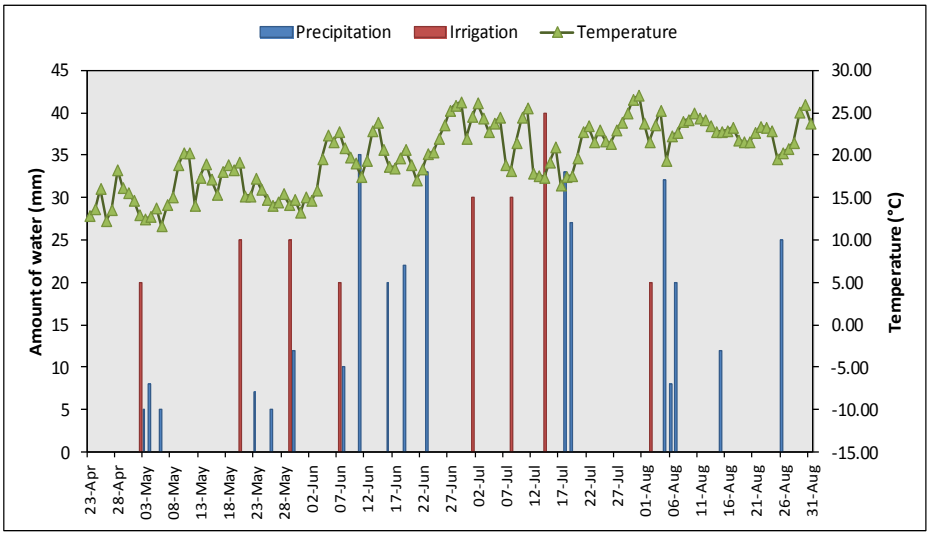


**Figure 1.** Irrigation schedules, irrigation water applied, and meteorological data for the experimental year (daily rainfall and daily average air temperature)

**Yield and yield components**

Unpredictable weather conditions in the region, first of all, amounts and distribution of precipitation, cause fluctuation in agricultural yields (Dragović, 2012; Vojnov *et al*., 2020; Vojnov *et al*., 2022). It is considered that generally, the potato is very sensitive to water stress (King and Stark, 1997; Pejić *et al*., 2014; Pejić *et al*., 2015), primarily due to its shallow roots (Singh, 1968; Opena and Porter, 1999; Onder *et al*., 2005; Ahmadi *et al*., 2011), even a short period of drought reduces tuber yield and quality (Vanloon, 1981; Miller and Martin, 1990; Kumar and Minhas, 1994). High yields of potatoes of excellent quality can be obtained only in conditions of optimal soil moisture (Bošnjak and Pejić, 1994; Ayas, 2013) when plants consume water for their needs or evapotranspiration. Pejić *et al* (2015) stressed that in the region of Vojvodina it is possible to achieve high and stable yields of potatoes, at the level of 50-60 t $ha^{-1}$ if a shortage of RAW in the soil, in the growing season, is eliminated by proper irrigation management.

According to the research results, irrigation, both SDI (58.06 t $ha^{-1}$) and SSDI (61.15 t $ha^{-1}$) had a significant effect ($p \leq 0.05$) on potato yield regarding the nonirrigated, control variant (38.33 t $ha^{-1}$), but differences in the yield obtained using SDI and SSDI were not significant (Table 1). Early studies conducted in different climate and soil conditions have also shown that irrigation significantly affects potato yield compared to rainfed production (Onder *et al*., 2005; Ayas, 2013; Cantore *et al*., 2014; Pejić *et al*., 2015; Rolbiecki *et al*., 2021). The obtained results are also consistent with researchers who reported that no statistical differences were found between surface and subsurface irrigation in potato yield (Phene, 1995; Weatherhead and Knox; 1998; Onder *et al*., 2005; El Mokh *et al*., 2014). Contrary to that, some studies indicated a significantly higher

yield of potato resulting from subsurface compared to surface drip irrigation (Petel and Rajput, 2007; Badr *et al*., 2010; Badr *et al*., 2012). The better performance of subsurface drip irrigation is explained by favorable soil water status in the root zone as well as more efficient utilization of nutrients from the limited wetted area. Findings and conclusions related to potato yield are identical for yield components; irrigation significantly affects all tested yield components ($p \leq 0.05$), except the percentage of tuber sized 35-40 mm compared to the nonirrigated variant (Table 1). Differences in the yield components obtained using SDI and SSDI were not significant. The obtained results are completely consistent with the results of Phene (1995), Weatherhead and Knox (1998), and Onder *et al*. (2005) who also reported no significant differences between surface and subsurface irrigation methods on potato yield components. However, the irrigation significantly affected all yield components compared to the rainfed conditions.

**Table 1.** Yield and yield components of potato

| Variant | Rep. | Yield (t ha$^{-1}$) | Tub. size <35 mm (%) | Tub. size 35-40 mm (%) | Tub. size >40 mm (%) | Tuber number plants$^{-1}$ | Plant yield (g plant$^{-1}$) | Mean tuber weight (g) |
|---|---|---|---|---|---|---|---|---|
| SDI | 1 | 57.86 | 0.00 | 2.7 | 97.3 | 7.48 | 1300 | 174.0 |
| | 2 | 59.18 | 0.00 | 4.1 | 95.9 | 7.65 | 1330 | 174.0 |
| | 3 | 57.13 | 0.00 | 1.2 | 98.8 | 8.46 | 1290 | 152.0 |
| | Aver. | **58.06$^{a}$** | **0.00$^{a}$** | **2.7$^{a}$** | **97.3$^{a}$** | **7.86$^{a}$** | **1310$^{a}$** | **166.7$^{a}$** |
| SSDI | 1 | 66.90 | 0.00 | 1.2 | 98.81 | 8.55 | 1510 | 176.0 |
| | 2 | 56.54 | 0.00 | 1.5 | 98.51 | 7.19 | 1270 | 177.0 |
| | 3 | 60.00 | 0.00 | 2.3 | 97.75 | 7.63 | 1350 | 177.0 |
| | Aver. | **61.15$^{a}$** | **0.00$^{a}$** | **1.7$^{a}$** | **98.4$^{a}$** | **7.79$^{a}$** | **1380$^{a}$** | **176.7$^{a}$** |
| Nonirrigated | 1 | 37.22 | 1.7 | 4.2 | 94.1 | 5.66 | 840 | 148.0 |
| | 2 | 38.32 | 3.3 | 2.6 | 94.1 | 5.67 | 860 | 152.0 |
| | 3 | 39.46 | 2.2 | 3.7 | 94.1 | 7.40 | 890 | 120.0 |
| | Aver. | **38.33$^{b}$** | **2.4$^{b}$** | **3.5$^{a}$** | **94.1$^{b}$** | **6.24$^{b}$** | **860$^{b}$** | **140.0$^{b}$** |

*Different letters in the same column denote statistically significant difference at $p \leq 0.05$

**Evapotranspiration, yield response factor, and water use efficiency**

Evapotranspiration (ET) represents the sum of water used by plants for transpiration (T) and water loss due to evaporation from plant and soil surfaces (E). Water used for plant evapotranspiration is influenced by a number of factors including the amount of water in the soil; it is the highest at the moisture of field capacity and it decreases with the decrease of water content in the soil (Vučić, 1976; Ferreira and Carr, 2002), the irrigation methods (Al-Jamal *et al*., 2001; Erdem *et al*., 2006), irrigation regimes (Onder *et al*., 2005; Pejić *et al*., 2014),

variety and length of growing season (Sharma *et al*., 1993), management practices (Fandika *et al*., 2016), environmental factors-atmospheric demand (Jones *et al*., 1984; Allen *et al*., 1998) and amount of crop cover (LAI) (Wright and Stark, 1990). Potato water demand for high tuber yield varied from 500 to 700 mm (Doorenbos and Kassam, 1979). Sharma *et al*. (1993) reported that potato plants need 500-600 mm of water throughout their life cycle. Bošnjak and Pejić (1995) reported seasonal ET of potato in the interval from 460 to 480 mm for the temperate climate conditions of the Vojvodina region. Aksić *et al* (2014) found that high and stable potato yield, in the conditions of south Serbia, could be reached if water consumption on evapotranspiration varied between 491 and 499 mm.

**Table 2.** Water balance of potato

| Elements | Planting to emergence | Early vegetative development | Tuber initiation | Tuber enlargement | Senescence | Total/ Average |
|---|---|---|---|---|---|---|
| | 23.04<br>13.05 | 14.05<br>4.06 | 5.06<br>3.07 | 4.07<br>31.07 | 1.08<br>31.08 | |
| $ET_o$ | 81 | 86 | 136 | 140 | 143 | **586** |
| $k_c$ | 0.5 | 0.7 | 1.1 | 0.9 | 0.7 | **0.8** |
| $ET_m$ | 41 | 61 | 150 | 126 | 100 | **478** |
| $ET_m$ (%) | 9 | 13 | 31 | 24 | 21 | **100** |
| Duration (days) | 21 | 22 | 29 | 28 | 31 | **131** |
| $ET_d$ | 2.0 | 2.8 | 5.2 | 4.5 | 3.2 | **3.4** |
| P | 18 | 24 | 120 | 60 | 97 | **319** |
| T | 15.1 | 16.2 | 20.9 | 21.6 | 22.9 | **19.3** |
| Δ | 0 | 0 | 0 | 0 | 0 | **0** |
| r | 0 | 0 | 0 | 0 | 0 | **0** |
| $ET_a$ | 18 | 24 | 120 | 60 | 97 | **319** |
| d | 23 | 37 | 30 | 66 | 3 | **159** |
| s | 0 | 0 | 0 | 0 | 0 | **0** |
| I | 20 (1) | 50 (2) | 50 (2) | 70 (2) | 20 (1) | **210** |

$ET_o$ – the reference evapotranspiration (mm), $ET_m$ – the maximum evapotranspiration – irrigated (mm), $ET_a$ – the actual evapotranspiration – rainfed (mm), $ET_d$ – daily evapotranspiration –irrigated (mm), P – rainfall (mm), Δ ± – inflow and outflow of water into the soil reserve (r), d – deficit of readily available water and s – surplus, percolated water

In the study period, the evapotranspiration rate in irrigation conditions ($ET_m$) and in rainfed, control variant ($ET_a$) was 478 mm and 319 mm respectively (Table 2). The highest evapotranspiration rate ($ET_m$) was recorded in the tuber initiation and the tuber enlargement part of the season amounted to 150 mm

(31%), and 126 mm (24%) respectively (Table 2). The highest value of average daily water use on evapotranspiration ($ET_d$) was detected in the tuber initiation at 5,2 mm, but the average value for the entire growing season was 3,4 mm (Table 2). A maximum $ET_d$ value of 7.2 mm was detected on 29 June, 68 days after planting by the end of the tuber initiation stage (Figure 2).

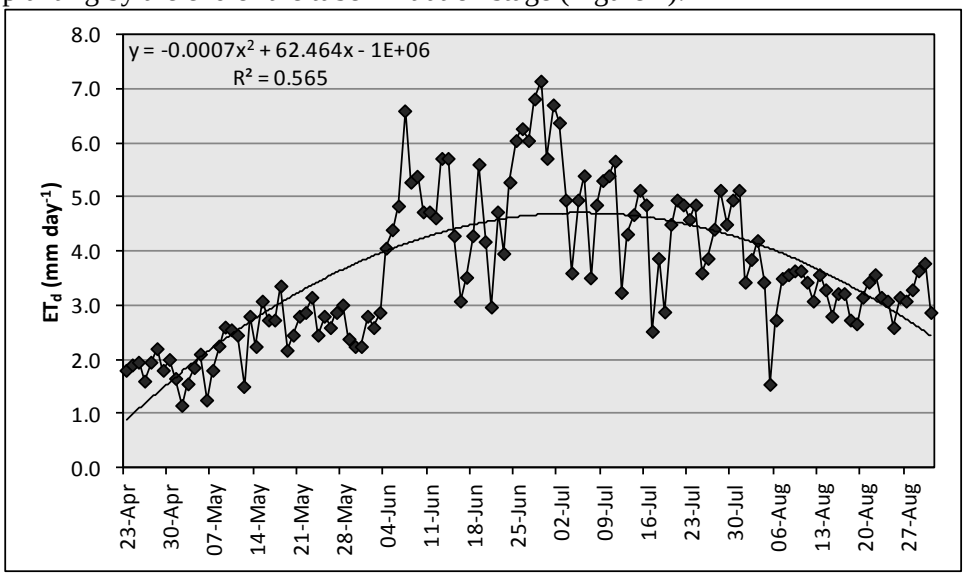


**Figure 2.** Daily water used on potato evapotranspiration

Obtained results are in agreement with Sharma *et al*. (1993) who reported that the water requirement of potato varies from 350-550 mm depending upon the length of the growing season, atmospheric demand, soil type, and crop variety. Onder *et al*. (2005) found, in the East Mediterranean Region of Turkey, that seasonal evapotranspiration of potato varied from 457 mm to 473 mm for potato irrigated by surface and subsurface drip irrigation respectively. The highest evapotranspiration rate ($ET_m$) recorded in the tuber initiation and the tuber enlargement part of the season clearly indicates that this period of potato vegetation is the most sensitive to water stress. Several studies have also confirmed that mentioned stages of potato development are the most sensitive to water stress (Doorenbos and Kassam, 1979; Kumar and Minhas, 1994; Yuan *et al*., 2003; Ashok, 2008; Begum *et al*., 2018). Shock *et al*. (1992) reported that adequate irrigation supply before and during tuber initiation increases the number of tubers per plant, but irrigation after tuber initiation stimulates tuber size (Eldredge *et al*., 1996). Karam *et al*. (2014) found out that treatment with deficit irrigation at the tuber bulking stage achieved a marketable yield 12% lower than that obtained in the well irrigated treatment. Obtained results are not in line with the findings of Faberio *et al*. (2001) who reported that tuber ripening is the growth stage that is most sensitive to water stress.

The maximum $ET_d$ value of 7.2 mm, recorded by the end of the tuber initiation stage is correlated with the fact that in that period the potato plants are

maximally developed and environmental factors, first of all, air temperature reaches the maximum values. These results are consistent with the information in the literature data. The same value of maximum $ET_d$ (7.2 mm) was recorded by Kumar *et al*. (2020) in the sub-humid sub-tropical region of India, 78 days after planting, during the mid-stage of the growing season. Bošnjak and Pejić (1995) have determined the average seasonal evapotranspiration of 3.5 mm and maximum daily evapotranspiration of 7-8 mm in soil and climate conditions of Vojvodina. Wright and Stark (1990) observed, in irrigated areas in Oregon and Washington, that potato reached a maximum $ET_d$ level of 8.5 mm just before effective full cover.

To compare results with other authors two different ways were used to compute WUE values. Howell (2001), Pejić *et al*. (2011a) indicated that care should be taken when comparing WUE values as many researchers have evaluated WUE in different ways (Viets, 1962; Bos, 1985; Stanhill, 1986; Payero *et al*., 2006; Molden, *et al*., 2010). It means that in climatic conditions where irrigation is supplementary WUE's calculation takes into account yields and plants evapotranspiration with and without irrigation (Bos, 1985; Erdem *et al*., 2006, Bajić *et al*., 2022), compared to arid regions where crop production cannot be realized in conditions of natural water supply. Thus in arid climate WUE's values are calculated as the ratio of yield and water added by irrigation or water used for plant seasonal evapotranspiration (Viets, 1962; Ati *et al*., 2012). As well Djaman *et al*. (2021) stressed that potato WUE strongly depends on the genetic material, management practices, irrigation regime, fertilizer rate, and other environmental conditions and all those should be taken into account when comparing results.

Regardless of the method of WUE calculation, no statistical differences were found between SDI and SSDI. IWUE values were 9.39 and 10.85 kg $m^{-3}$ ($Y_m$-$Y_a$/I) and 27.64 and 29.09 kg $m^{-3}$ (Y/I) for SDI and SSDI respectively. ETWUE values were 12.40 and 14.35 kg $m^{-3}$ ($Y_m$ - $Y_a/ET_m$ - $ET_a$) and 12.14 and 12.79 kg $m^{-3}$ ($Y_m/ET_m$) for SDI and SSDI respectively (Tab. 3). Onder *et al*. (2005) also reported that SSDI irrigation method did not offer a significant advantage for both yield and WUE compared to the SDI irrigation in early potato production under Mediterranean conditions. They determined IWUE values of 11.16 and 9.91 kg $m^{-3}$ for SDI and SSDI irrigation respectively. Based on the mentioned conclusions, they do not recommend the SSDI irrigation method due to its technical application difficulties.

Our results are in accordance with values reported by Aksić *et al*. (2014) who found out WUE values of potato (Y/ET) in the interval between 9.70 and 9.82 kg $m^{-3}$ on the variant of 30 kPa before irrigation in the conditions of south Serbia. IWUE values of potato reported in our study from 27.64 to 29.09 kg $m^{-3}$ for SDI and SSDI respectively were similar to 26.0 kg $m^{-3}$ reported by Rolbiecki *et al*. (2021) for drip irrigated potato in the temperate climate in the central part of Poland.

**Table 3.** Irrigation water use efficiency of potato

| Drip Irrigation | IWUE $Y_m$-$Y_a$/I (kg $m^{-3}$) | IWUE Y/I (kg $m^{-3}$) | ETWUE $Y_m$ - $Y_a/ET_m$ - $ET_a$ (kg $m^{-3}$) | ETWUE $Y_m/ET_m$ (kg $m^{-3}$) |
|---|---|---|---|---|
| SDI | 9.83 | 27.55 | 12.98 | 12.10 |
| | 9.93 | 28.18 | 13.12 | 12.38 |
| | 8.41 | 27.20 | 11.11 | 11.95 |
| | **9.39[a]** | **27.64 [a]** | **12.40 [a]** | **12.14[a]** |
| SSDI | 14.09 | 31.85 | 18.67 | 14.00 |
| | 8.68 | 26.92 | 11.46 | 11.83 |
| | 9.78 | 28.51 | 12.92 | 12.55 |
| | **10.85 [a]** | **29.09 [a]** | **14.35 [a]** | **12.79[a]** |

*Different letters in the same column denote statistically significant difference at $p \leq 0.05$

The yield response factor ($K_y$), for the total crop growing period, was 1,03 and 1.12 for SDI and SSDI respectively (Tab. 4). The value of $K_y$ in this study reveals that the relative yield decrease was nearly equal to the rate of ET deficit. Pejić *et al*. (2011a) reported that the accuracy of $K_y$ depends on having a sufficient range and number of values for Y and ET, and assumes that the relationships between Y and ET are linear over this range (Pejić *et al*., 2011b, Pejić *et al*., 2011c).

The obtained results agreed with the findings of Doorenbos and Kassam (1979), Ayas and Korukçu (2010), Mandal *et al*. (2018), Ayas (2013), and Kiziloglu *et al*. (2006) who found similar values of $K_y$ values for the total potato growing season. Darwish *et al*. (2006) found the $K_y$ value of 0.80 for processing potato for an entire growing period in the dry Mediterranean conditions of Lebanon.

**Table 4.** Evapotranspiration and yield response factor of potato

| Variant | $ET_m$ | $ET_a$ | $Y_m$ | $Y_a$ | $1\text{-}ET_a/ET_m$ | $1\text{-}Y_a/Y_m$ | $K_y$ |
|---|---|---|---|---|---|---|---|
| SDI | 478 | 319 | 58.06 | 38.33 | 0.33 | 0.34 | 1.03 |
| SSDI | 478 | 319 | 61.15 | 38.33 | 0.33 | 0.37 | 1.12 |

**Quality of processing potato**

No significant differences in the tested parameters of potato quality were found either between the irrigated variants in relation to the nonirrigated one, as well as between the SDI and SSDI irrigation treatment (Tab. 5).

The obtained results on the absence of statistical difference in dry matter content in potato between irrigated and nonirrigated variants are not in accordance with the results of many other authors who stated higher dry matter content on nonirrigated or deficit irrigated variants compared to irrigated variant (Kashyap and Panda, 2003; Karam *et al*., 2014).

**Table 5.** Quality of processing potato

| Variant | Replicates | Specific gravity (g $cm^{-3}$) | Dry matter content (%) | Sucrose (mg $g^{-1}$) | Glucose (mg $g^{-1}$) |
|---|---|---|---|---|---|
| SDI | 1 | 1.079 | 21.05 | 0.26 | 0.02 |
| | 2 | 1.086 | 22.25 | 0.64 | 0.05 |
| | 3 | 1.080 | 21.15 | 0.58 | 0.05 |
| | Average | **1.081[a]** | **21.48[a]** | **0.49[a]** | **0.04[a]** |
| SSDI | 1 | 1.085 | 21.98 | 0.68 | 0.07 |
| | 2 | 1.080 | 21.25 | 0.52 | 0.06 |
| | 3 | 1.077 | 20.55 | 0.35 | 0.04 |
| | Average | **1.080[a]** | **21.26[a]** | **0.52[a]** | **0.06[a]** |
| Nonirrigated | 1 | 1.077 | 20.6 | 0.48 | 0.03 |
| | 2 | 1.076 | 20.5 | 0.62 | 0.08 |
| | 3 | 1.076 | 20.46 | 1.08 | 0.11 |
| | Average | **1.076[a]** | **20.52[a]** | **0.73[a]** | **0.07[a]** |

*Different letters in the same column denote statistically significant difference at $p \leq 0.05$

## CONCLUSIONS

Based on the obtained results, it can be concluded that irrigation had a significant effect on potato yield compared to the nonirrigated variant the (38.33 t $ha^{-1}$) but differences in the yield obtained using the SDI (58.06 t $ha^{-1}$) and the SSDI (61.15 t $ha^{-1}$) were not significant.

Preference should be given to the SSSD irrigation as placing laterals can be done together with the sowing or planting of plants which can affect the uniform and timely emergence of plants. In the study period, seasonal evapotranspiration in irrigation conditions ($ET_m$) and in rainfed control variant ($ET_a$) was 478 mm and 319 mm respectively. IWUE values were 9.39 and 10.85 kg $m^{-3}$ ($Y_m$-$Y_a$/I) and 27.64 and 29.09 kg $m^{-3}$ (Y/I) but ETWUE values were 12.40 and 14.35 kg $m^{-3}$ ($Y_m$ - $Y_a$/$ET_m$ - $ET_a$) and 12.14 and 12.79 kg $m^{-3}$ ($Y_m$/$ET_m$) for SDI and SSDI respectively.

The yield response factor ($K_y$) for the total crop growing period, was 1.03 and 1.12 for SDI and SSDI respectively which indicates that potato can be grown without irrigation in the temperate climate of Vojvodina. These results will improve precise planing and efficient management of irrigation for potato in the region.

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***Gordan S. KARAMAN*** [1]

# FIRST DISCOVERY OF *NIPHARGUS DECUI* G. KARAMAN & SARBU 1995 (CRUSTACEA, FAM. NIPHARGIDAE) IN BULGARIA, WITH REMARKS TO ITS VARIABILITY (CONTRIBUTION TO THE KNOWLEDGE OF THE AMPHIPODA 328)

## SUMMARY

The subterranean species *Niphargus decui* G. Karaman & Sarbu 1995 (Crustacea Amphipoda, fam. Niphargidae) known so far only from type locality in Romania (Vama Veche village, 10 km south of Mangalia), is discovered at the first time in Bulgaria (subterranean waters of Shabla, Tolbuhin region). The species is redescribed and figured, and variability of the specimens from type-locality and these from Bulgaria is discussed. Morphological relation of this species regarding some other *Niphargus* species in Bulgaria and some adjacent regions is presented.

