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INFLUENCE OF ESSENTIAL OIL SPEARMINT (*MENTHA SPICATA* L.) CULTURE ON SOIL BIOGENICITY AND DETERMINATION OF ITS ANTIMICROBIAL ACTIVITY AGAINST *ESCHERICHIA COLI*

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Abstract

Soil microbiological and agrochemical indicators were analyzed during biological cultivation of spearmint, in greenhouse conditions, as main indicators of good plant development, studied for antimicrobial activity against Escherichia coli, by testing different variants of plant extracts (decoction, tincture, medicinal wine, medicinal vinegar, medicinal oil). The results of the agrochemical analysis show that spearmint does not have a major impact on the dynamics of macronutrients in the soil. While the biogenicity and activity of enzymes cellulase and catalase increased in the soils with spearmint culture compared to the no-vegetation control. Positive antimicrobial activity against the pathogenic microorganism Escherichia coli was reported for all variants of extracts of spearmint, differing for individual parts of the plant and individual variants of extracts. Root and whole plant extracts showed higher antimicrobial activity compared to leaf and stem extracts. The strongest antimicrobial activity of the plant extracts was found in the medicated oil and medicated vinegar variants and the weakest in the `decoction` variants. The choice of solvent and exposure time likely influence the diameter of the sterile zone.

Key words: *spearmint, soil microorganisms, cellulase, catalase, antimicrobial activity.*

INTRODUCTION

One of the sources of antimicrobial agents of natural origin can be traditional plants, which contain a whole range of pharmacologically active compounds with pronounced antimicrobial action. Currently, these compounds are of particular interest. Plants accumulate in their tissues an arsenal of protective substances necessary for survival in an extreme environment and in an aggressive neighborhood with pathogenic microorganisms. In this regard, the study of the antimicrobial potential of plants is extremely important for humans. Some plant metabolites, such as flavonoids, alkaloids and terpenes, have pronounced antimicrobial activity.

Plant oils and extracts have been used for a wide variety of purposes for many years. Recently, they have attracted widespread interest as a source of natural antimicrobial compounds. Essential oils and plant extracts are of particular interest because they are relatively safe and have well-pronounced antimicrobial properties. Traditionally, people have used crude extracts from various plant parts as medicinal agents. Plant extracts have also been used to treat infectious diseases caused by antibiotic-resistant microorganisms. In fact, herbal medicines have received much attention as sources of beneficial compounds because they are considered to have stood the test of time, and are relatively safe for human use and environmentally friendly. They are also economical and easily available. Last but not least, natural products are of great chemical diversity and could provide an opportunity to create new drugs. They can be used as a source of pure compounds or as standardized plant extracts. All or any part of the plant, such as bark, leaves, roots, seeds and stems, can be incorporated into the creation of a new pharmaceutical product, as most possess antimicrobial properties. Essential oils are produced from various plant parts (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) as secondary metabolites (Palazzolo et al., 2013). Mentha essential oil is mainly produced from the leaves of the plant (Hiruma, 1993; Monte et al., 2001; Gobert et al., 2002; Lorenzo et al., 2002; Marchese et al., 2005; Sartoratto et al., 2004; Bertini et al., 2005; Bieski, 2005; Pľuchtová et al., 2018).

Mentha spicata L. is a well-known herb of the Lamiaceae family, a rich source of essential oils that are widely used in the pharmaceutical industry, cosmetics, and as a spice in food preparation. It is distributed mainly in the temperate and sub-temperate zones of the world. It is widely cultivated for commercial essential oil production (İcan et al., 2002; Pandey et al., 2003; Lawrence, 2007). *M. spicata* has also gained increased scientific interest, primarily as an antimicrobial and antioxidant bioactive natural extract (Mata et al., 2007). *M. spicata* essential oil demonstrated moderate antioxidant activities as well as moderate to weak antimicrobial activities with the best sensitivity observed for Gram-positive bacteria due to its chemical composition of 44 unique oxidized monoterpene compounds (67.2%), followed by monoterpene hydrocarbons (20.8%) (Bardaweel et al., 2018). The main constituents of *M. spicata* essential oil are phenolic compounds such as carvone and limonene (Telci et al., 2010). According to Bardaweel et al. (2018) spearmint oil exhibited the strongest antibacterial activity against *Staphylococcus epidermis* and *Escherichia coli* and weaker antifungal activity against *Candida glabrata*. *M. spicata* essential oil showed a moderate level of antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli*, with *Listeria monocytogenes* being the most sensitive species (Shahbazi, 2015). Oxidized monoterpenes have apparently been reported as a potent antimicrobial agent in the composition of several essential oils (Knobloch et al., 1989). Furthermore, 1,8-cineole and sesquiterpenes have been shown to exhibit significant antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria (Knobloch et al., 1989; Baratta et al., 1998). However, since essential oils contain multiple components, their antimicrobial activity is rather due to additive, synergistic or antagonistic effects of the individual components (Bardaweel et al., 2018).