**Keywords**: Amphipoda, *Niphargus decui*, taxonomy, subterranean waters, Bulgaria, Romania.

## INTRODUCTION

The subterranean fauna of Bulgaria is only partially known, including Amphipoda also. Amphipods in Bulgaria settled numerous caves, springs and various types of the subterranean waters, (wells, subterranean lakes, etc.) from the sea shore to the high mountain springs and caves. Among them, family Niphargidae is the most numerous, presented in Bulgaria by almost 20 known taxa.

The most of *Niphargus* species discovered in Bulgaria are endemic for Bulgaria because of specific geological history, geomorphological, climatic, ecological and hydrological conditions. The partially knowledge of the subterranean Amphipoda fauna in the adjacent regions of Balkan limited more detailed recognition of taxonomical relation of Bulgarian subterranean Amphipoda fauna with that of surrounding countries.

During our study of Balkan subterranean Amphipoda fauna, the species *Niphargus decui* G. Karaman & Sarbu, 1995. not endemic, but new for the

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Bulgarian fauna, is discovered and redescribed in this work. By this way, the morphological variability of this species based on specimens from Bulgaria and from type-locality (Romania) is presented and new diagnosis of *N. decui* is made. Some morphological relations between this species and some more or less similar species from Bulgaria and adjacent regions is given.

## MATERIAL AND METHODS

The studied material was preserved in the 70% ethanol. The specimens were dissected using a WILD M20 microscope and drawn using camera lucida attachment. Body-parts were submersed in the mixture of glycerin and water for study and drawing by camera lucida; later transferred to Liquid of Faure as permanent slides. All illustrations were inked manually.

Some morphological terminology and setal formulae follow G. Karaman`s terminology (Karaman, G. 1969; 2012) for the last mandibular palpus article [A= A-setae on outer face; B= B-setae on inner face; C= additional C-setae on outer face; D= lateral marginal D-setae; E= distal long E-setae], and for propodus of gnathopods 1 and 2 [S= corner S-spine; L= lateral slender serrate L-spines; M= facial corner M-setae; R= subcorner R-spine on inner face]. Terms "setae" and "spines" are used based on shape, not origin. Our studies were based on the external morphology, ecology and zoogeography of animals.

## TAXONOMICAL PART

## AMPHIPODA SENTICAUDATA

### Family NIPHARGIDAE

### *NIPHARGUS DECUI* G. Karaman & Sarbu 1995
### Figures 1-6

**MATERIAL EXAMINED**:

BULGARIA: BU-11= Shabla, Tolbuhin region [nearly 65 km NE of Varna], 3.11.1978, sondage, 10 exp. (leg. L. Cvetkov);

.ROMANIA: S-5221: Vama Veche village, 10 km south of Mangalia, well, July 24, 1994, many specimens (holotype and paratypes) (leg. M. Sarbu).

**DIAGNOSIS.**

Body moderately slender, metasomal segments with 4 dorsoposterior marginal setae, urosomal segments 1-2 with dorsolateral spines; epimeral plates 1-3 slightly pointed, plates 2 and 3 with several subventral spines. Head with short lateral cephalic lobes, eyes absent, maxilla 1 inner plate with 2 setae, outer plate spines mainly with one or more lateral teeth; maxilliped with 6 distal spines on inner plate. Coxae 1-4 longer than broad, coxa 4 with ventroposterior lobe, coxa 5 much shorter than coxa 4.

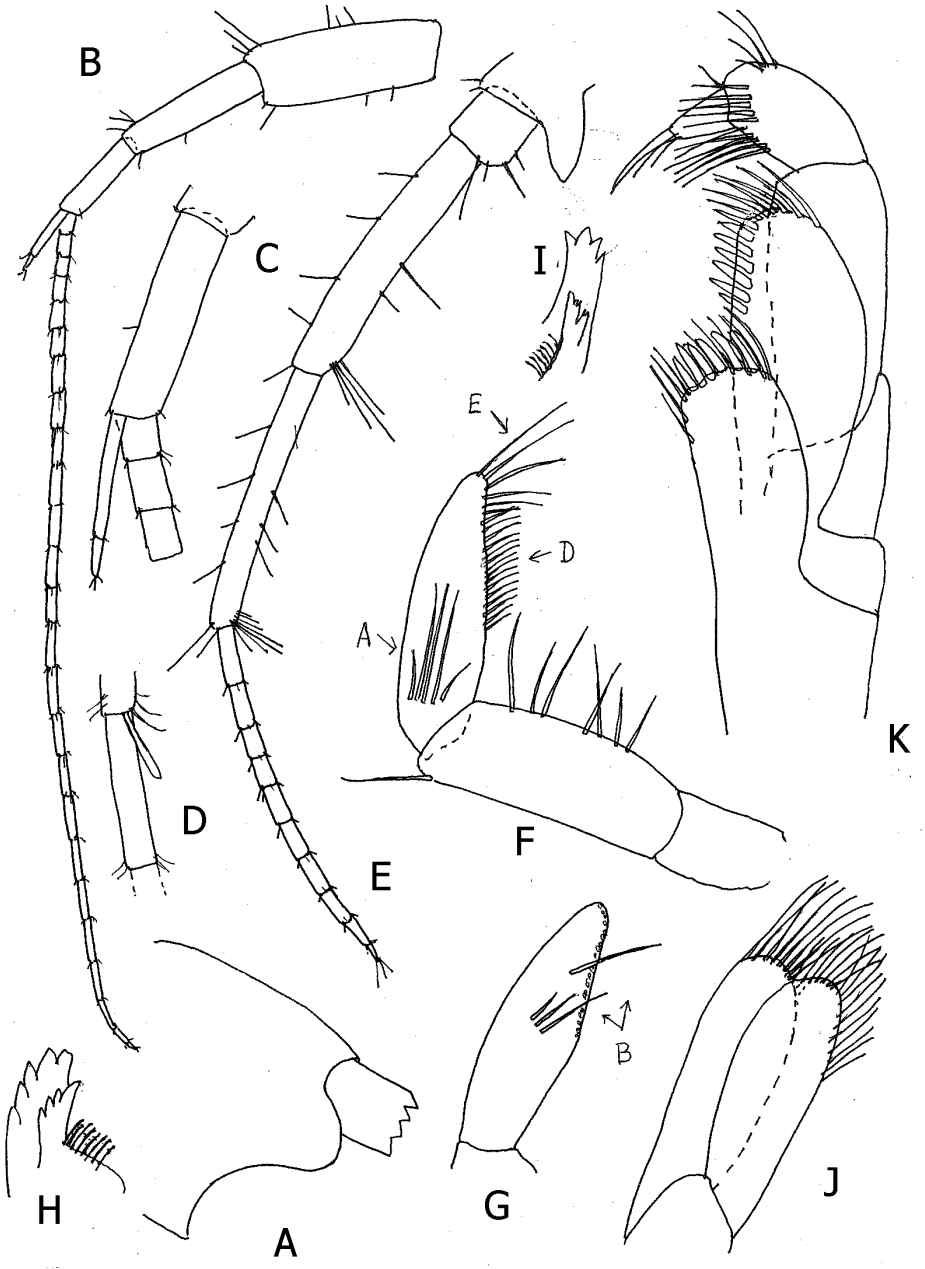


Fig. 1. *Niphargus decui* G. Karaman & Sarbu, 1995, Shabla, Bulgaria, female 7.0 mm: A= head; B= antenna 1; C= accessory flagellum; D= aesthetasc; E= antenna 2; F= mandibular palpus outer face (A= facial A-setae; D= marginal D-setae; E= distal E-setae); G= palpus article 3, inner face (B= facial B-setae); H= left mandible (incisor, lacinia mobilis, rakers); I= right mandible (incisor, lacinia mobilis, rakers); J= maxilla 2; K= maxilliped.

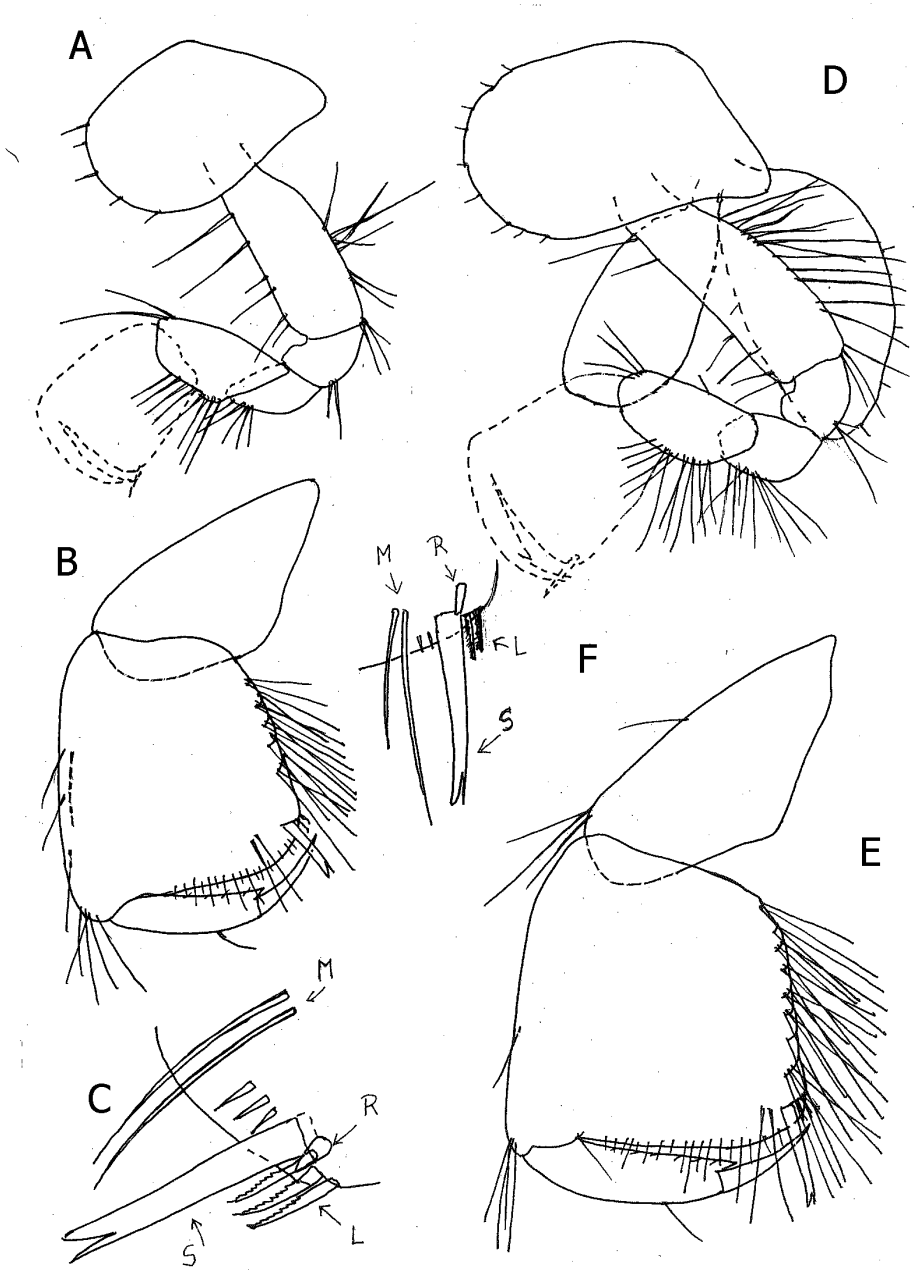


Fig. 2. *Niphargus decui* G. Karaman & Sarbu, 1995, Shabla, Bulgaria, female 7.0 mm: A-B= gnathopod 1, outer face C= distal corner of gnathopod 1-propodus (S= corner spine; L= lateral spines; M= corner facial M-setae; R= subcorner spine on inner face); D-E= gnathopod 2, outer face; F= distal corner of gnathopod 2-propodus (S= corner spine; L= lateral spines; M= corner facial M-setae; R= subcorner spine on inner face).

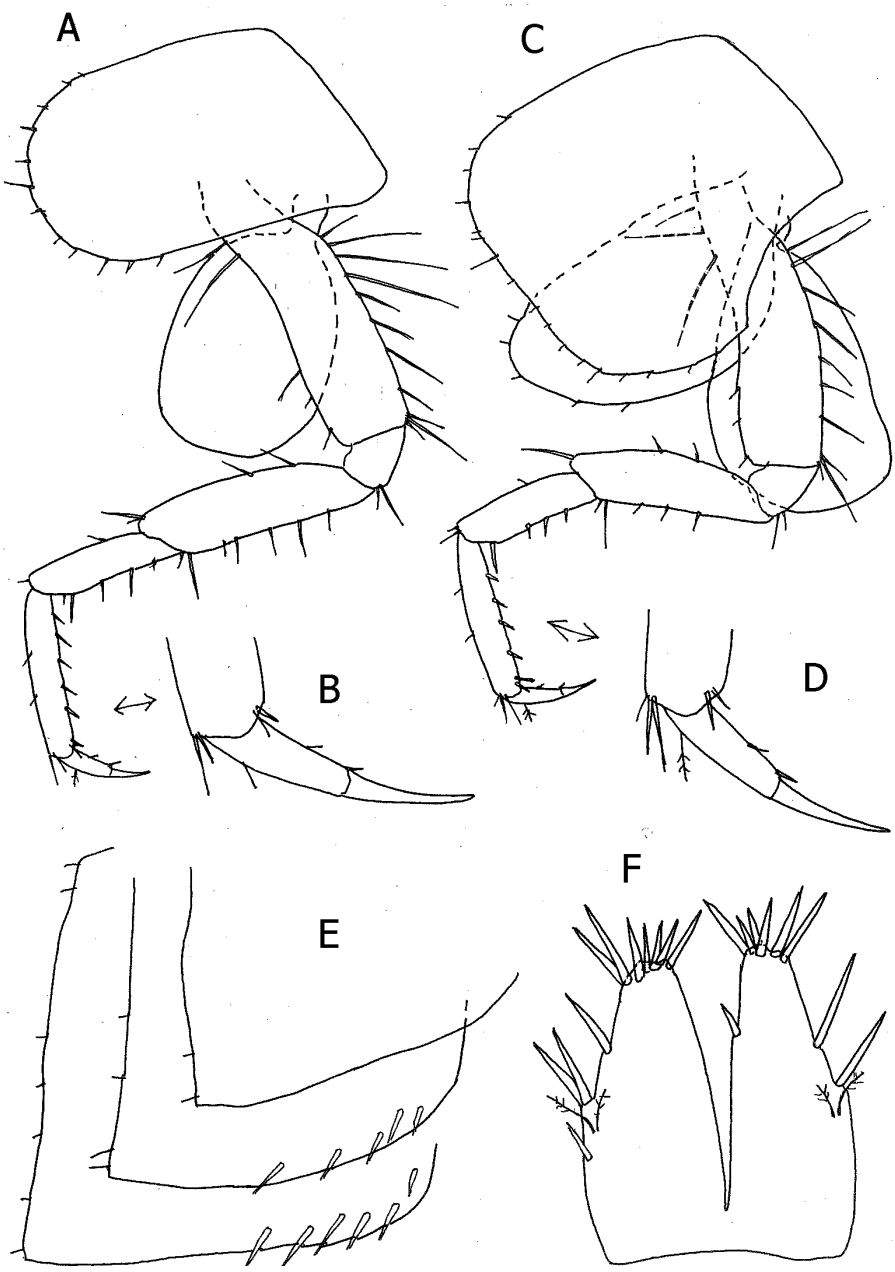


Fig. 3. *Niphargus decui* G. Karaman & Sarbu, 1995, Shabla, Bulgaria, female 7.0 mm: A-B= pereopod 3; C-D= pereopod 4; E= epimeral plates 1-3; F= telson.

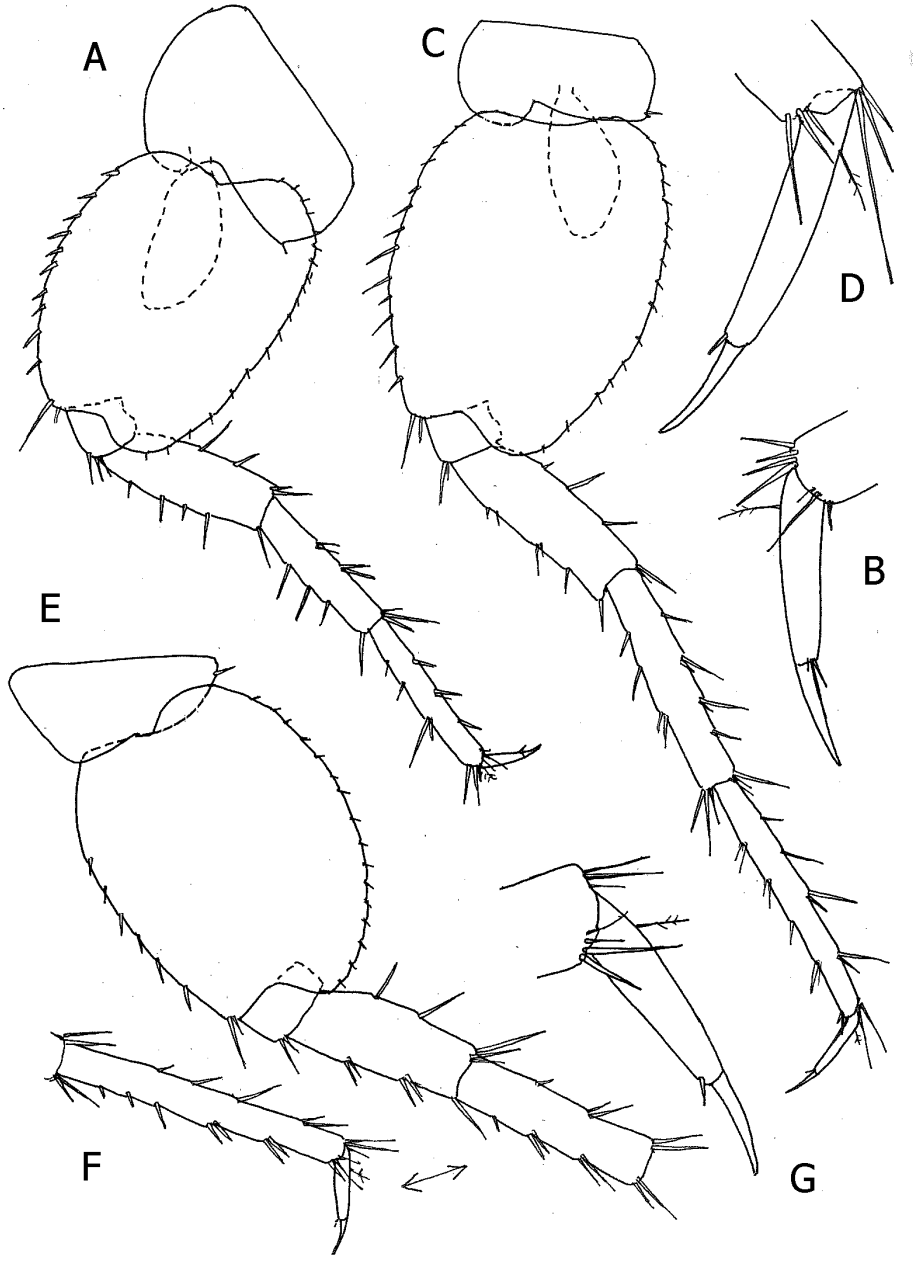


Fig. 4. *Niphargus decui* G. Karaman & Sarbu, 1995, Shabla, Bulgaria, female 7.0 mm: A-B= pereopod 5; C-D= pereopod 6; E-F= pereopod 7, female 6.9 mm

Gnathopods 1-2 *kochianus*-type, with dactylus bearing one median seta at outer margin. Pereopods 3-4 with slender dactylus bearing 1 or 2 slender spine-like setae at inner margin. Pereopods 5-7 with large ovoid article 2 with ventroposterior lobe, dactylus slender, with one slender spine at inner margin. Pleopods 1-3 with almost naked peduncle provided with 4-6 retinacula. Uropod 1 with nearly equal rami, uropod 2 inner ramus longer than outer one. Uropod 3 short and strong, spinose, second article of outer ramus very short. Telson deeply incised, with distal and marginal spines, facial spines absent. Coxal gills ovoid, larger in gnathopod 2 and pereopods 3-4, smaller in pereopods 5-6. Oostegites broad, with long marginal setae.

**DESCRIPTION**:

**Female 7.0 mm with oostegites from Shabla**: Body moderately slender, head with short rostrum and subrounded lateral cephalic lobes, ventroanterior sinus developed, eyes absent (fig. 1A). Mesosomal segments naked; metasomal segments 1-3 with 2+2 short dorsoposterior marginal setae (fig. 3E). Urosomal segment 1 with one dorsolateral strong spine on each side; urosomal segment 2 with 2 strong dorsolateral spines on each side, urosomal segment 3 naked (fig. 6D).

Epimeral plates 1-3 slightly pointed, with more or less inclined posterior margin bearing several short setae; epimeral plate 2 with 5 subventral spines, epimeral plate 3 with 6 subventral spines (fig. 3E).

Antenna 1 slender, slightly exceeding half of body-length; peduncular articles 1-3 progressively shorter (ratio: 53:42:27), covered by single short setae each (fig. 1B). Main flagellum very slender, consisting of 25 articles bearing single short setae (most of articles with one aesthetasc) (fig. 1D). Accessory flagellum slightly elongated, 2-articulated, reaching ¾ of last peduncular article (fig. 1C).

Antenna 2 shorter than antenna 1, relatively slender, peduncular article 3 short, with 4 short ventral setae; articles 4 and 5 of equal length; article 4 with bunch of long distoventral setae and several other setae along dorsal and ventral margin (the longest setae remarkably exceeding diameter of article itself); article 5 with distal dorsal and ventral group of setae, along both margins appear several shorter or longer setae. Flagellum slender, rather longer than last peduncular article, consisting of 10 scarcely setose articles (fig. 1E). Antennal gland cone short (fig. 1E).

Mouthparts well developed. Labrum broader than long (fig. 5A). Labium broader than long, inner lobes well developed, short; outer lobes entire, rounded distally (fig. 6A).

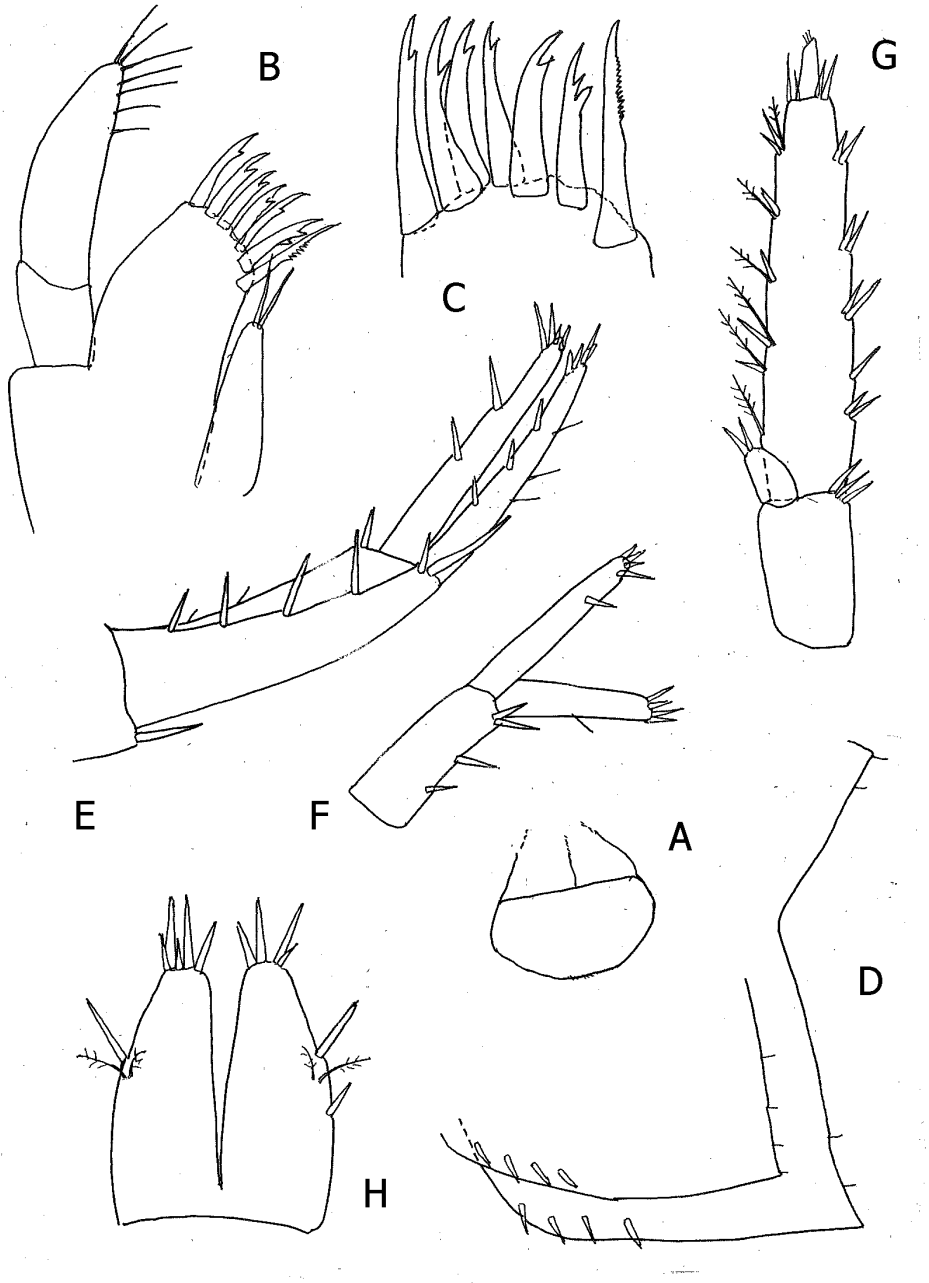


Fig. 5. *Niphargus decui* G. Karaman & Sarbu, 1995, Shabla, Bulgaria, female 7.0 mm: A= labrum; B-C= maxilla 1; E= uropod 1; F= uropod 2; G= uropod 3. Female 6.9 mm: D= epimeral plates 2-3; H= telson.

Mandibles with triturative molar. Left mandible: incisor with 5 teeth, lacinia mobilis with 4 teeth, accompanied by 8 rakers (fig. 1H). Right mandible: incisor with 4 teeth, lacinia mobilis serrate, accompanied by 7 rakers (fig. 1 I). Palpus mandibulae well developed, 3-articulate: first article naked; second article with 7 setae; third article subfalciform, scarcely longer than article 2 (ratio:79:71), bearing 16 marginal D-setae, 5-6 distal long E-setae, on outer face appear row of 5 medial A-setae (fig. 1F), on inner face are attaches 2 groups of B-setae (3+1) (fig. 1G).

Maxilla 1: inner plate long, with 2 distal setae; outer plate with 7 spines [5 spines with one lateral tooth, one spine with 2 lateral teeth, inner spine with finely serrate distal margin (fig. 5C); palpus 2-articulated, slightly exceeding distal tip of outer plate-spines and provided with 7 distal setae (fig. 5B).

Maxilla 2 with longer plates bearing distal setae, inner plate with distolateral marginal setae also (fig. 1J).

Maxilliped rather longer, inner plate strong, reaching outer tip of first palpus article, bearing 6 distal spines mixed with single setae; outer plate slightly shorter than palpus article 2 and bearing 10-12 distolateral strong spines and row of distal setae; palpus article 3 outer margin with bunch of setae, inner margin with setae; article 4 with one outer marginal median seta, at inner margin with 2 setae near basis of the nail; nail relatively long (fig. 1K).

Coxae 1-4 moderately long. Coxa 1 rather longer than broad (ratio: 53:46), ventroanterior corner subrounded, margin with 5 setae (fig. 2A). Coxa 2 distinctly longer than broad (ratio: 73:50), strongly convex margin with 9 setae (fig. 2D). Coxa 3 longer than broad (ratio: 86:54), margin with 11 setae (fig. 3A). Coxa 4 with well developed ventroposterior lobe, rather longer than broad (ratio: 89:85), margin with 16 short setae (fig. 3C).

Coxae 5-7 moderately short. Coxa 5 much shorter than coxa 4, bilobed, broader than long (ratio: 67:44), anterior lobe short (fig. 4A). Coxa 6 remarkably smaller than 5, bilobed (ratio: 54:28), anterior lobe small (fig. 4C). Coxa 7 entire, broader than long (ratio: 57:29) (fig. 4E).

Gnathopods 1 and 2 relatively small, with propodus smaller than corresponding coxa (fig. 2A, D). Gnathopod 1: article 2 with row of 6 long marginal setae along anterior margin and bunches of setae at posterior margin; article 3 with distoposterior bunch of 2 setae (fig. 2A); article 5 nearly as long as propodus, with 2 groups of setae at anterior margin and numerous setae at posterior margin. Propodus rather *kochianus*-type, longer than broad (ratio: 78:65), posterior margin with 5 transverse rows of setae; palm straight in the middle, convex at corner (fig. 2B), defined on outer face by corner S-spine accompanied laterally by 3 serrate L-spines and 2 facial subcorner M-setae (fig. 2C), on inner face by one subcorner R-spine. Dactylus reaching posterior margin of propodus, with one median seta at outer margin and 5-6 small submarginal setae along inner margin (fig. 2B).

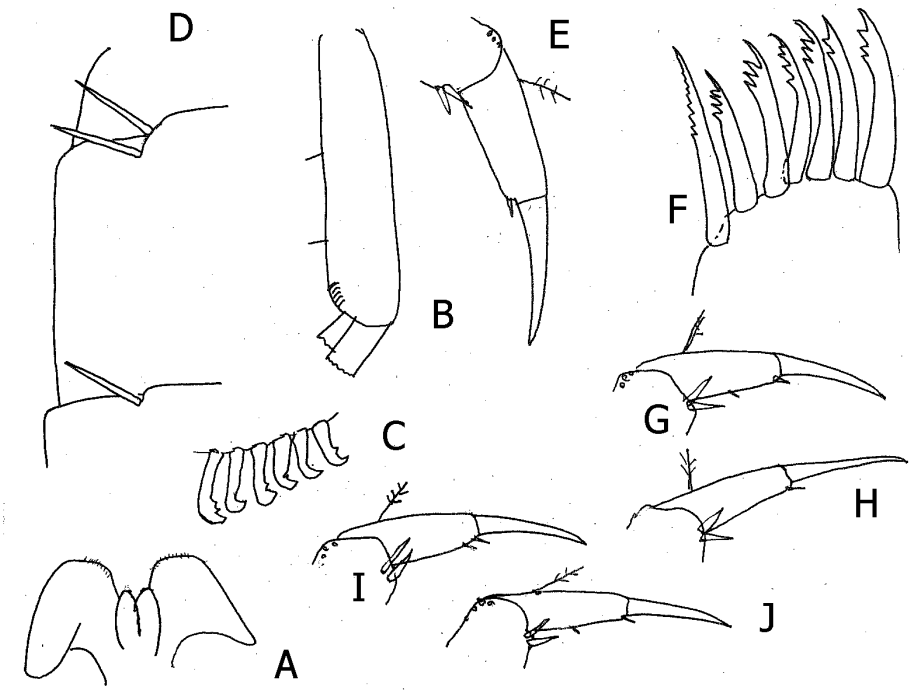


Fig. 6. *Niphargus decui* G. Karaman & Sarbu, 1995, Shabla, Bulgaria, female 7.0 mm: A= labium; B= pleopod 3; C= retinacula of pleopod 3; D= urosomites 1-2; E= pereopod 3 dactylus;
**Female 6.0 mm (paratype**), Vama Veche, Romania: F= maxilla 1 outer plate; G-H= left and right pereopod 3 dactylus; I-J= left and right pereopod 4-dactylus.

Gnathopod 2 distinctly larger than gnathopod 1: article 2 with row of shorter setae along anterior margin and numerous long setae along posterior margin; article 3 at posterior margin with distal bunch of 3 setae; article 5 almost as long as propodus, anterior margin with one median seta and distal bunch of setae, posterior margin with numerous setae (fig. 2D). Propodus trapezoid, rather longer than broad (ratio: 85:80), at posterior margin with 8 transverse rows of setae (fig. 2E); palm straight in the middle and convex at corner, defined on outer face by corner S-spine accompanied laterally by 2 serrate L-spines and 2 subcorner facial M-setae, on inner face with one subcorner R-spine (fig. 2F). Dactylus reaching posterior margin of propodus, at outer margin with one median seta, along inner margin with row of short submarginal setae (fig. 2E).

Pereopod 3 moderately slender, article 2 with several setae at anterior margin and row of long setae along posterior margin, setae diminishing towards distal part of article itself; article 3 with 2 distal posterior setae. Articles 4-6 of different length (ratio: 56:37:45); article 4 with 3-4 setae at anterior margin and

6-8 stronger setae along posterior margin; article 5 with 2 anterior marginal setae and 4-5 stronger setae along posterior margin (setae usually not exceeding diameter of article); article 6 with 3 single anterior marginal setae, and 6 short slender single spines at posterior margin (fig. 3A); dactylus slender, much shorter than article 6 (ratio: 25:45), at inner margin with 2 single slender spines, at outer margin with one median plumose seta; nail long, but rather shorter than pedestal (ratio: 35:40) (fig. 3B).

Pereopod 4 rather similar to pereopod 3: article 2 at anterior margin with 3 proximal long and 3 short setae in distal part, posterior margin with long setae; article 3 with distoposterior group of 2 setae. Articles 4-6 of different length (ratio: 47:37:44); article 4 along both margins with several short single setae; article 5 at anterior margin with one median and 2 distal short setae, at posterior margin with 4 single slender spines; article 6 at anterior margin with 2-3 short setae, along posterior margin with row of 5 single short spines (fig. 3C). Dactylus slender, shorter than article 6 (ratio: 33:44), at inner margin with 2 single slender spines, at outer margin with one median plumose seta (fig. 3D); nail shorter than pedestal (ratio: 33:38).

Pereopods 5-6 relatively short. Pereopod 5: article 2 very large, ovoid (ratio: 84:63), with well developed ventroposterior lobe; anterior margin strongly convex but not produced, bearing row of 11-12 short spines; posterior margin strongly convex, provided with row of nearly 19 short setae (fig. 4A); article 3 short, with 2 distal spines. Articles 4-6 of poorly different length (ratio: 46:44:47), bearing at both margins short and long slender spines (the longest spines are remarkably longer than diameter of article itself). Article 2 remarkably longer than article 6 (ratio: 84:47). Dactylus slender, remarkably shorter than article 6 (ratio: 19:47), at inner margin with one slender spine near basis of the nail, at outer margin with one median short plumose seta (fig. 4B), nail shorter than pedestal (ratio: 30:55).

Pereopod 6 distinctly longer than pereopod 5; article 2 ovoid, large, rather longer than broad (ratio: 96:72), ventroposterior lobe well developed (fig. 4C), anterior strongly convex margin with row of 10 slender spines, posterior remarkably convex margin with 16 short setae. Article 3 short, with distoanterior long spine-like seta and short seta. Articles 4-6 of unequal length (ratio: 52: 63:71), article 4 along anterior and posterior margin with single slender spines not exceeding width of article itself. Articles 5-6 along anterior and posterior margin with several slender spines of unequal length (the longest spines exceeding width of article itself, especially on article 7). Article 2 is remarkably longer than article 6 (ratio: 96:71). Dactylus slender, shorter than article 6 (ratio: 30:71), at inner margin with one slender spine near basis of the nail, at outer margin with one median short plumose seta (fig. 4D); nail shorter than pedestal (ratio: 32:73).

Pereopod 7 missing, and described here of **female 6.9 mm**: Article 2 very large and ovoid, longer than broad (ratio: 90:71), with well developed ventroposterior lobe, anterior convex margin with 7-8 spines, posterior strongly convex margin with 16 short setae (fig. 4E, F). Articles 4-6 of different length

(ratio: 47:55:81), along both margins with spines shorter or longer than diameter of articles themselves. Article 2 only rather longer than article 6 (ratio: 90:81). Dactylus shorter than article 6 (ratio: 30:81), at inner margin with one slender spine near basis of the nail, at outer margin with one median plumose seta (fig. 4G); nail shorter than pedestal (ratio: 57:30).

Pleopods 1-3 peduncle almost naked, that of pleopod 3 with 2 posterior marginal short simple setae (fig. 6B). Peduncle of pleopod 1 with 4 retinacula; that of pleopod 2 with 5 retinacula; peduncle of pleopod 3 with 6 retinacula (fig. 6C), at posterior margin with 2 short single setae.

Uropod 1: peduncle longer than rami, with dorsoexternal row of strong spines ~~and~~ dorsointernal row 1-2 spine-like setae and distal strong spine. Outer ramus as long as inner one, with 3 lateral and 4 distal spines, including 4 short simple setae at outer margin (fig. 5E); inner ramus with 2 lateral and 4 distal spines.

Uropod 2: peduncle rather shorter than inner ramus, bearing 4 spines; outer ramus shorter than inner ramus, bearing one lateral short simple seta and 4 distal spines;. inner ramus with 2 lateral and 4 distal spines (fig. 5F);

Uropod 3 short and strong, peduncle rather longer than broad (ratio: 40:25); inner ramus short, scale-like, with 2 distal slender spines. Outer ramus 2-articulate: first article along outer margin with 6 groups of spines, along inner (mesial) margin attached 6 single or paired spines mixed with single longer plumose setae (fig. 5G); second article shorter than width of first article proximal part, bearing 1-2 distal short simple setae.

Telson rather longer than broad (ratio: 85: 73), deeply incised, lobes tapering distally, with 6-7 distal spines each, along outer margin with 3 groups of spines (1-2-1), along inner margin with 0-1 short spine; facial spines absent; a pair of short plumose setae appears near the middle of outer margin (fig. 3F).

Coxal gills moderately large, on gnathopod 2 and pereopods 3-4 not exceeding distal margin of corresponding pereopod (figs.2D, 3A,C); gills on pereopod 5 and 6 remarkably shorter (fig. 4A, C).

Oostegites large, with short marginal setae (figs. 2D; 3C).

**MALES** from Shabla unknown.

**VARIABILITY (female only).**

**Female 6.9 mm:** Urosomal segment 1 with one dorsolateral spine, urosomal segment 2 with 2 strong dorsolateral spines on each side, urosomal segment 3 naked.

Telson lobes with 4-5 distal and 1-2 outer marginal spines (fig. 5H), mesial marginal and facial spines absent. Maxilla 1 like that in female of 7.0 mm, with 5 spines on outer plate bearing one lateral spine only, one spine with 2 teeth, inner spine finely serrate.

Epimeral plates 2 and 3 with 4 subventral spines, epimeral plate 3 slightly more pointed (fig. 5D). Pleopods 1-3 with elevated number of retinacula like that

in female of 7 mm. Peduncle of pleopod 3 with 3 short posterior marginal setae. Urosomal segment 1 with one dorsolateral spine, urosomal segment 2 with 2-3 dorsolateral spines on each side.

Supplementary short seta at inner margin of pereopods 3-4 dactylus absent (fig. 6E). Dactylus of pereopods 5-7 always without additional spine-like seta or spine.

The presence of that additional small spine-like seta on pereopods 3-4 was overlooked in previous description of this species from Romania, because is present on only nearly 10 % of specimens in both localities.

Oostegites in **female of 6.8 mm** are with long marginal setae.

**Female of 6 mm** (Vama Veche): Dactylus of pereopod 3 with 1-2 spine-like setae; both dactyls of pereopod 4 were with 2-spine-like setae (fig. 6G, H, I, J).

The specimens from Shabla are morphologically quite similar to these from Vama Veche except the different number of lateral teeth on maxilla 1 outer plate (fig. 6F); based on this difference only, we consider specimens from Shabla identical with these of Vama Veche (Romania).

**LOCUS TYPICUS:** Vama Veche, village, 10 km south of Mangalia, Romania.

**DISTRIBUTION:** Romania; Bulgaria (new)

**REMARKS AND AFFINITY**

In Bulgaria were known two species with additional spines on dactylus of pereopods, *Niphargus valachicus* Dobreanu & Manolache 1933 [loc. typ.: Bucharest, Romania], and *Niphargus bulgaricus* Andreev 2001 [loc. typ.: surface waters from the Bolata Marsh, Bulgaria, mentioned later (Vidinova et al., 2016) from the subterranean waters of Shabla Lake). Both species are characterized by presence of several spines on inner margin of dactylus in all pereopods, strongly pointed epimeral plates and large strongly inclined propodus of gnathopods 1-2 provided with several median setae along outer margin of dactylus.

Several *Niphargus* species from Bulgaria are with only one external median seta on dactylus of gnathopods 1-2 propodus, like *N. decui*:

*N. cvetkovi* Kenderov & Andreev 2015 [loc. typ.: water source "Cheshma Gorgoritsa" near the village Novi Han, E. of Sofia, Bulgaria] characterized by elevated number of retinacula and equal rami of uropod 1 in female, like these in *N. decui*, but provided with narrowed article 2 of pereopods 5-7, short coxae 1-4 and absence of ventroposterior lobe on coxa 4, not "*kochianus"* shape of propodus of gnathopods 1-2, etc.

*N. georgievi* S. Karaman & G. Karaman, 1959 [loc. typ.: 0urouchka peštera Cave near village Krochouna, Lovetsch Mt., Bulgaria], species with elevated number of retinacula, telson and uropod 1 like that in *N. decui*, but provided with narrowed article 2 of pereopods 5-7, strongly inclined palm of gnathopods 1-2 propodus, etc.

*Niphargus melticensis* Dancau & Andreev 1973 [loc. typ.: well in Sokolovo, Lowetch district, Bulgaria] with slender dactylus of pereopods, equal rami of uropod 1 in female, coxa 4 with ventroposterior lobe, article 2 of pereopod 7 broad, lobed, short uropod 3 similar to these in *N. decui*, but differs by 2 retinacula only, strongly narrowed propodus of gnathopods 1-2 and elongated article 5 of gnathopod 2.

*N. pecarensis pecarensis* S. Karaman & G. Karaman 1059 [loc. typ.: Pečara Dupka Cave near Belogradčik, Bulgaria) with equal rami of uropod 1 and elevated number of retinacula, differs by surrounded epimeral plates 1-3, short unlobed coxa 4, absence of lateral spines on telson, narrowed article 2 of pereopods 5-7, etc.

Very large ovoid article 2 of pereopods 5-7 is present in some species from subterranean waters of SW part of Balkan peninsula: *Niphargus asper* G. Karaman 1972 [loc. typ.: wells in Podgorica, Montenegro], *Niphargus numerus* G. Karaman & Sket 1990 [loc. typ. Vjetrenica Cave in Popovo polje, Bosnia and Herzegovina], *N. factor* G. Karaman & Sket 1990 [loc. typ.: cave Čavlinska pećina near Obrovac, Croatia], *N. brevirostris* Sket 1971 [loc. typ.: Ličko Lešće in Lika, Croatia], *Niphargus cymbalus* G. Karaman 2017a (loc. typ.: Glikorizo, Arta, Greece), *Niphargus fautor* G. Karaman 2017b [loc. typ.: Glikorizo, Arta, Greece), but all these species differs distinctly from *N. decui* by presence of 2 retinacula on pleopods 1-3.

In Romania are known various species with more or less broad lobed article 2 of pereopods 5-7, but never as large as that in *N. decui*. Many of these species are not described in detail to recognize clearly their taxonomical relations with other *Niphargus* species from Balkan (Carausu et al., 1955).

The broad article 2 of pereopods 5-7 indicated living of these animals in slowly running or not running subterranean waters.

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# CLIMATE INFLUENCE AND ESSENTIAL OILS COMPOSITION OF SALVIA OFFICINALIS IN POPULATIONS OF SOUTHERN ALBANIA

## SUMMARY

*Salvia officinalis* L. is an important medicinal and aromatic plant species belonging to Lamiaceae family, largely used in folk medicine and in culinary. This plant species is growing mainly wild in the mountain areas of Albania where the population's essential oils composition is affected by environmental factors and weather conditions. The aim of the present study is to provide data on the EO composition of the *S. officinalis* in Southern Albania, and on the influence of climatic conditions on foreground temperature and precipitations in a selected location on the variation of the EO components. The essential oils were extracted by hydro-distillation and analyzed by gas chromatography (GC-FID). Qualitative and quantitative variation in composition of essential oil was analyzed on yearly basis for five consecutive years. In total, 20 main compounds were identified representing 95.1% to 98.9% of the total EO. Monoterpenes were found to be the main group of components ranging from 87.8% to 95.5% of total EO with the oxygenated monoterpenes as the most abundant compounds. The chemical profile of *S. officinalis* grown wild in mountain area in Southern Albania was alpha-Thujone (29.9%) > Camphor (21.7%) > Cineole (12.1 %) > Camphene (7.9 %) > beta-Thujone (5.4%) > alpha-Pinene (4.6%) > alfa-Humulene (2.6%) > beta-Caryophyllene (2.5%). The temperature was positively correlated with, sesquiterpenes and negatively correlated with bicyclic monoterpenes, while the opposite was observed for precipitation. The ordination analysis results PCA explained 93% of total variance, camphene, camphor, alpha-pinene, cineole and beta-thujone were the most variable components among analyzed years.

**Keywords**: *Salvia officinalis*, essential oils, precipitation, temperature

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## INTRODUCTION

*Salvia officinalis* L. or sage is an outstanding member of Lamiaceae family, native to Mediterranean Basin. It is widely used in folk medicine, as culinary herb, in aromatherapy and food industry. Essential oils of this species have important biological activities such as antioxidant (Abdelkader *et al*., 2014) antimicrobial (Kačániová *et al*., 2021), antiviral, anti-inflamatory, anticancer, antidementia, hypoglycemic and hypolipidemic, etc (Ghorbani and Esmaeilizade, 2017). Different chemotypes of sage show big differences in a distinct compound, there are reported about 18 sage chemotypes (Schmiderer *et al.,* 2013). The use of the EO derived from medicinal plants depends on components availability, therefore the identification of chemotypes is rather important, also considering the increased demand for unique EO producing cultivars (Rathore *et al*., 2022).

There are several factors affecting chemical variability in plants including ecological conditions, development stage of the plant, geographical and climatic variation as well as genetic diversity (Figueiredo *et al*, 2008). Earlier studies reported variation of EOs compositions of sage populations grown in different locations and ecological conditions in Albania (Schmiderer *et al.,* 2013, Nuro *et al*., 2017, Papa *et al*., 2017), in different harvesting season (Hasa *et al*., 2021).

The cultivation of medicinal and aromatic plants has a long tradition in the agro-ecological conditions of Europe and Mediterranean area (Petrović *et al*., 2022). It was proved that several ecological factors such as phosphorus, potassium, organic matter, temperature and moisture are positively correlated with herbaceous species richness and play an important role in organic sage cultivation (Solomou *et al*., 2020). In addition, it is well known that the composition of essential oils and concentration of volatile compounds in Salvia sp. depends not only on genetic and seasonal factors but also on environmental conditions (Ramezani *et al*., 2020), although with the accelerating climate change this can potentially be problematic for the future production and use of these oils in the pharmaceutical and food industry (Karalija *et al.,* 2022). There are limited reports on the variation of chemical oil composition of medicinal plants under different climatic conditions. These studies demonstrate that the temperature and precipitation variation affected the essential oil yields and essential oil chemical composition in medicinal and aromatic plants as lavandin (Liao *et al*., 2021; Acimovic *et al*., 2022), chamomile (Gosztola *et al*., 2010) and sage (Radwan *et al.*, 2017).