Essential oils of *Mentha piperita*, *Mentha spicata* var. *crispa* and *Mentha arvensis*, have great antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes* and *Bacillus subtilis* (Horváth and Koščová, 2017; Bokhari et al., 2016; Ullah et al., 2012). *Mentha pulegium* shows activity against *Staphylococcus aureus* and *Enterococcus faecalis* (Aycan et al., 2015). In general, Gram-positive bacteria are more sensitive to the effects of essential oils than Gram-negative bacteria, due to significant structural differences in the cell wall of these two groups of bacteria (Horváth and Koščová, 2017; Cunha et al., 2018; Mancuso et al., 2019). This fact increases the interest in researching the antibacterial activity of rhododendron against Gram-negative bacteria. *Mentha spicata* and other *Mentha* species show activity against Gram-negative bacteria. *Mentha spicata* essential oil has significant antibacterial potential against biofilm cultures of *Vibrio* spp. (Snoussi et al., 2015). *Mentha longifolia* is active against *Salmonella typhimurium* (Heydari et al., 2015). *Mentha pulegium* inhibits the growth of *Pseudomonas* sp., *Escherichia coli* and *Pseudomonas aeruginosa* (Aycan et al., 2015; Moghtader et al., 2016; Sariri et al., 2011). When studying the antibacterial activity of essential oils from *Mentha piperita* and *Mentha spicata* against the species *Enterobacter cloacae*, *Salmonella* sp., *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, the same antibacterial activity against Gram-negative bacteria and varying antibacterial activity against Grm was found - positive bacteria (Pľuchtová et al., 2018). *Mentha spicata* essential oil showed good antimicrobial activity against Staphylococcus aureus and *Escherichia coli* due to the high concentration of carvone (67%) (Scherer et al., 2013).

The use of *Mentha* oils and essential oils in general as natural substances and therefore easily biodegradable may be a promising alternative to synthetic materials to combat increasingly common bacterial infections (Mancuso, 2020). The purpose of the present study is, on the one hand, to determine the influence of the essential oil culture of spearmint on the soil microflora, and on the other hand, to analyze the antimicrobial activity of the plant against the pathogenic bacterial species *Escherichia coli*.

MATERIALS AND METHODS

The experiment was carried out in the greenhouse conditions of the educational and experimental field of the Department of Plant Breeding at the Technical University - Varna.

Before planting the experiment, during the growing season and after harvesting the crop, soil samples were taken, for each of the options, to determine:

- ammonium nitrogen (NH_4–N) and nitrate nitrogen (NO_3–N) - photometric method;
- assimilable forms of phosphorus and potassium - Egner-Riem double-lactate method;
- Soil pH (water extract) - ISO 10390.

For the microbiological analysis, the method of dilution and triplicate inoculation of solid nutrient media was used with subsequent counting of colony-forming units (CFU) in 1 g abs. dry soil (Mishustin and Emtsev, 1989; Malcheva and Naskova, 2018; Nustorova and Malcheva, 2020). Systematic and physiological groups of aerobic microbes - bacilli and non-spore-forming bacteria (on ordinary agar), micromycetes (mold fungi) - on Chapek-Dox agar, actinomycetes and bacteria assimilating mineral nitrogen (on Actinomycetes isolation agar) were determined. The general microflora was determined. The mineralization coefficient was calculated according to the formula: bacteria assimilating mineral nitrogen/(non-spore-forming bacteria+bacilli) (Mishustin and Runov, 1957; Malcheva and Naskova, 2018).