Sage grows mostly wild in mountain areas in Albania, but several initiatives to cultivate it have recently taken place due to the increased demand for its essential oil (EO). Thus, the knowledge on the quality of EOs of sage populations and the effect of microclimatic conditions on their chemical composition, would be relevant for the sage cultivation initiatives and other related industries in the country. Therefore, the current study aims to study chemical polymorphism of natural populations of *S. officinalis* in Southern Albania, and to investigate the influence of climatic conditions on foreground

temperature and precipitation in the selected locations on the variation of the EO components.

## MATERIAL AND METHODS

**Sampling sites and plant material.** The areal parts of *Salvia officinalis* plants were collected from four populations grown wild in mountain area in Southern Albania year to year for five consecutive years 2017-2021 in the mid-June. The plant material was air dried in the dark and used to extract essential oils. The geographical position and localities of each sampling site are given in the table 1. In addition, key meteorological data as the average temperature and precipitation of the sample collection region was obtained by the database of IGJEUM (2017-2021). The minimum, maximum and the mean values of temperatures and rainfall registered in the sampling region was given in the Table 2 and Figure 1.

**The essential oil isolation.** The essential oils of the sampled wild sage populations were extracted from 50g air dried plant material using hydro-distillation using Clevenger type apparatus for 4 hours. Toluene (2 ml) was then added to the balloon for isolation of the essential oils, which were then dried over anhydrous sodium-sulphate ($Na_2SO_4$) and stored in sealed amber glass vials at +8$^oC$ prior further analysis.

**Table 1.** Sampling collection sites geographical coordinates

| Code | Latitude | Longitude |
|---|---|---|
| Sample 1-S1 | $40^0$ 33'11.34" | $20^0$7'51.608" |
| Sample 2-S2 | $40^0$15'7.351" | $20^0$58'8.166" |
| Sample 3-S3 | $40^0$35'35.32" | $20^0$10'6.852" |
| Sample 4-S4 | $40^0$17'8.375" | $20^0$35'27.8" |

**Analytical Gas chromatography analysis.** The analytical gas chromatographic analyses of isolated essential oils of sage were carried out on GC V 450 instrument equipped with a flame ionization detector and split-splitless injector, attached to aVF-1ms capillary column (30 m x 0.33 mm x 0.25 μm). The applied injector temperature was 280$^oC$ and FID temperature was held at 300°C. The essential oil (1 ul) diluted in Toluene was injected in split mode 1:50. The nitrogen was used as carrier (1 ml/min) and carrier gas flow rate was 25 ml/min. Hydrogen and air were flame detector gases with 30 ml/min and 300 ml/min, respectively. The oven temperature was programmed as follows: 40°C (held for 2 minutes) to 150$^oC$ (with 4°C/min), after that to 280°C with 10°C/min and held for 7 minutes. The identification of the essential oils compounds was based on the comparison of their Kovats indices (KI), their retention times (RT) to the data already available (Adams, 1995; David *et al*., 2010, Konig *et al*., 1999). A mixture of n-alkanes from n-octane ($C_8$) to eicosanes ($C_{20}$) was used in KI

calculation. The identified components were subjected to principal component analysis (PCA) to analyze the variability in each analyzed year, temperature, and rainfall levels.

## RESULTS AND DISCUSSION

The analysis of *S. officinalis* essential oil composition was carried out in five successive years. There were observed a difference of +1.2°C in the mean temperatures of the analyzed years, they ranged from 15.9°C (year 2020) to 17.5°C (year 2019). While it was observed great variation in the minimal temperature values, ranging from 0°C (year 2018) to 8°C (year 2021). Comparing rainfall among years, in the years 2018 and 2021 had significantly higher mean rainfall values compared to other years with mean values of 122.2 mm and 92 mm, respectively whereas in the year 2020 are recorded the lowest mean values of rainfall with 57.5 mm. While high variation resulted for the minimal rainfall values that ranged from 0 mm in the year 2017 to 10 mm in the year 2020.

In the June, the month when the collection of samples was carried out, the temperatures varied from 17°C (year 2020) to 26°C (year 2017), while the average rainfall ranged from 10 mm to 110 mm for the years 2017 and 2018, respectively (Table 2 and Figure 1).

**Table 2.** Yearly mean, minimal and maximal temperatures, and rainfall values in the sampling region for the period 2017-2021

| | 2017 | 2018 | 2019 | 2020 | 2021 |
|---|---|---|---|---|---|
| **Mean Temperature (°C)** | **16.3** | **16.7** | **17.5** | **15.9** | **17.1** |
| Min temp | 3 | 0 | 3 | 6 | 8 |
| Max temp | 30 | 36 | 29 | 26 | 27 |
| **Mean temp- June** | **26** | **25** | **26** | **17** | **23** |
| **Mean rainfall (mm)** | **79.3** | **122.2** | **78.2** | **57.5** | **92** |
| Min rainfall | 0 | 9 | 4 | 10 | 0 |
| Max rainfall | 400 | 233 | 260 | 170 | 400 |
| **Mean rainfall-June** | **10** | **110** | **50** | **20** | **30** |

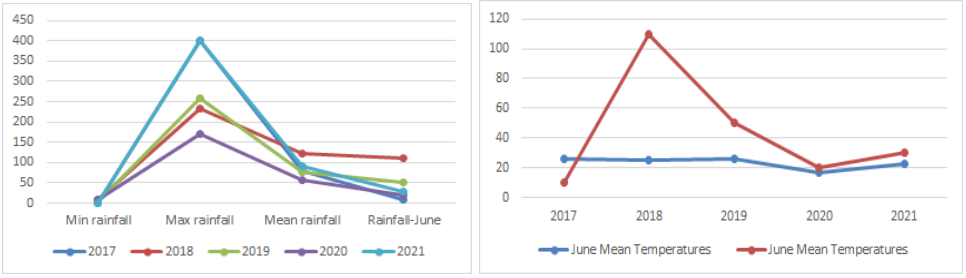


**Figure 1.** The mean values of temperatures and rainfall in June during five successive years, 2017-2021.

**Essential oils components**. The overall GC/FID analysis of EOs identified in total 120 different components. This study focused on the assessment of the variation of 20 most abundant components, which represented 95.1% to 98.9% of total yield of EOs. The peaks lower than 0.05% were not included into our analysis. The mean values (in percentage) of the main chemical components obtained from year-to-year analysis of the sampled population were given in the table 3.

Qualitative and quantitative assessment of EOs composition of all sample set calculated as the mean values of each of the components identified in five analyzed years suggested that the chemical profile of *S. officinalis* grown wild in mountain area in Southern Albania was: alpha-Thujone (29.9%) > Camphor (21.7%) > Cineole (12.1 %) > Camphene (7.9 %) > beta-Thujone (5.4%) > alpha-Pinene (4.6%) > alfa-Humulene (2.6%) > beta-Caryophyllene (2.5%) (Figure 2) Compared to the chemical profiles reported in previous studies for sage grown in other regions in Albania (Nuro *et al*., 2017), the sage populations under study had lower concentration of alpha and beta-Thujone and higher levels of Cineole and Camphor, the same was observed for other components as bicyclic monoterpenes. These differences suggests that populations in Southern Albania might be a different chemotype. In addition, the differences in chemical composition and components concentration of sage grown in Southern Albania compared to EOs composition of sage grown in other regions might be the reason of preferential and wider use on the folk medicine of sage grown in Southern region. Comparative analysis of our data with the reported chemical profiles of sage grown in other Balkan and Mediterranean countries (Khedher *et al.*, 2017; Oniga *et al*., 2010; Damyanova *et al*., 2016; Miguel *et al*., 2011; Bernotiene *et al*., 2007; Awen *et al.*, 2011; Perry *et al.*, 1999) did not show significant differences.

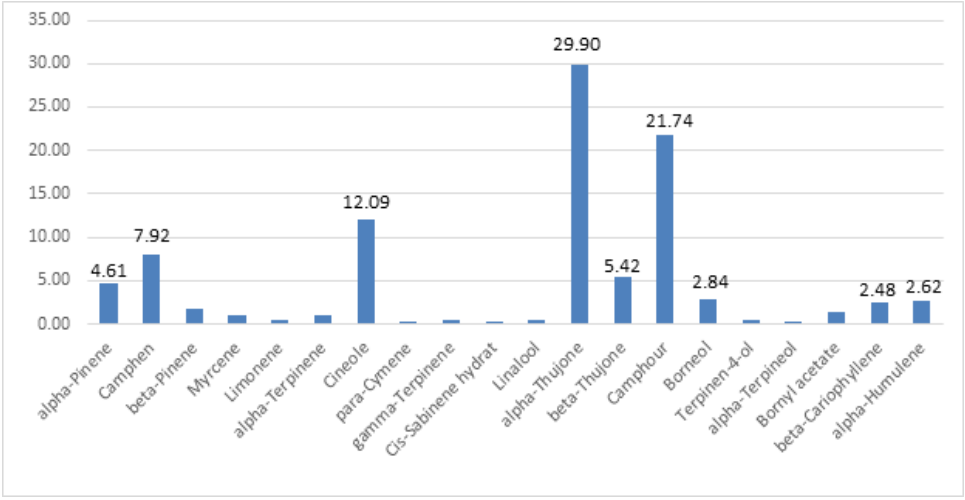


**Figure 2.** Profile of main compounds for *Salvia officinalis* from Southern Albania

The essential oil composition varies within a country depending on the microclimate conditions suggesting the significance of tracking the climate-related conditions and their effect on plants (Karalija *et al*., 2022). Thus, the analysis of essential oil components of four natural populations of *Salvia officinalis* was carried out for five consecutive years 2017-2021 in order to investigate the variation of Essential Oils (EOs) chemical components in relation of the temperature and precipitation variations. Some components such as: alpha-Thujone, beta-Thujone, Camphor, Cineole, and Camphene were present in all analyzed samples independently from the climate changes. As it was shown in the Figure 3, these components are found in the first line of chromatogram, due to their low boiling points. In addition, the characteristic smell of sage is attributed to the presence of these chemical components.

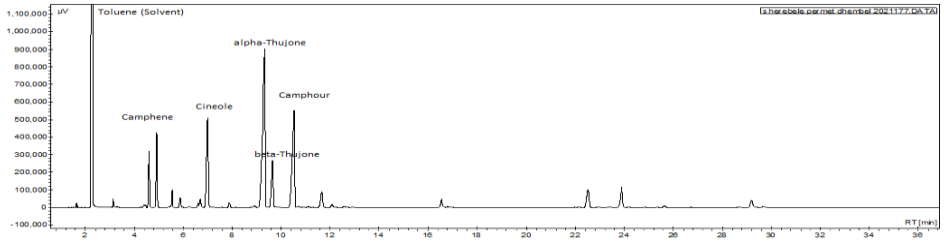


**Figure 3.** Chromatogram of *Salvia officinalis* essential oil (year 2021)

The results showed that the EOs of sage populations collected in mountain area of Southern Albania (Table 3) presented two major chemical groups of terpenes: monoterpenes and sesquiterpenes ranging from 87.8% (year 2018) to 95.5% (year 2019) and 3.3% (year 2019) to 7.25% (year 2018), respectively.

The monoterpenes fraction comprised the oxygenated monoterpenes (72.3 – 75.7%) > bicyclic monoterpenes (12.1 – 16.6%) > sesquiterpenes (3.3 – 7.3%) > monocyclic monoterpenes (1.3 – 2.8%) > aliphatic monoterpenes (1.0 – 1.2%) > aromatic monoterpenes (0.1 – 0.3%).

Oxygenated monoterpenes such as alpha and beta-Thujones, Camphor, Cineole, Borneol, Bornyl acetate, Linalool, Terpinen-4-ol and alpha-Terpineol were found in all samples. The highest level of these components in sage EO (75%), was observed in the EOs sampled in the years 2017, 2019 and 2021. These components were in lower concentration in the EOs of samples collected in the years 2018 and 2020, where they constitute 73.2% and 72.4%, respectively. Nevertheless, no significant variations were observed for the concentration of oxygenated monoterpenes through the analyzed samples. Similarly, other studies on lavandin reported that differences in climate conditions through years have not impacted hydrolate composition variations (Acimovic *et al*., 2022)

Bicyclic monoterpenes as alpha and beta-Pinene, Camphene and cis-Sabinene hydrate, represented the second major group of compounds identified in our sample set. This group of compounds was found in higher amount in the samples collected in the year 2019 (16.2%) and in the year 2020 (16.6%),

whereas the lowest concentration of bicyclic monoterpenes was obtained in the samples collected in the years 2018 (12.1%) and 2021 (12.5%), in which the level of precipitations was higher than in the other years. Being highly volatile, the level of bicyclic monoterpenes concentration in EO is affected by both the temperature variation and level of precipitation.

**Table 3.** Variation of chemical composition (%) of essential oil of *Salvia officinalis* populations, average values per year

| | Rt | Year 2017 | Year 2018 | Year 2019 | Year 2020 | Year 2021 |
|---|---|---|---|---|---|---|
| **alpha-Pinene** | 4.32 | **5.52 ± 1.25** | **3.65 ± 1.66** | **4.81 ± 1.33** | **4.82 ± 0.92** | **4.23 ± 0.87** |
| **Camphene** | 4.41 | **7.40 ± 2.24** | **7.02 ± 1.14** | **9.58 ± 1.73** | **9.57 ± 1.37** | **6.01 ± 0.44** |
| beta-Pinene | 5.22 | 2.10 ± 0.48 | 1.30 ± 0.43 | 1.65 ± 0.71 | 2.13 ± 0.44 | 1.74 ± 0.53 |
| Myrcene | 5.34 | 1.20 ± 0.33 | 0.97 ± 0.31 | 1.10 ± 0.40 | 1.06 ± 0.32 | 0.95 ± 0.28 |
| Limonene | 6.41 | 0.13 ± 0.04 | 0.30 ± 0.09 | 1.21 ± 0.49 | 0.28 ± 0.05 | 0.26 ± 0.04 |
| **α-Terpinene** | 6.47 | **0.81 ± 0.27** | **0.73 ± 0.24** | **1.04 ± 0.31** | **1.50 ± 2.17** | **0.95 ± 0.19** |
| **Cineole** | 6.73 | **12.90 ± 4.26** | **11.51±3.45** | **12.42± 3.49** | **12.13 ± 2.49** | **11.48 ± 3.22** |
| para-Cymene | 7.33 | 0.10 ± 0.49 | 0.10 ± 0.04 | 0.11 ± 0.03 | 0.33 ± 0.82 | 0.26 ± 0.08 |
| gamma-Terpinene | 7.98 | 0.32 ± 0.67 | 0.41 ± 0.06 | 0.33 ± 0.05 | 0.98 ± 0.04 | 0.07 ± 0.02 |
| Cis-Sabinene hydrate | 8.13 | 0.10 ± 0.03 | 0.11 ± 0.03 | 0.14 ± 0.04 | 0.11 ± 0.03 | 0.47 ± 0.09 |
| Linalool | 8.44 | 0.25 ± 0.06 | 0.25 ± 0.9 | 0.21 ± 0.06 | 0.17 ± 0.05 | 0.99 ± 0.04 |
| **α-Thujone** | 9.12 | **29.92 ± 4.22** | **31.37±3.64** | **26.7 ± 3.29** | **27.27 ± 4.38** | **34.21 ± 4.37** |
| **β-Thujone** | 9.21 | **5.64 ± 0.93** | **5.58 ± 1.07** | **5.71 ± 1.66** | **4.28 ± 1.22** | **5.87 ± 1.64** |
| **Camphor** | 10.52 | **22.50 ± 2.16** | **19.53±4.36** | **25.30± 3.82** | **24.13 ± 3.47** | **17.24 ± 1.43** |
| **Borneol** | 11.78 | **2.10 ± 0.71** | **2.73 ± 0.84** | **3.14 ± 0.44** | **2.35 ± 0.72** | **3.86 ± 1.43** |
| Terpinen-4-ol | 12.21 | 0.52 ± 0.06 | 0.51 ± 0.14 | 0.51 ± 0.14 | 0.48 ± 0.18 | 0.39 ± 0.09 |
| α-Terpineol | 14.43 | 0.10 ± 0.03 | 0.22 ± 0.06 | 0.12 ± 0.02 | 0.20 ± 0.05 | 0.20 ± 0.04 |
| **Bornyl acetate** | 16.95 | **1.20 ± 0.33** | **1.52 ± 0.42** | **1.41 ± 0.37** | **1.33 ± 0.55** | **1.50 ± 0.81** |
| **β-Caryophyllene** | 22.44 | **2.44 ± 0.83** | **1.94 ± 0.84** | **1.61 ± 0.59** | **3.24 ± 0.70** | **2.16 ± 1.31** |
| **α-Humulene** | 24.65 | **2.72 ± 0.91** | **2.30 ± 0.82** | **1.71 ± 1.43** | **1.14 ± 1.53** | **3.19 ± 2.42** |
| **Total** | | **97.98** | **95.07** | **98.85** | **97.51** | **96.03** |
| Σ Monoterpene | | 92.82 | 87.82 | 95.52 | 93.12 | 90.68 |
| Σ Monocyclic monoterpenes | | 1.26 | 1.45 | 2.58 | 2.76 | 1.28 |
| Σ Bicyclic monoterpenes | | 15.12 | 12.08 | 16.18 | 16.63 | 12.45 |
| Σ Aliphatic monoterpenes | | 1.20 | 0.97 | 1.11 | 1.06 | 0.95 |
| Σ Oxygenated monoterpenes | | 75.14 | 73.22 | 75.54 | 72.34 | 75.74 |
| Σ Aromatic monoterpenes | | 0.11 | 0.12 | 0.11 | 0.33 | 0.26 |
| Σ Sesquiterpenes | | 5.16 | 7.25 | 3.33 | 4.38 | 5.35 |

The maximum values of temperature variation impact was also observed in sesquiterpenes, which have high boiling points. At the year 2018, when the highest maximal temperature and mean precipitation were registered, the

sesquiterpenes had high concentration. Sesquiterpenes (beta-Caryophyllene and alpha-Humulene) highest concentration was obtained from samples of the year 2018 (7.3%) and in lowest percentage was obtained in the samples collected in the 2019 (3.3%) (Table 3) The ratio of monoterpenes and sesqiuterpenes fractions in essential oils changes with the variation of the temperature (Usano-Alemany *et al.*, 2014).

Monocyclic monoterpenes (alpha and gamma-Terpinene and Limonene) showed remarkable differences concentrations in EOs which resulted higher in the years 2019 (2.6%) and 2020 (2.8%) compared to the years 2017 and 2021 (1.3%). The concentration of Aliphatic monoterpenes (Myrcene) and aromatic monoterpenes (para-Cymene) were very low (≤ 1). The total amount of alpha-Thujone and beta-Thujone in our samples varied from 31.6% (year 2020) to 40.1% (year 2021). Whereas Cineole varied from 11.5% (year 2018, 2021) to 12.9% (year 2017). Camphor lowest concentrations were observed in the years 2018 and 2021 with 19.5% and 17.2%, respectively while the highest camphor concentration was observed in samples collected in the year 2019 with a value of 25.3%. Results suggested that camphor is affected by level of precipitation, it was in higher amount in the EO collected in years with lower levels of rainfall. The results are in line with the Radwan *et al*., (2017), that reported an increase of the camphor concentration and of the total monoterpenes in sage under drought stress. However, several compounds did not correlate with the temperature and precipitation in neither their yearly mean values nor with the mean weather conditions recorded on June, period when samples were collected.

**Principal Component Analysis.** The twenty main identified compounds of sage EO were subjected to principal component analysis (PCA) to analyze their variability in the different analyzed years, in relation with yearly temperatures and rainfall values.

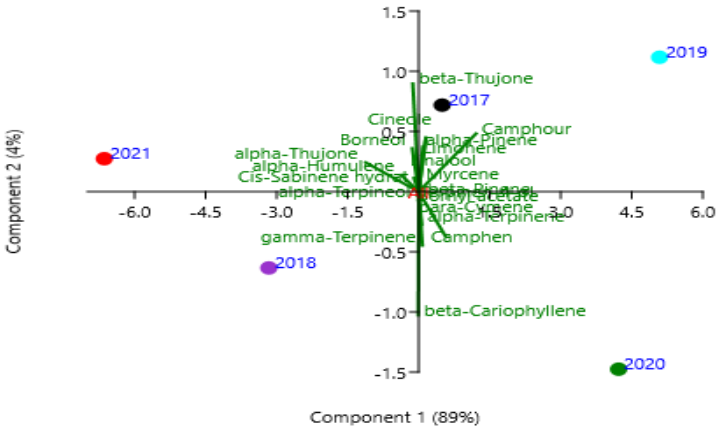


**Figure 4.** Principal component analysis (PCA) of the essential oil composition of S. officinalis variation in five analyzed years

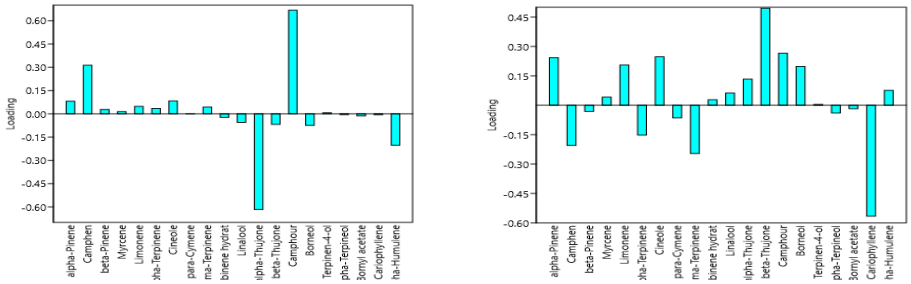


**Figure 5.** Loading plot of principal component analysis PC1 loading plot (left) and PC2 loading plot (right)

The ordination analysis results for two main components (PC1 and PC2) were given in the figure 4, the data points of the variables with similarities were grouped in the same cluster in PCA.

The main two principal components mutually accounted for 93% of total variance. The yearly variation of the essential oil compounds based on the first major principal component (PC1), which accounted for 89% of the total variation. The analysis demonstrated that camphene and camphor had positive loadings (values ≥0.3), whereas alpha-thujone and alpha-humulene had negative loadings (values ≤0.3). This result also is supported by the higher camphor and camphene concentration in the EO collected in the years 2017 and 2019 and 2020, positioned also in the right of the PC1 axis compared with their concentrations in the EOs of years positioned in the left side of the PC1 axis (2018 and 2021) (Table 3, Figure 4). The yearly variation of the essential oil compounds based on the second major principal component (PC2), which accounted for 4% of the total variation, showed positive loading (values ≥0.3) of alpha-pinene, cineole, beta-thujone, and negative loading (values ≤0.3) of gamma-terpinene and beta-caryophyllene (Figure 4 and Figure5). The concentration of alpha-pinene and cineole had highest concentration in the EO of the year 2017 and lowest concentrations in the EOs of the year 2018 in which the maximum values of mean precipitation were recorded (Table 1 and Table 3). The highest concentration of beta-Thujone were obtained in the EO of the year 2019 with higher level of the mean temperature and precipitation compared to the year 2020 in which lowest beta-Thujone concentration were obtained (Table 3), supporting the year differentiation from the PC2 presented in the scatterplot (Figure 4) and indicating that this compound might be influenced by both temperature and rainfall values.

## CONCLUSIONS

This study shows the qualitative and quantitative differences on overall content and relative abundance of compounds of essential oil of *Salvia officinalis* grown wild in Southern Albania and their variation affected by weather conditions such as temperature and precipitation through five successive years. GC/FID analysis of the essential oil of sage in this location contains higher

concentration of alpha-Thujone, beta-Thujone, Camphor, Cineole, and Camphene. The most variable components among analyzed years were camphor, alpha-pinene, cineole and beta-thujone. It was observed that bicyclic monoterpenes and sesquiterpenes were more affected by the climate variations than other groups of compounds. The study provided new insight for the quality of essential oils and the effect of microclimatic conditions on its chemical composition, relevant for the sage cultivation initiatives and industries.

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# CHANGES OF SEVERAL METABOLIC PARAMETERS OF SOYA INOCULATED WITH PHYTOPATHOGENS AT APPLICATION NANOCHELATES

## SUMMARY

In greenhouse experiments changes in several metabolic parameters soybeans plants after inoculation by *P. savastanoi* pv. *glycinea* 9190, *X. axonopodis* pv. *glycines* 9192, BCMV, *A. laidlawii* on background pre-treatment by nanochelates (NHs) of V, Ge, Cu, Mo have been shown. The phenolic compounds content increased in leaves at inoculation by phytopathogens on background pre-treatment by NHs and without it. The content of $H_2O_2$ in leaves increased in variants: BCMV (+VNHs, GeNHs, CuNHs), 9192 (+VNHs, CuNHs), 9190 (+GeNHs, CuNHs), *A. laidlawii*+MoNHs. But it decreased in the variants: BCMV + MoNHs, *A. laidlawii* + VNHs, 9190 + VNHs, 9192 (+ GeNHs, CuNHs) compared to controls. The peroxidase activity increased in variants: *A. laidlawii*, 9190, GeNHs + 9190, GeNHs + 9192, CuNHs, CuNHs (+ phytoplasmas, 9192), MoNHs (+ BCMV, phytoplasmas, 9190, 9192) with decreased catalase activity. The content of ABA in the leaves increased in variants: 9190, 9192 (+ VNHs, CuNHs), GeNHs + 9190, but the IAA content reduced. However, on variants MoNHs (+9190, 9192), GeNHs + 9192, *A. laidlawii*, *A. laidlawii* (+ GeNHs, CuNHs, MoNHs) the content of both phytohormones was decreased. The content of IAA and ABA in BCMV-infected leaves without treatment was increased, but on background of NHs – it decreased

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significantly. The $F_v/F_m$-value increased in almost all variants, except for CuNHs + BCMV. The photosynthetic rate ($R_{Fd}$-value) at inoculation plants with viral, bacterial and phytoplasmas pathogens on background treatment of MoNHs; bacterial strain on background of pre-treatment of GeNHs; phytoplasmas on background of VNHs have been increased. Thus, pre-treatment by nanochelates had regulatory effect on soybean plant metabolism helping increase its resistance to phytopathogens was shown. Thus, pre-treatment by nanochelates had regulatory effect on soybean plant metabolism but the pre-treatment of MoNHs was more effectiveness thanks not only intensification resistance against pathogens but and the assimilation of $CO_2$ increased.

**Keywords**: *Xantomonas axonopodis pv. glycines, Pseudomonas savastanoi pv. glycinea, Acholeplasma laidlawii var. granulum,* BCMV, nanochelates

## INTRODUCTION

Soybeans are one of the most popular oilseeds grown worldwide. Among the world's soybean producers are Brazil, the United States and Argentina (Shahbandeh, 2022).

Factors that negatively affect the yield and quality of soybeans, in addition to risky climate change, are infections with fungi, bacteria, viruses, phytoplasmas and other phytopathogens. In addition, the gradual expansion of sown areas and production of this crop has led to a sharp increase in the number of diseases (Mammadov *et al*., 2018). It should be noted that various phytopathogens effect on plant metabolism by including specific virulence factors. Thus, it is known that effector proteins with enzymatic functions, such as the SUMO XopD protease, play an important role in the interaction of *Xanthomonas* with host plants. In addition, *Xanthomonas* expresses a unique class of type III effectors, including the AvrBs3 family, which simulation plant transcriptional activators and manipulate plant transcripts (Kay & Bonas, 2009; Büttner & Bonas, 2010). AlgU has been shown to play a critical role in the pathogenesis of *Pseudomonas savastanoi* by regulating many virulence factors (Nguyen *et al*., 2021). Meanwhile, the existence of a common regulatory mechanism for different species of bacteria has been reported. Thus, the pathogen *Pseudomonas savastanoi* enters to the host plant through a secretion system of type III, which is regulated by a two-component system RhpRS. This system depends on the mechanisms of phosphorylation and nutrition and can switch between the regulation of virulence and metabolism, which is widespread among different species of bacteria (Xie *et al.*, 2019).

Other harmful pathogens transmitted by insect vectors and have limited metabolism – phytoplasmas. There is a wide range of phytoplasmas, and they cause many symptoms in different host plants, but legumes often have weak symptoms on the leaves and infertility of flowers (Sharman, 2016). Genomic analysis of phytoplasmas isolated from soybean plants with signs of "flower bud deformation" showed the presence of genes such as auxin response factor 9 (ARF9) and the transcription factor domain of the Forkhead family (FHA), which

are directly involved in plant development in various ways such as hormonal regulation, plant morphology, embryogenesis and DNA repair (Kale *et al*., 2016).

Plant viruses are submicroscopic obligate parasites that consist of RNA or DNA particles coated with a protein coat. Their reproduction depends on the cellular mechanism of their hosts and cannot occur outside it (Soybean viruses, 2019; Gergerich & Dolja, 2006). Viral infections also pose a serious threat to the productivity and quality of soybeans. It is known that main passes of plants virus diseases transmitted are insects: soybean aphids, thrips, bean leaf beetle and infected seeds (Soybean viruses, 2019). About twenty-seven viruses are known to be potentially dangerous to plants today. Soybeans often have viruses such as Soybean mosaic virus (SMV), Bean pod mottle virus (BPMV), Alfalfa mosaic virus (AMV), Soybean dwarf virus (SbDV), Soybean vein necrotic virus (SVNV), Tobacco ringspot virus (TRSV), and Tobacco streak virus (TSV) *et al* (Soybean viruses, 2019). Some of these viruses have a wide range of host plants. Yep, Bean common mosaic virus (BCMV) causes significant loss of bean productivity but can also infecting of soybeans (Lee *et al*, 2015; Kyrychenko & Prylipko, 2020). In research by Zhou *et al*. (2014) identified 30 isolates of BCMV that are capable of infecting soybeans. The main tactics for controlling viral infections are natural or artificial resistance to viruses, the use of uninfected seeds, control of weed reservoirs of viruses, control of insect vectors of plant viruses (Hill & Whitham, 2014, Gergerich & Dolja, 2006). Plants induce a multicomponent protective response to pathogenic infection, involving the expression of many protective genes encoding enzymes and protective metabolites. During pathogenic infection, there is a noticeable accumulation of protein associated with pathogenesis (PR), in a place away from the source of infection. At the same time there is an accumulation of salicylic acid and $H_2O_2$ at the site of infection to regulate systemic acquired resistance (SAR) in plants. The multistage process of the plant protection mechanism against phytopathogens includes host cell death, necrosis, accumulation of phenolic compounds, cell wall modification, and synthesis of specific antimicrobial compounds (Chowdhary *et al.*, 2021).

It should be noted that all the above pathogens can significantly inhibit the productivity of soybean plants. However, there are currently no effective chemical measures to control the diseases caused by these pathogens. The treatment of biologically active substances created with the use of nanotechnologies can be modern alternative methods of control of these pathogens. Nanoparticles of Au, Ag, alumina (Al), Se, titanium dioxide ($TiO_2$), silicon dioxide ($SiO_2$), copper oxide (CuO), zinc oxide (ZnO), calcium oxide (CaO) and magnesium oxide (MgO) have a proven antimicrobial action (Karimi, 2019; Filipović *et al*., 2021). Antimicrobial activity of Ag-Cu, complex Co-Cu-Zn-Fe-Mn-Mo-Mg, biological substance of I-Se, and each nano chelates of V and Ge, have been shown (Huliaieva *et al*., 2020). Known that $MoO_3$ nanoparticles has high antimicrobial activity against gram-negative and gram-positive bacteria, *Candida albicans* and *Aspergillus niger* and the ability to remove free radicals

(Fakhri & Nejad, 2016). However, the effect of different nanoparticles on the metabolism of cultivated plants requires in-depth study. Therefore, the aim of our work was to study the effect of pre-sowing treatment with nanochelates Ge, V, Cu, Mo and inoculation of soybean plants with phytopathogenic bacteria, mycoplasmas and viruses on its several metabolic parameters.

## MATERIAL AND METHODS

Experimental soybean plants of the variety Artemida were grown in a greenhouse on the Institute of Microbiology and Virology of NASU of D.K. Zabolotny territory. Before sowing, soybean seeds were treated by soaking in a solution of nanochelates: V (2.25 μg / l), Ge (3.75 μg / l), Cu (4.5 μg / l) and Mo (8 μg / l). Nanoparticles were obtained by erosion-explosive method, producer: "Nanomaterials and Nanotechnologies" society (Ukraine).

In research used pathogens: *Xantomonas axonopodis* pv. *glycines* IMV B-9192 (soyabean bacterial pustule), *Pseudomonas savastanoi pv. glycinea* IMV B-9190 (bacterial blight of soybean), *Acholeplasma laidlawii* var. *granulum* 118 (IMV BM – 34) (pale-green dwarf of cereals), Bean common mosaic virus (BCMV). Artificial inoculation of plants with *A. laidlawii* was conduct by prick of suspension in soybean stem. Artificial inoculation of plants with bacterial pathogen conducted by applying by brush of bacterial suspension on the leaves repeatedly pierced with a needle in 4–6 leaf phase. Bacterial and phytoplasmas strains was obtained from off Ukrainian collection of microorganisms of Institute of Microbiology and Virology of NASU of D.K. Zabolotny. BCMV-inoculation of soybean plants was carried out in the phase of two leaves, by applying a brush on soybean leaves freshly prepared virus-containing material after pre-dusting with carborundum. Sheme of experiments: 1 – Control; 2 – *A.laidlawii* (phytoplasmas) inoculation; 3 – BCMV-inoculation; 4 – *X. axonopodis* pv. *glycines* 9192 inoculation; 5 – *P. savastanoi pv. glycinea* 9190 inoculation; 6 – pre-sowing treatment VNHs; 7 – VNHs+phytoplasmas; 8 – VNHs+BCMV; 9 – VNHs+ *X. axonopodis* pv. *glycines* 9192; 10 – VNHs+ *P. savastanoi pv. glycinea* 9190; 11 – GeNHs; 12 – GeNHs+phytoplasmas; 13 – GeNHs+BCMV; 14 – GeNHs+ *X. axonopodis* pv. *glycines* 9192; 15 – GeNHs+ *P. savastanoi pv. glycinea* 9190; 16 – CuNHs; 17 – CuNHs+phytoplasmas; 18 – CuNHs+BCMV; 19 – CuNHs+ *X. axonopodis* pv. *glycines* 9192; 20 – CuNHs + *P. savastanoi pv. glycinea* 9190; 21 – MoNHs; 22 – MoNHs+phytoplasmas; 23 – MoNHs+BCMV; 24 – MoNHs+ *X. axonopodis* pv. *glycines* 9192; 25 – MoNHs+ *P. savastanoi* pv. *glycinea* 9190. Repetition in the experiment triple.

The content of phytohormones – IAA and ABA were determined by quantitative Spectro densitometric thin layer chromatography (Biliavska *et al.*, 2017). Sampling of plant material (leaves) was performed 12 days after inoculation with phytoplasmas and BCMV and 6 days after inoculation by bacterial strains 9091 and 9192. Plant specimens of common bean with symptoms BCMV were tested by RT-PCR for the presence of BCMV. Total RNA was extracted from 0.5 g of leaf tissue by a previously reported method. The RT was

performed using Reverta kits (AmpliSens), according to the manufacturer's instruction. The work used primers that allow to identify a fragment of the nucleotide sequence encoding the protein gene of the envelope with the following nucleotide sequences: direct primer 5'-ttcggacgtcgtgagtgtta-3 'and reverse 5'-cccgagtccacattaattcc-3', the size of the amplification product was 391 bp (Kyrychenko & Kovalenko, 2018; Kyrychenko *et al*., 2019; Kyrychenko & Prylipko, 2020). For PCR, 2 μL of the template genomic DNA was amplified in a 25-μL of total volume containing 1 × reaction buffer, 5 pmol of each primer, 0.3 mM dNTPs, 1.25 U of TaqDNA polymerase and nuclease-free water. Reactions were performed under the following conditions: 3 min denaturation at 95°C, thermal cycling for 35 cycles (1 min at 94°C, 1 min at 60°C and 1 min 30 s at 72°C), ending with the final extension at 72°C for 5 min. PCR products of amplification were visualized in 1.5 % agarose gels with TBE buffer and ethidium bromide (0.5 mg $mL^{-1}$).

Quantitative detection of phytohormones was performed using a scanning spectrodensitometer "Sorbfil". Determination of the content of soluble polyphenols was performed by the method of Folin and Ciocalteu (Folian & Ciocalteu, 1927) in the modification of Singleton and Rossi (Singleton & Rossi, 1965), which is based on the reaction of phenols with Folin & Ciocalteu reagent (it produces a blue color which absorbs at 765 nm). The total content of phenol was determined 27 days after inoculation with phytopathogens. The activity of antioxidant enzymes – catalase (EC 1.11.1.6) and non-specific peroxidases (EC 1.11.1.7) in seedlings was determined in one sample. The catalase activity expressed in the amount of $O_2$ formed by the action of the enzyme for 1 min per 1 g of wet wt. (ml of $O_2 \cdot g^{-1} \cdot min^{-1}$). The activity of nonspecific peroxidases was studied by the method of Boyarkin. Peroxidase activity was expressed in arbitrary units per 1 $g^{-1} \cdot c^{-1}$ of wet wt. (Huliaieva *et al.*, 2018). Analysis of the enzymatic activity of catalase and peroxidase was performed 27 days after inoculation with phytopathogens. The hydrogen peroxide content was determined by the method of Bellincampi *et al*. (2000), estimating the color intensity of compounds formed with xylenol orange spectrophotometrically.

The photochemical activity of soybean leaves was measured by the biophysical method of induction of chlorophyll fluorescence using a portable device "Floratest" (Huliaieva *et al*., 2018). Measurements were performed one month after inoculation with experimental strains.

Induction fluorescence of parameters: minimum chlorophyll fluorescence ($F_0$); $F_v = F_m - F_0$ – the variable fluorescence; $\frac{Fv}{Fm}$ – maximum quantum yield of primary photochemistry; decrease ratio $R_{Fd} = \frac{Fm-Ft}{Ft}$, which, when measured at saturation irradiance is directly correlated to the net $CO_2$ assimilation rate (PN) of leaves (Lichtenthaler *et al*., 2007; Misra *et al*., 2012).

For statistical analysis was used computer programs of Microsoft Excel. On diagrams shown the arithmetic mean errors.

## RESULTS AND DISCUSSION

In greenhouse experiments we observed the appearance of symptoms at artificial infection: yellowing at the edges leaf (on phytoplasmas-infected soybean plants) (fig. 1A), the green vein banding, which become slightly darker than the areas between the veins and leaf malformation, which usually exhibit a downward curling or cupping (on BCMV-infected soybean plants) (fig. 1B, 1C). Symptoms soybean bacterial pustule begin as small, light green spots with raised centers and gradually the lesions can grow together into large irregular brown areas (fig. 1E, 1F). Symptoms bacterial blight of soybean have look angular lesions, which begin as small yellow to brown spots on the leaves, witch centers of the spots will turn a dark reddish-brown and dry out, yellowish green "halo" will appear around the edge (fig. 1F, 1G).

By the RT-PCR analysis of BCMV viruses was conducted in leaves common bean with BCMV-symptoms (fig. 1D), that used for inoculation soybean plants (fig. 2). The expected sizes of amplifying DNA fragments for BCMV were 391-base pair (bp) (Kyrychenko & Prylipko, 2020).

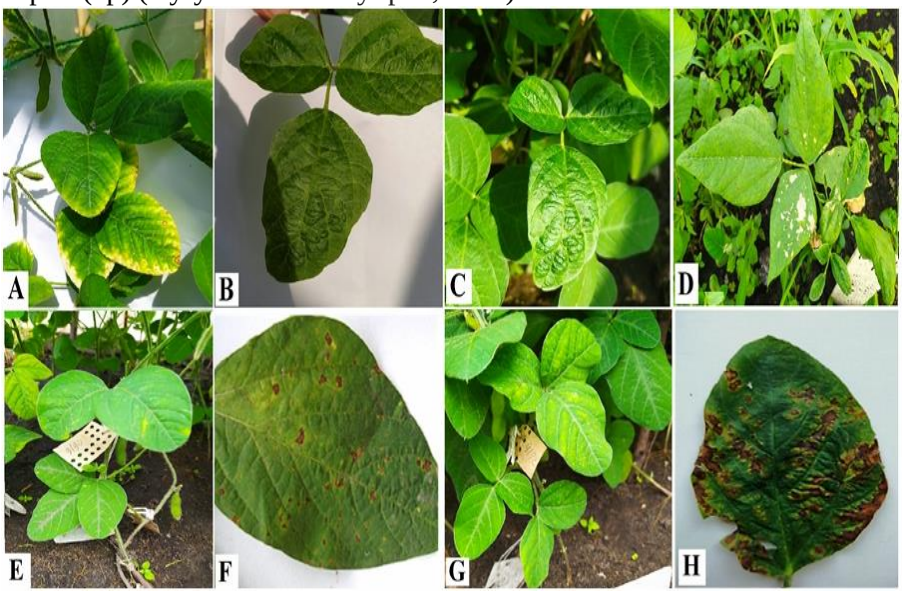



**Figure 1.** Appearance of symptoms

It is known that the infection of plants with phytopathogens and action of other stressors in cellular compartments (chloroplasts, mitochondria, peroxisomes) form active forms of oxygen (ROS), which serve as signaling molecules and participate in protection against pathogens (Shetty *et al*., 2008), but with excessive accumulation and cause cell death (Sharma *et al*., 2012; Suzuki *et al.*, 2012; Anjum *et al.*, 2016; Huang *et al*., 2019). The formation of ROS occurs not only under stressful conditions, but also in a result of electronic transport activity, in PSI and PSII (Foyer, 2018; Sharma *et al*., 2012). It should be noted that ROS are also involved in the regulation of important metabolic

processes associated with growth and development (Anjum *et al*, 2016; Damiani *et al*., 2016; Suzuki *et al.*, 2012; Dvořák *et al*., 2021). Research by Damiani *et al.* (2016) established the participation of ROS and NO in the establishment of symbiosis between legumes and rhizobia. Due to the above, the regulation of ROS in cells is an important metabolic process. It is known that ROC is regulated by so-called enzymatic (superoxide dismutase (SOD), catalase (CAT), guaiacolperoxidase (GPX), ascorbate-glutathione cycle enzymes (AsA-GSH), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR)) or non-enzymatic antioxidants (Ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, phenols) (Huang *et al*., 2019; Sharma *et. al*., 2012; Das & Roychoudhury, 2014). Typically, an increase in antioxidant activity indicates on the activation of ROS neutralization by increasing its content under oxidative stress, as well as increasing the resistance of plants to stressors (Sharma *et al*., 2012; Podgórska *et al*., 2017). Non-enzymatic antioxidants such as phenolic compounds are known to protect plants from phytopathogens due their antimicrobial properties and other important functions: involved in growth and development, including hormone production, osmoregulation, UV protection, etc. (Wallis & Galarneau, 2020; Chowdhary *et al*., 2021). Plants are accumulated phenols to delay the growth of microbial pathogens and limit their spread in the infected area (Chowdhary *et al.*, 2021). Research by Maddox *et al*. (2010) indicated inhibition of the growth of gram-negative phytopathogenic bacteria, which causes diseases of many crops – *Xylella fastidiosa*, under the action of minimal inhibitory concentrations of 12 phenolic compounds. The antiviral activity of phenolic compounds has also been confirmed (Chowdhary *et al*., 2021). However, the induction of phenolic metabolism in plants may be a response to multiple stresses (Sharma *et al*., 2012).

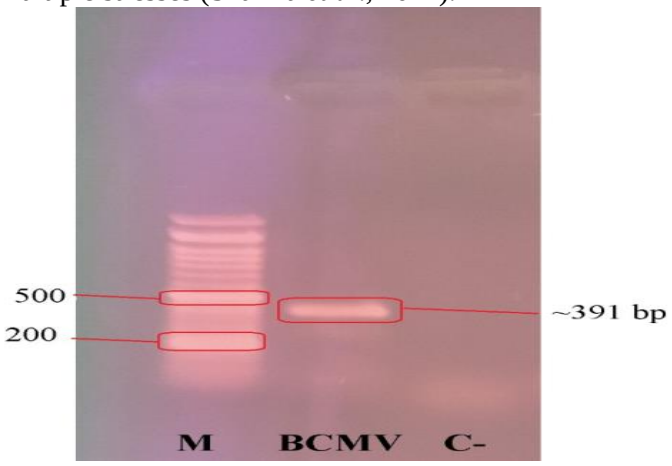



**Figure 2.** RT-PCR detection of BCMV viruses in common bean plants have been grown in greenhouse condition: M-molecular marker (CSL-MDNA-50BP DNA Ladder RTU), BCMV (from common bean leaves with BCMV symptoms), C- – negative control

Our studies have shown an increase in the content of phenolic compounds as at inoculated by phytopathogens: *P. savastanoi pv. glycinea* 9190 (by 52,6%) > *X. axonopodis* pv. *glycines* 9192 (by 43,9%) > B3MK (by 37,2%) > phytoplasmas (by 25,9%), so and at pre-sowing treatment by nanochelates: CuNHs (by 52,6%) > GeNHs (by 35,4%) > MoNHs (by 22,4%) > VNHs (by 9,7%) (fig. 3a). Moreover, content of phenolic compounds increased most significantly on variants: inoculation by bacteria on background of pre-sowing treatment of GeNHs, MoNHs, CuNHs; the BCMV-inoculation on background of pre-treatment of GeNHs, CuNHs, MoNHs; inoculation soybean by phytoplasmas on background of pre-treatment of CuNHs, VNHs, MoNHs (see fig. 3a). Thus, increase in phenolic compounds in leaves experimental variants are indicates on plant resistance increased to pathogenic infecting.

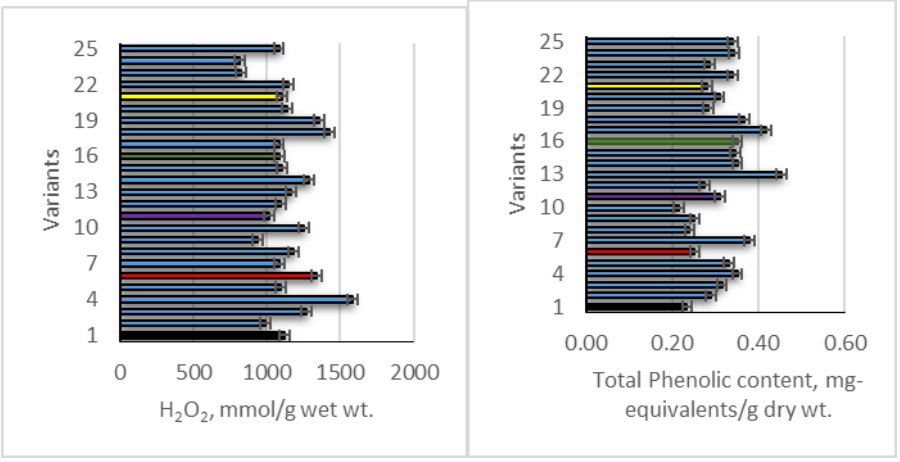
Another well-known ROS that does not belong to free radicals and performs important regulatory, protective and signaling functions is $H_2O_2$. Generated in cells in a result of various stressors, including the penetration of pathogens, as well as in the process of metabolic transformations (redox reactions, photorespiration, electron transport chain), hydrogen peroxide easily penetrates biological membranes (Slesak *et al*., 2007; Sharma *et al*., 2012; Niu & Liao, 2016; Smirnoff & Arnaud, 2019; Petrov & Breusegem, 2012). It is also known that $H_2O_2$ at low concentrations acts as a signaling molecule, participating in the regulation of certain biological processes and can increase plant resistance to pathogens. However, at high concentrations hydrogen peroxide – can inactivate enzymes, including the Calvin cycle – up to 50% (at concentrations of 10 μm), as well as SOD, protein kinases, phosphatases, transcription factors containing thiolate residues, oxidize amino acids, cysteine methionine and others (Sharma *et al*., 2012).

According to our data, the content of $H_2O_2$ decreased in the leaves at pre-treatment of GeNHs and increased at pre-treatment of VNHs, while at pre-treatment of MoNHs was at the control level (fig. 3b).

Research by Shin *et al*. (2004) indicated that the level of $H_2O_2$ in Arabidopsis in the leaves increased slightly after 6 hours but doubled 30 hours after potassium starvation. Similar tendencies were observed in maize plants (Shin *et al*., 2004). However, even low concentrations of non-essential metals, such as Cd, Pb, Hg, can disrupt physiological and biochemical processes in plants and cause oxidative stress. It is also known that metals that have redox activity (Fe, Cu) increase the level of ROS directly, and those that do not its (Cd, Hg, Zn) increase ROS indirectly (Cuypers *et al*., 2016). However, in our study we considered the dual function of ROS in plant metabolism and their role in increasing plant resistance, particularly at infection with phytopathogens. Because it is known that one of the early responses to pathogen infecting under the conditions of its recognition is an oxidative burst with significant formation of ROS and the product of its dismutation – $H_2O_2$ in the apoplast of plants (Sharma *et al*., 2012; Shetty *et al*., 2007). Research by Shetty *et al*. (2007) showed that hydrogen peroxide ($H_2O_2$) increases the resistance of wheat plants depending on

the biotrophic or the necrotrophic of phase life cycle: inhibits it's at the biotrophic faze cycle but promotes its transition to necrotrophic phase or development of necrotrophic pathogens.

The inoculation soybean by pathogens influenced on the content of hydrogen peroxide in different ways: its content increased at the inoculation of BCMV and *P. savastanoi* pv. *glycinea* 9190 and it decreased – at the inoculation *of X. axonopodis* pv. *glycines* 9192 and phytoplasma compared to control (fig. 3b). This may be due to different sensitivity of plants to these pathogens.




a b

**Figure 3.** Total phenolic content (a) and $H_2O_2$ content (b) in soybean leaves under the influence of various factors:1 – Cntrol; 2 – phytoplasmas; 3 – BCMV; 4 – 9190; 5 – 9192; 6 – VNHs; 7 – VNHs+phytoplasmas; 8 – VNHs+BCMV; 9 – VNHs+9190; 10 – VNHs+9192; 11 – GeNHs; 12 – GeNHs +phytoplasmas; 13– GeNHs +BCMV; 14 – GeNHs +9190; 15 – GeNHs +9192; 16 – CuNHs; 17 – CuNHs +phytoplasmas; 18 – CuNHs +BCMV; 19 – CuNHs +9190; 20 – CuNHs +9192; 21 – MoNHs; 22 – MoNHs +phytoplasmas; 23 – MoNHs +BCMV; 24 – MoNHs +9190; 25 – MoNHs +9192.

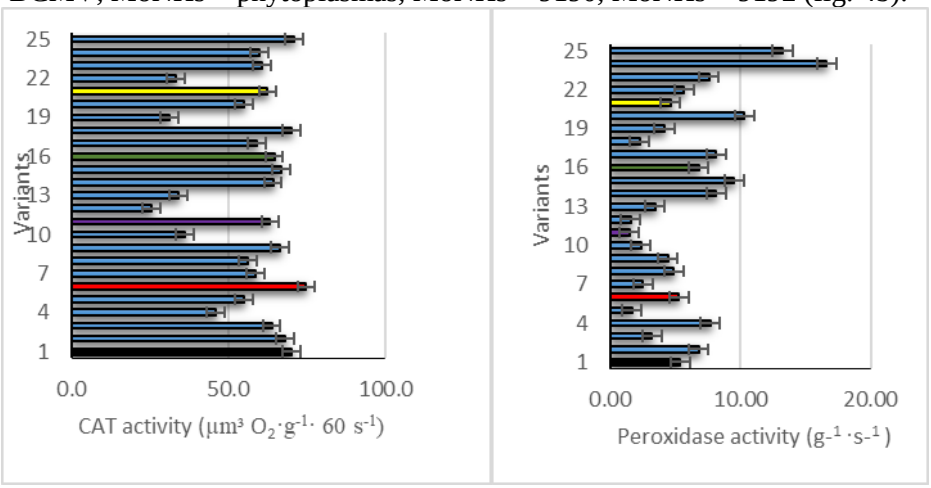
The largest decrease in the content of hydrogen peroxide in the leaves was observed on variants: the pre-treatment of MoNHs + BCMV, the pre-treatment of VNHs + phytoplasmas, the pre-treatment by VNHs + *P. savastanoi* pv. *glycinea* 9190, the pre-treatment both GeNHs, CuNHs with inoculation of *X. axonopodis* pv. *glycines* 9192.

It has been shown that, especially, flavonoids and phenylpropanoids are oxidized by peroxidase, and act in $H_2O_2$-scavenging, phenolic/AsA/POD system. But main enzymes that regulate the content of $H_2O_2$ in plants are catalase (CAT), ascorbate peroxidase (APX) and peroxidase type III (PRX) (Sharma *et al*., 2012; Smirnoff & Arnaud, 2019).

From a month after inoculation with phytopathogens increased in the CAT activity only in the variant pre-treatment of VNHs was shown. Known,

CAT activity may increase or decrease in depending on intensively and type of stress. It known that stresses that reduce the rate of protein turnover also may reduce CAT activity. (Sharma *et al*., 2012).

In all other variants decreased CAT activity except for the variants: inoculation with phytoplasma, VNHs + 9190, GeNHs + 9192, CuNHs + BCMV. While, on variant MoNHs + 9192 its value was equal the control level (fig. 4a). The peroxidase activity increased on variants: phytoplasmas, 9190, GeNHs + 9190, GeNHs + 9192, CuNHs, CuNHs + phytoplasmas, CuNHs + 9192, MoNHs + BCMV, MoNHs + phytoplasmas, MoNHs + 9190, MoNHs + 9192 (fig. 4b).




a b

**Figure 4.** CAT (a) and peroxidase activity (б) in soybean leaves under the influence of various factors: 1– Control; 2– phytoplasmas; 3– BCMV; 4 – 9190; 5 – 9192; 6 – VNHs; 7 – VNHs+phytoplasmas; 8– VNHs+BCMV; 9 – VNHs+9190; 10 – VNHs+9192; 11– GeNHs; 12– GeNHs +phytoplasmas; 13– GeNHs +BCMV; 14 – GeNHs+9190; 15– GeNHs +9192; 16– CuNHs; 17– CuNHs +phytoplasmas; 18 – CuNHs +BCMV; 19 – CuNHs +9190; 20 – CuNHs +9192; 21– MoNHs; 22– MoNHs +phytoplasmas; 23– MoNHs +BCMV; 24– MoNHs +9190; 25– MoNHs +9192.

The role of reactive oxygen species (ROS) as a secondary messenger in the responses of several plant hormones, including stomatal closure (ABA), root gravitropism (IAA), seed germination (GA), lignin biosynthesis (JA), programmed cell death (GA), hypersensitivity reactions (SA), and osmotic stress (SA), is known. Particularly, it is known that ABA-induced increase of $H_2O_2$ cytosolic levels cause to stomatal closure (Sharma *et al*., 2012). Abscisic acid (ABA) is a known phytohormone that regulates various aspects of plant metabolism, including adaptive responses to abiotic and biotic stresses as well as in the regulation of seed development and germination (Seo & Marion-Poll, 2019). The basal ABA is essential for proper chloroplast biogenesis, central metabolism, and expression of cell-cycle genes on the cellular level (Brookbank

*et al.*, 2021). Auxin is also an important phytohormone involved in the regulation of plant growth and development, as well as in the signaling pathways of interaction between viruses and plants, affecting the development of the disease (Zhao & Li, 2021). It has been shown that plant viruses belonging to different families have developed different strategies to disrupt auxin signaling (for replication, systemic trafficking, transmission and development of viral symptoms), namely: (a) changing the intracellular localization of Aux/IAA, (b) preventing degradation of Aux/IAA by stabilizing or (c) inhibiting the transcriptional activity of ARF (Müllender *et al.*, 2021).

In our investigation was detected the decrease ABA content in soybean leaves at inoculation by phytoplasmas both of plants without treatment and plants with pre-treatment by NHs. The IAA content at inoculation by phytoplasmas decreased (by 85,0%) on variant without treatment and with treatment by GeNHs (by 25,9%), CuNHs (by 99,7%), MoNHs (by 94,4%), except for pre-treatment of VNHs, where concentration it increased by 85,5% (fig. 4). At inoculation by BCMV the content IAA and ABA increased by 5,8 and 75,3% accordingly. On variants with inoculation BCMV on background of pre-treatment by NHs both IAA and ABA content decreased in accordingly: VNHs (by 90,9 and 21,4%), GeNHs (11,1 and 21,4%), CuNHs (by 99,8 and 99,9%), MoNHs (71,9 and 92,3%) (fig. 5).

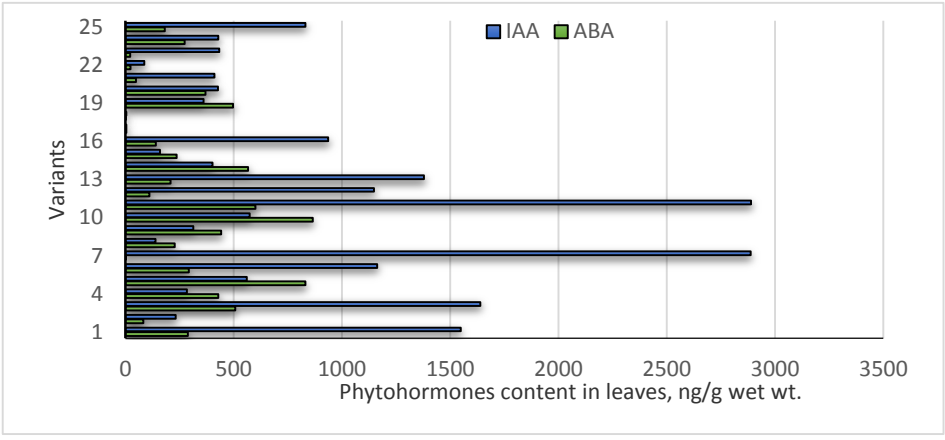



**Figure 5.** IAA and ABA content in soybean leaves under the influence of various factors: 1 – Control; 2 – phytoplasmas; 3 – BCMV; 4 – 9190; 5–9192; 6 – VNHs; 7 – VNHs+phytoplasmas; 8 – VNHs+BCMV; 9 – VNHs+9190; 10 – VNHs+9192; 11 – GeNHs; 12 – GeNHs +phytoplasmas; 13– GeNHs +BCMV; 14 – GeNHs +9190; 15 – GeNHs +9192; 16 – CuNHs; 17 – CuNHs +phytoplasmas; 18 – CuNHs +BCMV; 19 – CuNHs +9190; 20 – CuNHs +9192; 21 – MoNHs; 22 – MoNHs +phytoplasmas; 23 – MoNHs +BCMV; 24 – MoNHs +9190; 25 – MoNHs +9192

At inoculation with bacterial strains 9190 i 9192 the ABA content in leaves increased by 48,3 and 187,8% accordingly, hereat IAA content decreased

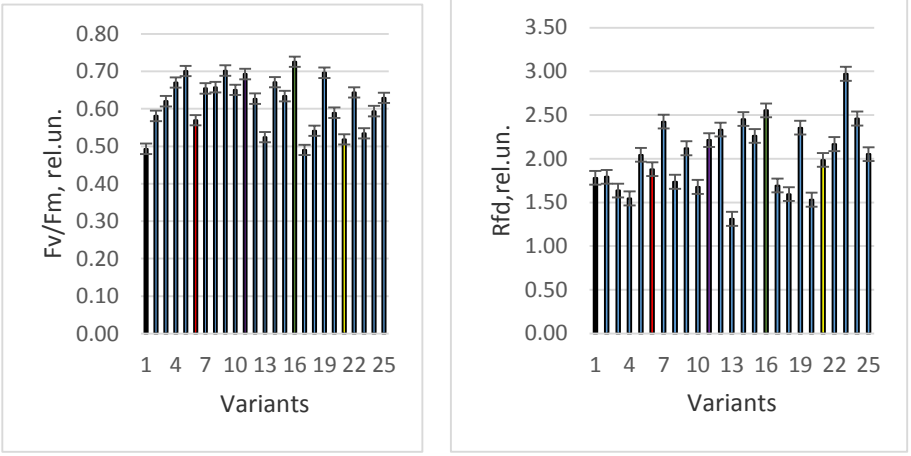
by 81,7 and 63,9% accordingly. At inoculation soybean plants by bacterial strains 9190, 9192 with pre-treatment VNHs, CuNHs and on variant with 9190+GeNHs similarly tendency changed to phytohormones content was shown. But in leaves on variants: GeNHs+9192 and MoNHs (+9190, 9192) content both IAA (by 89,7%) and ABA (18,1%) decreased.

It is known that ABA has remarkable impacts on plant defense against various pathogens, particularly bacteria, fungi, viruses. In particles, increased ABA at viral infection in plant suggested callose accumulation at PD or cell walls, RNA silencing. ABA generally affects several genes in the RNA silencing pathway, perhaps representing an important tool by which ABA tunes plant responses to different incentives (Alazem & Lin, 2017; Zhao & Li, 2021). Though ABA is active player in plant antiviral immunity, Pasin *et al*. (2020) reported, there the genus *Potyvirus* comprises a self-controlled RNA plant virus that may is evaded antiviral response of plants by controlled to the release of a downstream functional RNA-silencing suppressor and viral replication. It is also known that viral infection causes the simultaneous formation of synergistic or antagonistic phytohormones, disrupting hormonal induction, which correlates with the appearance of symptoms, active replication, movement, and systemic viral infection (Zhao & Li, 2021). It has been shown that at Arabidopsis–Pseudomonas interaction infection ABA double play – a positive role in pre-invasive stomatal immunity, but it plays a negative role in post-invasive PAMP triggered immunity and effector-triggered immunity. *P.syringae* TTSEs can upregulate ABA biosynthesis and/or signaling, potentially to suppress post-invasive immunity (Cao *et al*., 2011).

Thus, increased ABA content in leaves at BCMV-infection are indication suggested to promotion resistibility soybean plant. In same time, increased ABA at bacterial infected plants suggested to post-invasive exasperation of disease. However, we observed changes in phytohormonal status at pre-sowing treatment of plants NHs without infecting and with inoculation with phytopathogens, which indicates regulatory function NHs in plant metabolism.

The method of chlorophyll *a* fluorescence induction (CFI) is an important diagnostic tool in biological research, which reflects the influence of biotic and abiotic factors on the plant and its tolerance to stress (Lichtenthaler *et al*., 2007; Misra *et al*., 2012, Maxwell & Johnson, 2000; Rahimzadeh-Bajgiran *et al*., 2017; Stirbet *et al*., 2018; Guidi *et al*., 2019; McAusland *et al*., 2019; Zavafer *et al*., 2020; Huliaieva *et al*., 2018; Huliaieva *et al*., 2020a). First, we focused on determining changes in the $F_v/F_m$ parameter is the maximum quantum yield of PSII. This parameter is widely used as an indicator of photosynthetic efficiency of plants, their response to stress. Its suppression is associated with photoinhibition of PSII activity due to inhibition of PS II repair (inhibition of protein synthesis) by ROS, formed by excessive both reduction of the primary electron acceptor of PSII plastoquinone $Q_A$, or recombination of charges between the acceptor and donor sides of PSII (Guidi *et al.*, 2019).

The investigation of changes in the parameters of the maximum quantum yield of PSII under the action of the studied factors was shown an increase in its value in almost all experimental variants, except CuNHs + BCMV, where its value was equal to the control (fig. 6a). Therefore, the increase in the value of the parameter is indicates the effective control and utilization of free radicals in the photosynthetic apparatus of experimental plants.




a b

**Figure 6.** FCI-parameters Fv/Fm (a) i $R_{Fd}$ (b) in soybean leaves under the influence of various factors: 1 – Control; 2 – phytoplasmas; 3 – BCMV; 4 – 9190; 5 – 9192; 6 – VNHs; 7 – VNHs+phytoplasmas; 8 – VNHs+BCMV; 9 – VNHs+9190; 10 – VNHs+9192; 11 – GeNHs; 12 – GeNHs +phytoplasmas; 13 – GeNHs +BCMV; 14 – GeNHs +9190; 15 – GeNHs +9192; 16 – CuNHs; 17 – CuNHs +phytoplasmas; 18 – CuNHs +BCMV; 19 – CuNHs +9190; 20 – CuNHs +9192; 21 – MoNHs; 22 – MoNHs +phytoplasmas; 23 – MoNHs +BCMV; 24 – MoNHs +9190; 25 – MoNHs +9192

We determined another indicator parameter that is closely correlated with net $CO_2$ assimilation – the chlorophyll fluorescence decrease ratio – $R_{Fd}$ (Lichtenthaler *et al*., 2007). The $R_{Fd}$ parameter used in any investigation for evaluation different effects on the rate of photosynthesis: salt-induced changes in the photosynthetic apparatus (Stefanov *et al*., 2022), for an evaluation of hot air stressed on photosynthetic apparatus of maize (Park *et al*., 2016), for assessed in low and high temperatures resistance in a seasonal study of the acclimation of *Pterocephalus lasiospermus* (Perera-Castro *et al*., 2018), effect high-light stress on photosynthetic apparatus (Lichtenthaler & Burkart, 1999), in monitoring of salinity, temperature, and drought stress in grafted watermelon (Shin *et al*., 2021), for assessors of waterlogging stress on the photosynthetic apparatus of mulberry leaves (Rao *et al*., 2021) and other. It is worth noting that, in investigation of Perera-Castro *et al*. (2018) under influenced the lethal temperatures (LT50) on *Pterocephalus lasiospermus*, $R_{Fd}$ resulted to be a more sensitive indicator

parameter for low and high temperature treatments, since thermic resistance estimated with $R_{Fd}$ parameter was never higher than those estimated with $F_v/F_m$ was shown (Perera-Castro *et al*., 2018).

Our studies revealed inhibition of $R_{Fd}$ in the leaves of BCMV and 9190 infected plants. The photosynthetic rate ($R_{Fd}$-value) was equal to control variant at inoculation by phytoplasmas and lightly increased at inoculation with 9192 (fig. 6b). The $R_{Fd}$-value on variants with background of pre-sowing treatment by NHs increased in accordance: CuNHs (43,5%) > GeNHs (24,3%) > MoNHs (11,6%) > VNHs (5,6%).

The $R_{Fd}$-value changed in variants: in BCMV-infected soybean leaves on background of pre-treatment of VNHst was equal to control, at pre-treatment of GeNHs, CuNHs decreased and at pre-treatment of MoNHs it increased. In plants infected with phytoplasmas with background pre-treatment of VNHs, GeNHs i MoNHs the photosynthetic rate ($R_{Fd}$-value) increased, but at pre-treatment of CuNHs made tendency to decreased.

The Chl fluorescence decrease ratio ($R_{Fd}$) (net $CO_2$ assimilation) increased with infection by both bacterial pathogens with background pre-treatment of GeNHs and MoNHs. The $R_{Fd}$-value in soybean plant leaves on the background both pre-treatment of VNHs and CuNHs with 9190 infection – increased and with 9192 infection – decreased (see fig. 6b).

Thus, the infection soybean plants with phytopathogens in different ways influenced on Chl fluorescence decrease ratio ($R_{Fd}$): at BCMV and *P. savastanoi* pv. *glycinea* 9190 – decreased, but at inoculation by phytoplasmas equal to control and at inoculation with *X. axonopodis* pv. *glycines* 9192 lightly increased. This is indicating that $R_{Fd}$-value dependents on the phase of infection, plant resistance and the type of pathogen. In same time, pre-treatment of soybean by nanochelates contributed to increased $R_{Fd}$-value in variants of soybean plants without infected with pathogens. The $R_{Fd}$-value on background of MoNHs application with BCMV, bacterial, phytoplasmas infected soybean plants, on background pre-treatment of GeNHs at infection by both bacterial and phytoplasmas pathogens, on background pre-treatment of VNHs at infected with phytoplasmas have been increased. Thus, the use of nanochelates of Mo was the most effective.

## CONCLUSIONS

The phenolic compounds content in soybean leaves increased in all variants: at pre-treatment by nanochelates, at inoculation plants by phytopathogens, at infected by phytopathogens on background pre-treatment by nanochelates.

The inoculation soybean with pathogens influenced on the content of hydrogen peroxide in different ways: its content increased at the inoculation of BCMV and *P. savastanoi* pv. *glycinea* 9190 and it decreased – at the inoculation of *X. axonopodis* pv. *glycines* 9192 and phytoplasma compared to control.

The CAT activity in tissue leaves increased only on variants pre-treatment VNHs. In same time, peroxidase activity of tissue leaves increased in variants: phytoplasmas and 9190 inoculation, GeNHs + 9190, GeNHs + 9192, CuNHs, CuNHs + phytoplasmas, CuNHs + 9192, MoNHs + BCMV, MoNHs + phytoplasmas, MoNHs + 9190, MoNHs + 9192.

The content ABA increased on variants: with BCMV-inoculation, *P. savastanoi* pv. *glycinea* 9190-inoculation, *P. savastanoi* pv. *glycinea* 9190-inoculation with pre-treatment of VNHs, CuNHs, GeNHs, *X. axonopodis* pv. *glycines* 9192-inoculation, *P. savastanoi* pv. *glycinea* 9190-inoculation with pre-treatment of VNHs, CuNHs, pre-treatment VNHs. But ABA content decreased on variants: BCMV-inoculation on background of pre-treatment nanochelates, *A.laidlawii*-inoculation, *A.laidlawii*-inoculation with pre-treatment of nanochelates, 9192-inoculation on background of pre-treatment of GeNHs, with inoculation both 9190, 9192 on background of pre-treatment of MoNHs.

The IAA content increased on variants: with BCMV-inoculation, at phytoplasmas-inoculation on background of pre-treatment VNHs. But IAA content decreased on variants: with 9190 and 9192 -inoculations (and with background pre-treatment of nanochelates), phytoplasmas-inoculation (with background pre-treatment by GeNHs, CuNHs, MoNHs), with BCMV-inoculation with background pre-treatment of nanochelates.

Besides the photosynthetic rate ($R_{Fd}$-value) increased at infection bacterial pathogens on background pre-treatment of GeNHs and MoNHs. The bacterial infection on background of pre-treatment by VNHs and CuNHs had a different effect on the $R_{Fd}$-value. It increased in *P. savastanoi* pv. *glycinea* 9190 infection plants and decreased in *X. axonopodis* pv. *glycines* 9192 infection plants.

Thus, pre-treatment by GeNHs, MoNHs, VNHs, CuNHs had regulatory effect on soybean plant metabolism and helping to increase its resistance to infecting with phytopathogens were shown.

In same time, using of pre-treatment of MoNHs before inoculation of soybean plants by pathogenic bacteria, BCMV and *A.laidlawii* promoted increase not only resistance to pathogens but increased the assimilation of $CO_2$.

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# EVALUATION OF SOIL QUALITY INDICATORS IN ORGANIC FERTILIZATION AS AN ALTERNATIVE TO SUSTAINABLE AGRICULTURE

## SUMMARY

Sustainable agriculture is the yield of agricultural products with good quantity and quality, but with care for the soil in the future. Nowadays, agriculture practices have led to humus reduction in the cultivated soil layer, even in sustainable soils. Applying the principles of the circular economy, composting and organic fertilization are ways to increase the organic matter in the soil. However, whether entirely organic fertilization is an alternative to sustainable agriculture is the aim of this article. The following soil quality indicators are assessed – organic carbon; total nitrogen; ratio C:N. The results present vegetation experience with two scenarios. The first scenario is with Luvisol (LV) and the second is with Fluvisol (FL). Each scenario has an addition of various organic ameliorants. The period of experiment covers the composting period and two vegetation cycles - lettuce and spinach aftereffect. The evaluation is against control variants and limits values. The two soils have different physicochemical characteristics. Luvisol has a higher total carbon content and total nitrogen than Fluvisol. In the end, the 1st soil has increased the SOM up to 4% compared to the second soil, where the improvement is no more than 2%. Accordingly, the nitrogen content is higher in Luvisol than in Fluvisol. In conclusion, we can say that organic fertilization alone is not an alternative to sustainable land management principally because nitrogen depletes after the first growing season in both scenarios of the experiment.

**Keywords**: manure, composting, organic carbon, total nitrogen, ratio C:N

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## INTRODUCTION

Soil organic matter (SOM) is one of the most important soil characteristics. SOM turnover plays a crucial role in soil ecosystem functioning and global warming. SOM is critical for stabilising soil structure, retaining and releasing plant nutrients and maintaining water-holding capacity, thus making it a key indicator not only for agricultural productivity but also for environmental resilience. The decomposition of SOM further releases mineral nutrients, thereby making them available for plant growth while better plant growth and higher productivity contribute to ensuring food security (Van der Wal *et al.,* 2017). Over the last ten years, SOM has been gradually reduced in EU countries. The intensive and continuous exploitation of arable land that can have led to a reduction of organic matter in the soil is reflected in the EU Soil Strategy as one of the most important issues (Atiyeh et al., 2002). Arable land is a source rather than a depot of organic carbon. It's part of SOM which is why some explain as it is the hidden potential (Gougoulias *et al.*, 2014). In Central Europe, the soil organic carbon content is mainly between 3-6%, and in Southern Europe, there are many regions where the soil organic carbon content is 0-2% (FAO, 2017).

The share of the arable area is an important indicator of the impacts of farming on soil organic matter dynamics for it is known that arable areas can deplete soil carbon stocks rapidly (Gobin *et al.*, 2011).

The humus substances in soils are co-concentrated in the one-meter soil layer. Depending on the climate, the microbiological activity and the type of materials transformed into humus, the amount and composition of the soil organic matter for different soils vary widely (Filcheva, 2007). On the territory of Bulgaria, 14 soil groups have been determined. The average content of humus state in the arable horizons in Bulgarian soils is low (Filcheva, 2015).

Preservation of soil organic matter is a potential means of reducing greenhouse gas emissions. The introduction of plant residues, manure, compost and organic sludge in arable land ranks first among the most recommended for maintenance and increase in soils. Applying the principles of the circular economy, composting and organic fertilization are ways to increase the organic matter in the soil. Plant hydrolysates are effective organic fertilizer additives to improve soil fertility. They are a good prospect for the evolution of more efficient methods with application in crop and vegetable production. They are the basis for the production of ecologically clean crops. Compost can be used as a fertilizer in connection with nutrition, but also as an improver of soil texture, and as a microbial additive to increase enzyme activity (Atiyeh *et al.*, 2002; Diacono and Montemurro, 2011; Kaleem Abbasi *et al.*, 2015; Steger *et al.*, 2007). Some authors believe that composts are soil improvers rather than fertilizers, due to the high content of organic matter and lower contents of nitrogen, phosphorus and potassium compared to mineral fertilizers (Khater, 2012). Optimal fertilizer management and efficient nutrient utilization are key to maximizing higher-quality yields (Fageria, 2009; Hariadi *et al.*, 2015). The use of organic fertilizers directly affects the improvement of soil fertility, because it does not increase the

content of nutrients directly, but acts as a slow-release fertilizer, providing N, P and K plants more optimally (Purbajanti *et al.*, 2016). However, mature composts increase SOM much better than fresh and immature composts due to their higher level of stable carbon. Nitrogen release in most organic improvers is slow and depends on the processes of mineralization in the soil (C/N ratio), and its absorption by plants affects several physiological processes, and morphological characteristics of yield (Tiemens-Hulscher *et al.*, 2014).

The study aims to determine the effectiveness of organic improvers (organic fertilization) on two soil types by determination of the main components - organic carbon and nitrogen, and the ratio between them for a period covering three of their measurements - composting and two vegetation cycles.

## MATERIAL AND METHODS

**Experimental Set.** The goal was achieved by using the following methodological approach, including: first, the technical stage of mixing the soil with the organic materials for more than 30 days before the Vegetation experiments (VEs) with culture lettuce Lactuca sativa and then spinach aftereffect. VEs contain two scenarios, each with a different soil type. The soils Cinnamon Forest Soil and Alluvial-meadow soil are associated with Chromic Luvisol (LV) and Eutric Fluvisol (FL) by World References Base (WRBSR, 2014). 1st scenario LV has 9th variants vs 2nd scenario FL has 7th variants. They're given below:

**Variants 1st scenario**

I.1 Luvisol (LV) control
I.2 LV + 5% ready compost *
I.3 LV +10% ready compost
I.4 LV + 15% ready compost
I.5 LV +10% compost, 2 months
I.6 LV + 10% fresh manure (1)
I.7 LV + 10% ready vermicompost
I.8 LV + 10% wastewater treatment plant (WWTP) sludge (2)
I.9 LV + 10% other compost from poultry farm waste
*Ready compost contains components (1) +(2) +straw

**Variants 2nd scenario**

II.1 Fluvisol (FL) control
II.2 FL + 10% vermicompost
II.3 FL + 10% agro biofertilizer (traditional compost)
II.4 FL + 5% agro biofertilizer (uni granules)
II.5 FL + 10% agro biofertilizer (uni granules)
II.6 FL + 15% agro biofertilizer (uni granules)
II.7 FL + 5% (100 g) dry starting product for agro biofertilizer

Each scenario in VEs contains three repetitions. Two-kilogram vessels were used. Organic improvers are weightily mixed into the soils. Both soil types

and organic amendments were examined for many analytical parameters – pH (ISO 10390: 2011), CEC (Ganev and Arsova, 1980), and content of NPK etc. Mainly after the composting period, the changes in the composition and content of SOM, as well as in the supply of nitrogen, phosphorus and potassium were observed. After the two vegetation periods, the yields of lettuce and spinach were followed, as well as some of their quantitative and biometric indicators. This publication shows unpublished data on organic carbon (Filcheva, 2015), total nitrogen (ISO 11261:2005) and their ratio for evaluating organic fertilization efficiency. Data are the average of three measurements and are shown graphically as mean and standard deviation relative to the control variant without organic additive. Data were statistically calculated by descriptive statistics and ANOVA factor analysis.

## RESULTS AND DISCUSSION

The figures from 1 up to 3 represent the average change in total organic carbon content, total nitrogen in % and the ratio between them compared to the control variant after a period of composting and two vegetation cycles (on left with Luvisol and on right with Fluvisol).

**Changes in the content of organic carbon.** The soils have low SOM due to intensive agricultural use. In LV, OC is 1.30% or as SOM 2.24%, in FL, OC - 0.73% or as SOM 1.26%. All input organic materials have the potential to increase the content of SOM in the soil according to the content of total organic carbon, which varies from 8.19 to 20.20%, and the average for all used materials is 15.4 ± 4.1%.

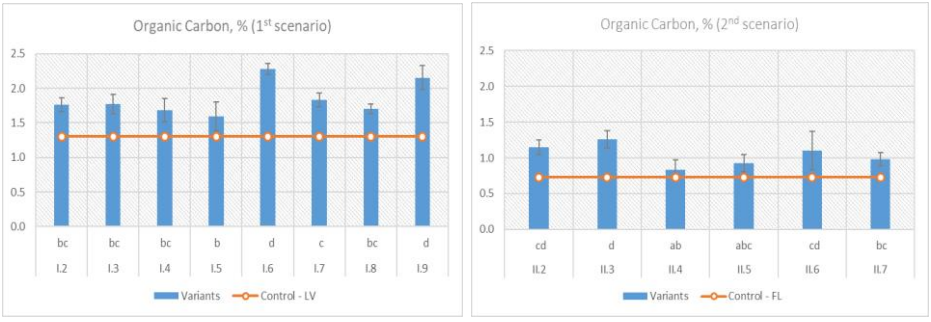


**Figure 1.** Changes in total organic carbon over the study period in both scenarios

Figure 1 shows that the content of organic carbon is highest when applying fresh manure (I.6) and mature compost from a poultry farm (I.9). For the second soil, it is compost, in the traditional form of Agrofertilizer (II.3). The F-test in the ANOVA show that there is a statistically significant difference between the mean organic carbon from one level of treatment to another at the 95.0% confidence level because P-value of the F-test is less than 0.05.

The increase in organic carbon in the LV was more than in the FL. The increase is from 18 to 70% compared to the control variant for LV and the second soil from 9 to 69%. The change is from medium to high organic carbon for LV

and, in the FL, from low to medium. The results correspond very well with the report on the application of organic amendments increases organic carbon by up to 90% compared to unfertilized soil and up to 100% compared to chemical fertilizer treatments (Diacono and Montemurro, 2011). We can say that the best agronomic performance of compost will be at the highest application rates and frequencies

Our published data explain changes in the content and composition of the organic matter of these two soils when adding the organic additives with gradation (5, 10 and 15%), about rates of organic amendments in this experiment. These are variants I.2 to I.4 for LV and II.4 to II.6 for FL. Genetic differences between soils may explain the results of organic carbon change. The main difference between the two soils is the sorption capacity, which shows a different content of colloids in them. Chromic Luvisols contain more colloids than Eutric Fluvisols, respectively 28.3 and 15.6 cmol.kg$^{-1}$. A good agriculture practice is adding slightly acidic compost to the neutral Eutric Fluvisols (Hristova *et al.*, 2021). In conclusion, our results may put with the connection with carbon sequestration in the agricultural soils of Europe and draw an important decision (Freibauer *et al.*, 2004). It said that efficient carbon sequestration in agricultural soils demands a permanent management change and implementation concepts adjusted to local soil, climate, and management features to allow the selection of areas with high carbon sequestering potential.

**Changes in soils by the content of total nitrogen.** Input soils have the following supply of total nitrogen: it's medium for LV and low for FL. Organic amendments in 2nd scenario have more nitrogen (2.4±0.4%) compare to the 1st scenario the nitrogen varies from 1.36 in poultry compost (I.9) up to 2,5% in fresh mature and WWTP sludge (variants I.6 and I.8). After applying organic amendments total nitrogen content increased from 33 to 65% in LV and the FL from 24 to 44%. There was no significant increase in option I.5 for LV and option II.5 in FL. The qualitative characteristic for this increase was medium to high total nitrogen in LV and low to medium for FL. There is a good correlation between the total amount of nitrogen in organic materials and their amount in the variants of the two soils.

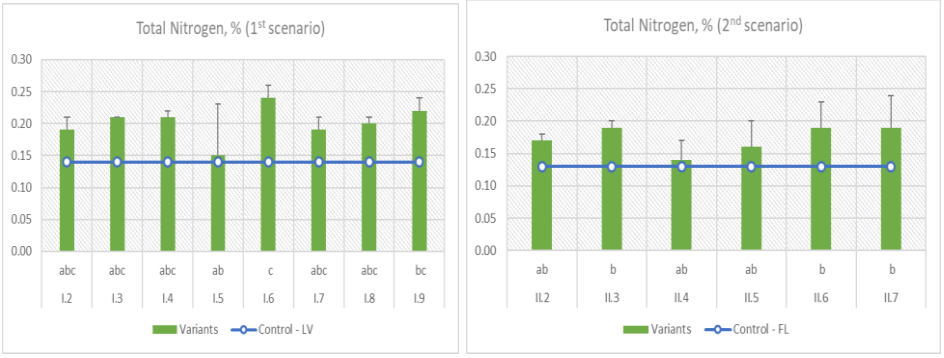


**Figure 2.** Changes in total nitrogen over the study period in both scenarios

Figure 2 shows the total nitrogen content, the largest amount remaining as a stock after the manure application (I.6) in the first soil type. All variants except 5% uni granules (II.4) are higher in the second soil type than the control. The F-test in the ANOVA shows that there is not a statistically significant difference between the mean total nitrogen from one level of treatment to another at the 95.0% confidence level because the F-ratio is equal to a ratio of the between-group estimate to the within-group estimate.

The assessment of organic fertilization is related to the increase of organic carbon and the main nutrients imported - nitrogen. For example, Flavel *et al.* (2006) found that all amendments released inorganic N at concentrations that would be high enough to warrant a reduction in inorganic N fertilizer application rates. Another author conducted a study with two levels of organic addition: the first low of 33 Mg $ha^{-1}$ to provide the N requirement of a given crop and a high amount of 268 Mg $ha^{-1}$ that maximized the benefit to the soil. The authors demonstrated that the % degradation of organic matter from sewage sludge and cow manure for the first year was 24 and 37, respectively (Tester, 1990).

Within this experiment, there are already published data for both scenarios that have a connection to the nitrogen cycle (Vasileva *et al.*, 2021; Vasileva *et al*., 2022; Hristova *et al*., 2021). The publication on the influence of initial substrates and composts on the growth and yield of Lettuce (Lactuca sativa L.) found that the type of organic enhancer affects growth indicators and yield of lettuce. Maximum yield was reported with the use of vermicompost (I.7) and fresh manure (I.6) (Vasielva *et al*., 2021).

The publication which covered the results of the 2nd scenario of the experiment shows relationships between the application of composts and changes in photosynthetic plastid pigments (Vasielva et al., 2022). The main conclusion is an increase in % Uni Granules has been shown to lead to a higher content of total nitrogen in the soil ($R^2$=0.988), but no increase in uptake and accumulation of nitrogen in lettuce leaves was observed. On the face of it, there is a significant correlation between the content of nitrogen and chlorophyll in lettuce ($R^2$=0.965).

In summary of the topic, which covers both variants, it can be said that the experiment is indicative of the development of good agricultural practices because both tested scenarios demonstrated strategic advantages and disadvantages. In the first case, Luvisols and traditional mature are a suitable combination of slightly acidic soil with alkaline organic fertilizer, they fix the organic carbon, but the soil nitrogen isn't enough in the post-vegetation period. In the second case, Fluvisols and granulated mature are the combinations of neutral pH soil with slightly acid granular compost. That scenario positively affects the nitrogen nutrition of plants but does not fix organic carbon very well. This raises working hypotheses to improve the nitrogen supply in the first case and increase the organic carbon in the second case (Hristova *et al.*, 2021).

**Changes in soils by the ratio C:N.** Figure 3 shows that C:N ratio is proportional to their amount in both scenarios - higher in the first than in the

second. Fisher's least significant difference (LSD) procedure shows no statistically significant differences between any pair of means at the 95.0% confidence level.

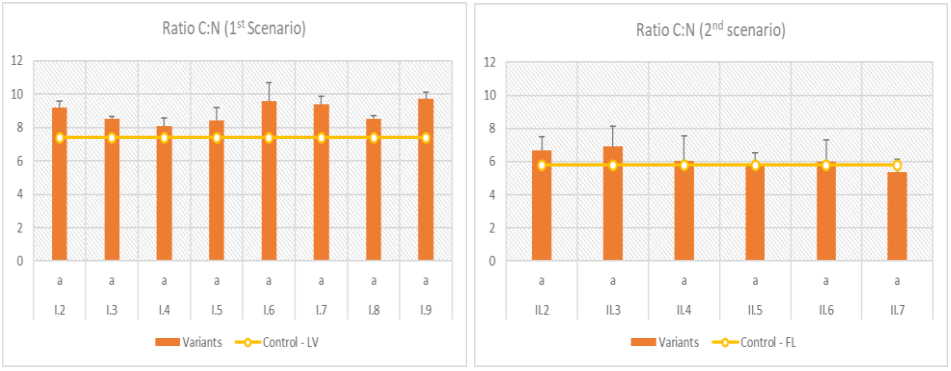


**Figure 3.** Changes in ratio C:N over the study period in both scenarios

The carbon-to-nitrogen ratio has two aspects in research with organic amendments: agrochemical and soil science. In soil C:N ratio is an indicator of the enrichment of humus with nitrogen. In soil diagnostics, nitrogen enrichment is very high when the ratio value is below 7. In our case, it is the soil in the second scenario because the ratio has a lower rate (Filcheva, 2015).

In our experiment, added different organic additives don't vary by C:N ratio. Organic materials have C:N ratio is 9.4 in traditional mature and 7.2 in granulated mature. Many authors comment that the C:N ratio in organic amendments indicates nitrogen mineralization and there are many outcomes on the kinetics of C and N mineralization by simulated dynamic models (Hadas *et al.* 2004; Lazicki *et al.*, 2020). The models effectively predict N evolution during crop residue decomposition in soil and the conclusion is that higher humification occurred in substrates with lower C:N ratios and we can say that our result covers this hypothesis (Nicolardot *et al.*, 2001).

Ratio C:N is highest in the compost from the poultry farm - variant I.9, and in variant II.2. Higher carbon content and little nitrogen (i.e. very high C:N ratio) variant I.9, in there is no adequate amount of nitrogen, microbes are deprived of their tools to break down carbon compounds or in this variant, the composting process is very it will be delayed. In variant II.2, we have higher nitrogen and low carbon content (i.e. very low C:N ratio). In this case, having more nitrogen than the microbes need to break down the carbon compounds will release more ammonia into the atmosphere.

Important conclusions we found are that applying organic amendments with a C:N ratio of 20 and higher reduced crop biomass and increased soil mineral nitrogen, while amendments with a C:N ratio of 10 had the opposite effect. These results suggest that crop cultivation is suitable for preventing N leaching. Applying organic amendments does not pose a risk of N leaching compared to mineral fertilizers and liquid manure (van der Sloot *et al.*, 2022).

Our research also covers the hypothesis at a C:N ratio of up to 10 and proved that nitrogen is accumulated in plants, but after that supply in the soil is insignificant.

Our conclusion based on this and other experiences (Mitova and Dinev, 2017; Hristova *et al.*, 2018) has relation to published data from an analysis of long-term experiments on the effects of combined application of organic additives and fertilizers on crop yield and soil organic matter comparing outcomes. They demonstrate and we agree with them that adding organic matter (alone or in combination with fertilizers) increases SOM content and their total nutrient management compared to fertilizers applied alone. However, benefits vary between organic and organic + fertilizers depending on the type of land use (Wei *et al.*, 2016).

## CONCLUSIONS

The soil evaluation indicators - total carbon, total nitrogen and the ratio between them in the studied two soil types with the application of different additives show a change directly control variants as mean value and standard deviation for the stage including composting and two vegetation cycles. Changes in organic carbon content are statistically proven, while changes in nitrogen content are not. As a result, C:N ratio is not indicative in this case.

The experiment is indicative of the development of good agricultural practices because both tested scenarios demonstrated strategic advantages and disadvantages. These raise working hypotheses to improve the nitrogen supply in the first case and increase the organic carbon in the second case. In conclusion, we can say that organic fertilization alone is not an alternative to sustainable land management principally because nitrogen depletes after the first growing season in both scenarios of the experiment.

## ACKNOWLEDGMENTS

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# SEED-BORNE AND SEED-TRANSMITTED VIRAL DISEASES AND THEIR EFFECT ON YIELD OF DIFFERENT SOYBEAN GENOTYPES

## SUMMARY

In 21st century soybean is a strategic legume for world agriculture. The key factor in increasing soybean production is the average yield growth, which in 2018 and 2021 in Ukraine was 3 t/ha as in the EU. From 2007 to 2017 the soybean yield in Ukraine was 2 t/ha; from 1997 to 2006 it was only 1 t/ha. Unlike, in the EU since 1999 the yield was 3 t/ha, and only in 2003, 2007 and 2012 it was 2 t/ha. For Ukraine not the least role in this situation is played by the reduction of quality of soybean sowing material due to seed infections. It is known that the quality of soybean seeds is significantly deteriorating due to infection by soybean mosaic virus (SMV), which circulates in all soybean growing regions worldwide. The aim of the study was to investigate SMV seed infection in sowing material and its effect on the yield of different soybean genotypes. DAS-ELISA showed SMV presence in a small seeds fraction of several soybean genotypes in both seedlings (embryos and cotyledons) and plants grown from this seeds. The weight of virus-infected seeds was 137 mg compared to healthy (204 mg). It was found that the percentage of seed-transmitted SMV infection is from 1.9 to 10.5% in some of investigated genotypes. It has been proven that seed spotting does not always indicate the presence of virus. Phylogenetic analysis of the SMV *CP* gene fragment was performed. Percent of nucleotide and amino acid sequence identity of Ukrainian SMV isolate with isolates from other countries was established.

**Keywords**: soybean, genotypes, soybean mosaic virus, seed infection

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## INTRODUCTION

Today, soybean is a strategic legume crop of world agriculture of the XXI century, which is the focus of world agricultural science and production. The key factor in increasing soybean production is the growth of the average yield, which in Ukraine in 2018 and 2021 was 3 t/ha, ie reached the EU level. From 2007 to 2017, the yield was 2 t/ha, and from 1997 to 2006 was only 1.0 t/ha in Ukraine. Soybean growing technology needs constant improvement. Given the growing demand for soybeans, the modern market of varieties is extremely diverse and attractive. Soybean yield is greatly influenced by the seed quality, namely the defeat of various diseases. For Ukraine, the reducing in soybean seed quality due to seed infections plays an important role in this situation.

It is known that the quality of soybean seeds significantly deteriorates by soybean mosaic virus (SMV), which circulates in all soybean regions of the world (Bowers and Goodman, 1979; 1991; Domier *et al.,* 2007; Hajimorad *et al.,* 2017). SMV infection leads to significant crop losses. The degree of damage to the crop is dependents on the host genotype, virus strain and virulence, time of the infection and the developmental stage at which soybean plants become infected (Shigemori, 1991). SMV is naturally transmitted by aphids in a non-persistent manner and via infected seeds.

Investigations made by Bashar (2015) shown that SMV transmission by seeds occurs due to infection of the embryo. However, SMV was also found to be present in all seed parts: in seed coat, germ root and cotyledons - 23%, 18% and 33% respectively. From 105 viruses transmitted by seeds, 36 (34.3%) were transmitted by seeds from bean hosts. Of the 35 economically important viruses and one viroid transmitted by seeds, 10 belong to potyviruses, including SMV. Many viruses founded in seeds collected from infected plants but are not transmitted to next generation, i.e. they are seed-borne but not seed-transmitted (Pagán, 2022). The question arises why 2021 was not so high in soybean yield, ie did not reach 3 t / ha with relatively sufficient soil moisture in some soybean crops, in particular in the Vinnytsia region. For the first time in Ukraine, we have shown that transgenic soybean varieties are also affected by the soybean mosaic virus (Mishchenko *et al.,* 2019). It was found that, despite genetic modifications, the yield of SMV infected plants was significantly reduced (Mishchenko *et al.,* 2019; 2018a). When plants were infected by SMV, soybean yields decreased in both farms of Kyiv and Poltava regions by 35.0-65.7%, respectively (Mishchenko *et al.,* 2019). Significant reduction in soybean yield (2.6 times) under SMV infection was found in Poltava region in the conditions of very dry 2017 year (hydrothermal coefficient, HTC = 0.53) compared to 2016 (HTC = 0.99). It was found that infection of plants by SMV (isolates SKP-16 and SGP-17) caused changes in biochemical parameters (protein content, basic fractions of spare proteins, etc.) (Mishchenko *et al.,* 2018a). Two unique amino acid substitutions (Ser → Cys and Lys → Ala) have been identified in the fragment of capsid protein gene of seed-transmitted SMV isolate named SKS-18 that may be

involved in seed transmission of the SMV and other important functions of the viral infection cycle (Mishchenko *et al.,* 2018b).

Therefore, the aim of this study was to estimate the presence of seed transmission of SMV to plants next generation, to determine "seed-transmitted" or "seed-borne" infection and impact of the virus on productivity and yield of different soybean genotypes.

## MATERIAL AND METHODS

Seed SMV infection was determined in the seed of 2021 yield. Various soybean genotypes, grown on the fields of Vinnytsia region (Kordoba, Kea, Kofu, Medok, Viola, Volta, Avatar, Ezra) and Poltava region (Niagara and Neptun of different reproductions, AKAE, Kordoba 3, Kofu-1, Kofu-2) were investigated. It should be noted that incorrect seed sampling at this stage can lead to erroneous test results. In our experiment, the seeds were divided into fractions by size, degree of spotting and 100 seeds of each fraction of the studied soybean samples were selected.

Viruses detection was performed in germinated for three day at 24$^{o}$ C soybean seeds and plants grown from them in greenhouse (growing-on test) by DAS-ELISA using commercial antibodies against SMV, BYMV, AMV manufactured by Loewe (Germany) in three replicates. Samples of healthy soybean were used as negative controls. Commercial SMV, BYMV, AMV preparations (Loewe, Germany) were used for positive controls. The reaction results were recorded on the Termo Labsystems Opsis MR (USA) reader with Dynex Revelation Quicklink software at wavelength of 405 nm. Samples with absorbance values that exceeded the negative control at least three times were considered positive (Crowther, 1995).

RT-PCR was used to confirm the SMV presence in samples. GeneJET Plant RNA Purification Mini Kit (Thermo Scientific, USA) was used to extract total RNA. cDNA synthesis was performed using RevertAid Reverse Transcriptase (Thermo Scientific, USA) and SMV-specific oligonucleotide primers for amplifying a 469bp fragment of viral coat protein gene were used. Amplification steps using Dream Taq Green PCR Master Mix (Thermo Scientific, USA) were performed using a Genetic Research Instrumentation LTD thermocycler (UK).

The obtained sequence of the *CP* gene of Ukrainian SMV isolates from soybean (MG940992) was compared with the sequences available in the NCBI GenBank database using the BLAST program. 33 SMV isolates from different countries were used for the analysis. Nucleotide and amino acid sequences were aligned using Clustal W. Phylogenetic trees for the 469 nt fragment of *CP* gene of SMV isolates were constructed in MEGA 7 by the Neighbor Joining method (Kumar *et al.,* 2018) using the Jukes–Cantor model with 1000 bootstrap replicates to estimate the statistical significance of each node.

To accurately determine the possible seed transmission of SMV, ELISA was performed in soybean germinated seeds and leaves of 14-day soybean plants

grown from these seeds. In the first stage of SMV detection was performed in 3-day seedlings. Then these seedlings were sown in the soil in a laboratory greenhouse. Phenological observations of plants were carried out throughout the growing season until the formation of beans, namely, the number of plants with symptoms of SMV infection on the leaves and plant productivity. In the second stage, ELISA was used to determine SMV antigens in the leaves of two-week-old soybean plants. The results of SMV antigens in the leaves of two-week-old soybean plants grown from SMV-infected seeds were considered reliable. Percent of virus seed transmission (ST) calculated using the formula: $ST= (n\times100)/N$, where n - number of virus infected plants (confirmed with symptoms presence, ELISA and RT-PCR), pcs; N - total number of plants grown from virus infected seeds under laboratory conditions, pcs. Several series of experiments with different soybean genotypes and in different years were carried out in order to accurately establish seed transmission of SMV, namely "seed-transmitted" and "seed-borne" and their impact on plant productivity and yield.

Germination, seed weight and plant productivity were determined by the conventional ISO weighting method (Pask *et al.,* 2012). In previous years (2016-2019), 30 soybean cultivars from five regions of Ukraine were studied.

Statistical analysis of experimental data was performed according to the parametric criteria of the normal distribution of variants, the standard deviation of the mean values - according to the generally accepted method using the computer database management program MS EXCEL.

## RESULTS AND DISCUSSION

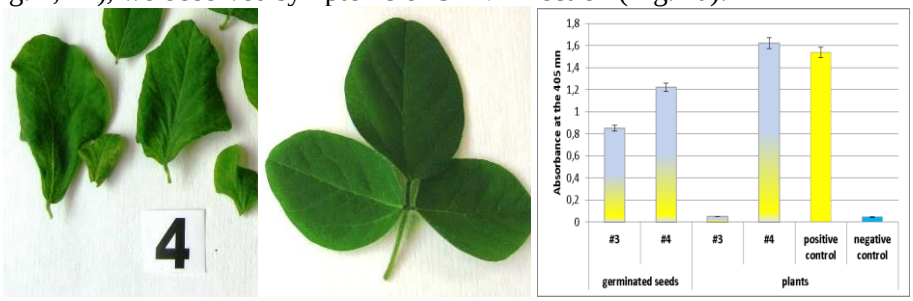
Soybean seeds of two varieties (KEA and Kordoba) were divided into different fractions by color and size. In fig. 1 presents soybean seeds of KEA variety.


**Figure 1.** Seeds of variety KEA: 1 –without spotting; 2 – “butterfly” spotting; 3 – small size and spotted; 4 –big spots

Average weight of seeds was 0.205 g, 0.196 g, 0.137 g, and 0.197, respectively. In germinated seeds of Kordoba variety there were no antigens of SMV (data not shown). DAS ELISA and RT-PCR confirmed SMV presence in germinated KEA seeds #3, 4 and in dry germinated seeds (Fig. 3). To determine seed borne or seed-transmitted infection we revealed the growing-on test was

done. The remaining germinated seeds were planted in the soil in laboratory greenhouse and observations of plant growth and the appearance of viral symptoms were done.

On soybean KEA plants grown in greenhouse from seeds with big spots (Fig. 1, #4), we observed symptoms of SMV infection (Fig. 2a).




**a** **b** **c**

**Figure 2**. SMV symptoms on leaves of soybean plants var. KEA grown from infected spotted seeds #4 (a) and healthy (b); c - detection of SMV by DAS ELISA in germinated seeds and plants of var. KEA

There no symptoms of SMV infection in soybean KEA plants grown in greenhouse from small size and spotted seeds (Fig. 1,#3). DAS-ELISA confirmed the absence of the virus in this sample (Fig. 2c). So, this was seed-borne infection. So, DAS ELISA showed the presence of SMV antigens in soybean KEA plants grown from SMV infected seeds with big spots (Fig. 2c). Thus, experiment shown that SMV was presented in sprouts and cotyledons of small size and spotted seeds (seed-borne infection) and seeds with big spots (seed-transmitted infection). The presence of root nitrogen-fixing nodules in healthy variants of KEA soybeans in contrast to the SMV infected was quite important and interesting, which is clearly visible visually in Fig. 3. The total weight of a healthy plant exceeded twice that of virus infected, namely: for a healthy plant 0.338 g, and 0.164 g for the SMV-infected. Each variant had 50 plants.

**a** **b**

**Figure 3.** Root system of soybean var. KEA: a – healthy with nodules; b – seed-transmitted SMV infection

Results about reduction of root nodules in soybean due to SMV infection are also demonstrated in India (Mandhare & Gawade, 2010).

In case with soybean seeds from Poltava region, seeds were also divided into several groups due to size and color. ELISA revealed SMV in germinated seeds of 5 soybean genotypes: Niagara P-35 (spotted big seeds); Niagara 34 (white seeds of various sizes); Neptun 24 (white seeds of various sizes); Neptun P 25 (both white and spotted seeds of various sizes) (Fig. 4).

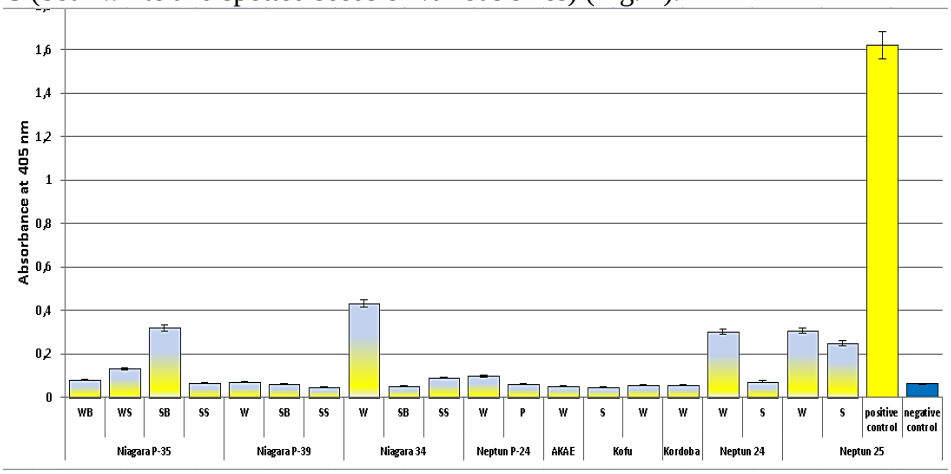



**Figure 3.** Detection of SMV in soybean seed by DAS-ELISA: WB – white big seeds; WS – white small seeds; SB –spotted big seeds; SS – spotted small seeds; W - white seeds of various size; S - spotted seeds of various size


a b c d

**Figure 4.** Seeds germination (a,b) and grown soybean plants (c,d): a,c - var. Neptun 24, white (seed-borne infection); b, d – var. Niagara P39, spotted (healthy), 25th February, 2022

There are no SMV was detected in plants grown from these infected seeds (data not shown). So, the infection is seed-borne. Despite this, in some samples seed-borne infection was revealed to reduce seed germination as seed-transmitted (Fig. 4). In Neptun 24, seed germination under SMV-infection was reduced in 28% -30 %.

In 2016-2019 seeds were collected by the presence of characteristic spotting of various degrees (Fig. 5a, 5b). In some cases seeds were without spotting (i.e. varieties Sultana, Ustya) but in field conditions we observed SMV infection symptoms.

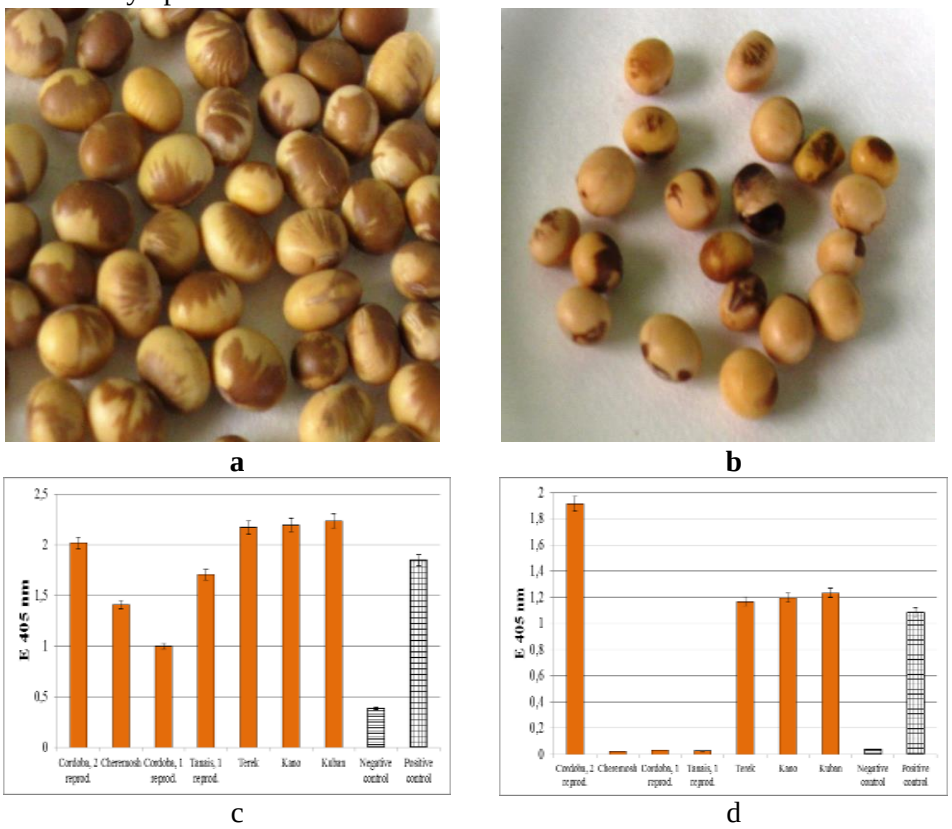



**Figure 5.** Soybean seeds (a,b) and SMV detection by ELISA (c,d): a – var. Medok (healthy); b –var. Kuban (seed-transmitted SMV infection), Poltava region 2016; c - in 3-days-old seed sprouts; d - in 2-week-old soybean plants, grown from SMV-infected seeds

DAS-ELISA results showed that SMV antigens are presented in 3-days-old sprouts of 7 soybean cultivars: Cheremosh, Tanais 1st reproduction, Cordoba 1st reproduction (Lviv region), Cordoba, 2nd reproduction (Sumy region), Terek, Kano and Kuban (Poltava region) (Fig. 5 b). In plants of 4 of them (Cordoba 2, reproduction, Terek, Kano and Kuban) were presented SMV (seed-transmitted) (Fig. 5d, Table 1). SMV symptoms presented also on the Fig. 6.

Our results showed that SMV seed infection (both seed-transmitted and seed-borne) don`t significantly affect the germination rate comparing with virus-free seeds and this parameter was different depending the tested soybean cultivar (Table 1).


a b c

**Figure 6.** SMV induced symptoms: a –var. Hutoryanochka, 21 July 2016; b – var. Kano, seed-transmitted, 10th August 2017; c – Kano, 6th September 2017

**Table 1.** Results on SMV seed transmission

| Variety | Germination, Lab/field,% | SMV transmission | | Dry weight of 1 plant (g) | Soybeans (pcs) per 1 plant (greenhouse) |
|---|---|---|---|---|---|
| | | type | From seed to plant, % | | |
| Volta* | 96/91 | seed-borne | 0 | 0.98 | 2.5 |
| Niagara P 39* | 93/90 | 0 | 0 | 1.16 | 3.5 |
| Neptun P 23* | 72 /69 | seed-borne | 0 | 0.95 | 2.3 |
| Neptun P 24* | 72/ 70 | seed-borne | 0 | 0.94 | 2.5 |
| Niagara – P 35* | 90/88 | seed-borne | 0 | 0.97 | 3.0 |
| Niagara – P 34* | 87/82 | seed-borne | 0 | 0.89 | 2.2 |
| Kofu-1* | 87/90 | 0 | 0 | 1.15 | 3.6 |
| Kofu -2* | 84/81 | 0 | 0 | 1.23 | 3.7 |
| Medok* | 93/90 | 0 | 0 | 1.20 | 3.8 |
| Viola * | 95/92 | 0 | 0 | 1.20 | 4.0 |
| Arika* | 94/93 | 0 | 0 | 1.20 | 3.6 |
| Ezra* | 95/92 | 0 | 0 | 1.15 | 3.7 |
| Kordobe, 1st reproduction, Vinnytsia region* | 95/94 | 0 | 0 | 1.20 | 3,9 |
| КЕА, Vinnytsia region* | 90/86 | seed-transmitted | 1.9 | 0.95 | 2,0 |
| Kordoba-2, Sumy region ** | 92/88 | seed-transmitted | 3.3 | 7.85 | 6.0 |
| Kordoba 2nd reproduction, Poltava region | 95/94 | 0 | 0 | 10.05 | 8.2 |
| Terek, Poltava region, 2016** | 89/84 | seed-transmitted | 10.5 | 6.15 | 5.0 |
| Kano, Poltava region** | 92/88 | seed-transmitted | 2.8 | 7.22 | 5.5 |
| Kuban, Poltava region** | 89/85 | seed-transmitted | 7.1 | 7. 43 | 6.0 |
| Tanais, Lviv region** | 94/91 | seed-borne | 0 | 9.80 | 9.0 |
| Cheremosh, Lviv region ** | 94/90 | seed-borne | 0 | 9.75 | 9.0 |

* winter greenhouse; **spring greenhouse

Liu *et al*. (2022) observed that SMV-infected spotted seed did not change germination rate of susceptible soybean seeds (cultivar NN1138-2). For example, Mandhare and Gawade received the opposite results. They are showed significant

reduction in seed germination (18 to 33%) in SMV infected soybean of different varieties (Mandhare & Gawade, 2010). But it should be mentioned that in our experiment we are showing that despite the fact that SMV doesn't critical affect germination, it reduces dry weight of plants and number of soybeans (pcs) per 1 plant (Table 1). Similar results indicating severe impact of SMV on soybean yield were received by Mandhare and Gawade, which are reported about significant reduction in plant height, number of nodules / plant, 1000 seed weight, seed germination (%) and seedling vigour index in virus infected plants of soybean varieties as compared to control (Mandhare & Gawade, 2010).

Phylogenetic analysis of 459 nt of *CP* gene sequence of soybean isolate named as KHUTP– 16 (Ac. No in the NCBI GenBank MG940992) showed that KHUTP–16 clustered with isolates UA1Gr, Ar33, Lo3, VA2 from Ukraine, Iran and USA (Fig. 7) and shares identity 98.1% by nucleotide sequence and 95.8% amino acid sequence.

Thus, our studies (ELISA, RT-PCR, visual symptoms) on seedlings and plants grown from infected seeds showed that a variety of seed spots do not always indicate its infection with SMV. In our vegetation, laboratory and field studies, seed-transmitted SMV infection was detected in Kuban, Cordoba, Kano, Terek and KEA soybean varieties. Seed-borne SMV infection was detected in genotypes Cheremosh, Tanais, Niagara and Neptun of 1$^{st}$ and 2$^{nd}$ reproductions. SMV was not detected in any of the variants in intact plants older than two months.

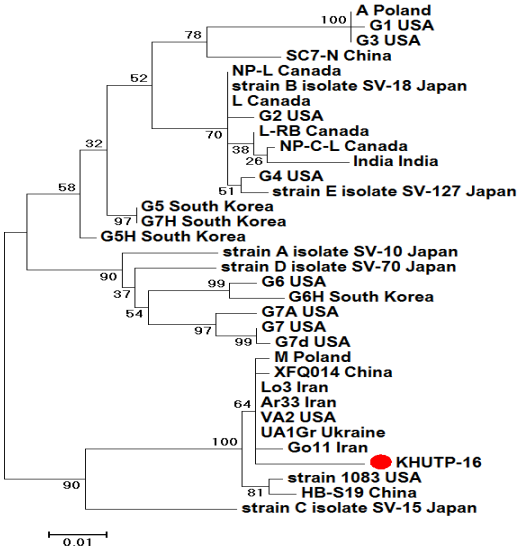



**Figure 7.** Neighbour-joining tree based on nucleotide sequences of 469 nt *CP* gene fragment of SMV isolates. Jukes-Cantor model was performed. The scale bar shows the number of substitutions per base. The studied Ukrainian isolate is marked with red circle

Data on a significantly different rate of seed transmission of SMV isolates are also shown in other countries: 0-2.78% in Poland (Jezewska *et al*., 2015), 0-43% in the United States (Domier *et al*., 2007), 0-67% in Brazil (Porto *et al*., 1975). Bowers and Goodman have shown that both SMV strains transmitted by seeds and those that do not have this ability penetrate the germ of soybean seeds, but only SMV isolates that remain infectious are transmitted through seeds (Bowers and Goodman, 1979). The involvement of CP in this process has been linked to such different abilities for SMV virions stability. It has also been suggested that changes in the amino acid sequence of the HC-Pro protein may affect the protein's ability to assemble the virion or its ability to suppress post-transcriptional gene silence (PTGS) (Domier *et al*., 2007). Summing up the results of multi-series studies, it was found that the highest percentage for seed-transmitted SMV infection have varieties of Poltava region and selection - Terek - 10.5%. Other genotypes (Niagara, Neptun) are also grown in Poltava region but only "seed-borne".

It has been shown that despite the presence of the virus in seeds, isolates do not always have the ability to move from seed to plant, i.e. they are "seed-borne" but not "seed-transmitted". It was found that most SMV isolates do not have the ability to move from seed to plant, the percentage of transmission for them is 0%. Similar results about low per cent of SMV transmission to the leaves of newborn seedlings were demonstrated by Liu and co-authors in China (Liu *et al.*, 2022) comparing to the previous studies which indicated seed transmission of the isolate SMV-SC3 as 0% to 13.68% in various domestic soybean cultivars (Song *et al*., 2015). Liu *et al.* have showed that isolate SMV-SC3 is replicated in the seed coat (brown spot rate was about 44.98%) and had infected the hypocotyl and cotyledon with rate of 1.67% (n=539) of the seeds in the embryo (Liu *et al.*, 2022). It is shown that 5 SMV isolates circulating in Ukraine are transmitted from seed to plant; the percentage of seed transmission for them is 1.9% - 10.5% depending on the genotype of soybean plants and the properties of the virus isolate. Information about different host genotypes and SMV infection is summarized in the review (Usovsky *et al.*, 2022).

## CONCLUSION

Five SMV Ukrainian isolates are transmitted from seed to plant with rate of seed transmission of 1.9% -10.5%. The genotypes of the 2021 harvest from Vinnytsia and Poltava regions have a lower content of "seed-transmitted" compared to 2015. Only 7 isolates from the 60 tested (in winter greenhouse) had a "seed-borne SMV infection", which led to a decrease in germination by 13 % - 28 % and did not significantly affect plant productivity and yield. Therefore, the harvest in Ukraine has increased to 3 t / ha in recent years. In some farms, the yield was slightly lower due to lack of moisture, especially in the flowering and tying phases of beans, which simply fell off during drought. Based on the results obtained, it is necessary to constantly monitor the presence of soybean mosaic virus in the field for seed collection (seed sowing material).

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Material and methods explain how the study was carried: the organism(s) studied; description of the study site, including the significant physical and biological features, and the precise location (latitude and longitude, map, etc); the

experimental or sampling design; the protocol for collecting data; how the data were analyzed. In this section also should be provided a clear description of instruments and equipment, machines, devices, chemicals, diagnostic kits, plants/animals studied, technology of growing/housing, sampling sites, software used etc.

**- RESULTS** followed by **DISCUSSION**

Results and Discussion may be combined into a single section (if appropriate) or it can be a separate section.

The results objectively present key results, without interpretation, in an orderly and logical sequence using both text and illustrative materials (tables and figures).

The discussion interpret results in light of what was already known about the subject of the investigation, and explain new understanding of the problem after taking results into consideration.

The International System of Units (SI) should be used.

**- CONCLUSIONS**

The conclusion should present a clear and concise review of experiments and results obtained, with possible reference to the enclosures.

**- ACKNOWLEDGMENTS**

If received significant help in designing, or carrying out the work, or received materials from someone who did a favour by supplying them, their assistance must be acknowledged. Acknowledgments are always brief and never flowery.

**- REFERENCES (LITERATURE)**

References should cover all papers cited in the text. The in-text citation format should be as follows: for one author (Karaman, 2011), for two authors (Erjavec and Volk, 2011) and for more than two authors (Rednak *et al*., 2007). Use semicolon (Rednak *et al*., 2012; Erjavec and Volk, 2011) to separate multiple citations. Multiple citations should be ordered chronologically. The literature section gives an alphabetical listing (by first author's last name) of the references. More details you can find in the Annex to the INSTRUCTIONS TO AUTHORS / Bibliographic style on the web page of the Journal: www.agricultforest.ac.me.

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