To isolate *Escherichia coli*, a solid culture was made on Endo agar. Typical *E. coli* colonies on Endo agar are dark red with a metallic sheen (Malcheva and Naskova, 2020). Certified reference material was used: *Escherichia coli* WDCM 0090 VT000904.

The agar diffusion method was used to determine antimicrobial activity (Nustorova and Malcheva, 2020). The volume of inoculated extract in each well was 60 µl.

The catalase activity of soil microorganisms was determined by the manganometric method (Khaziev, 1976).

In the laboratory experiment to determine the cellulose-decomposing activity, soil with a thickness of about 7 mm was poured into a Petri dish with a diameter of 10 cm, maintaining 60% PPV/maximum field moisture content/. In each Petri dish, 3 strips of sterile filter paper measuring 10/50 mm are placed on the soil and cultivated at 25°C. During 10 days, the area of the degraded cellulose is recorded with a standard mesh. Average values from the three bands are calculated.

The following variants of spearmint extracts were prepared (Table 1).

Table 1. Extract variants

Variant	Method of preparation of the extract	Parts of the plant used
Decoct	A decoction (potion) is the liquid obtained by boiling the chopped plant product with the necessary solvent, usually water. Recommended for roots, flowers, leaves, twigs, fruits. The extractive solution is filtered while hot.	roots leaves stems whole plant
Tincture	Therapeutic substances are extracted from the chopped herbs by soaking with ethyl alcohol at a concentration of 30%, usually for a time varying between 8-10 days. The operation is carried out in well-closed glass containers. Shaking, for good extraction, is necessary throughout the extraction period.	roots leaves stems whole plant
Medicinal wine	The extraction is carried out in a weak hydroalcoholic environment, at a slightly acidic pH. For preparation, pre-crushed herbs are soaked for 7-10 days in wine (of good quality and well stabilized), after which the preparation is filtered.	roots leaves stems whole plant
Medicinal vinegar	It is obtained by extracting the active substances from herbal drugs with wine vinegar. For preparation, previously crushed herbs are soaked for 7-10 days in vinegar (good quality), after which the preparation is filtered.	roots leaves stems whole plant
Medicinal oil	It is a form of soaking the herbs in olive oil. The duration of soaking is 4-6 weeks. Store in tightly closed glass bottles, in a dark and cool place.	roots leaves stems whole plant

RESULTS AND DISCUSSIONS

An agrochemical analysis of soil samples was made before the sowing of spearmint and at the end of the vegetation of the plant species (Table 2).

Table 2. Agrochemical analysis

Variant	pH	Macronutrients			
		NH_4 mg/kg	NO_3 mg/kg	P_2O_5 mg/100 g	K_2O mg/100 g
Control (no vegetation)	7.26	4.03	8.24	17.1	17.7
Mentha spicata L.	7.21	4.01	5.04	14.0	15.1

After the sowing of spearmint, the values of the digestible forms of N, P and K slightly decrease, but comparing with the limit values for stocking of the soil with available nitrogen compounds, mobile phosphates and digestible potassium, it can be concluded that the soil is poorly stocked with nitrogen, but it has a good degree of storage in terms of phosphorus and potassium, and the soil reaction is slightly alkaline, relatively favorable for the development of gorse.
The biogenicity and individual groups of microorganisms (bacteria, actinomycetes, micromycetes) of the studied variants are presented in Table 3.

Table 3. Quantity and qualitative composition of soil microorganisms (CFU x 103/g abs. dry soil)

Variant	Total micro-flora	Non-spore-forming bacteria	Bacilli	Actinomycetes	Micromycetes	Bacteria assimilating mineral nitrogen	Minera-lization coefficient
Control (before placing the trial, no vegetation)	3259.2	2667.2	400	57.6	134.4	4920	1.60
Mentha sativa L. (at the end of the growing season)	3932.8	2860	452.4	282	338.4	5460	1.65

The results show that the biogenicity of the soils is higher in the variant with vegetation compared to the control (no vegetation). This trend applies to the individual studied groups of microorganisms and, accordingly, to the general microflora. The rate of decomposition of organic matter in soils correlates with the amount of microorganisms.
In both variants, non-spore-forming bacteria take the main share in the composition of the general microflora, followed by bacilli, and the least represented are micromycetes (mold fungi) and actinomycetes (Figures 1 and 2).

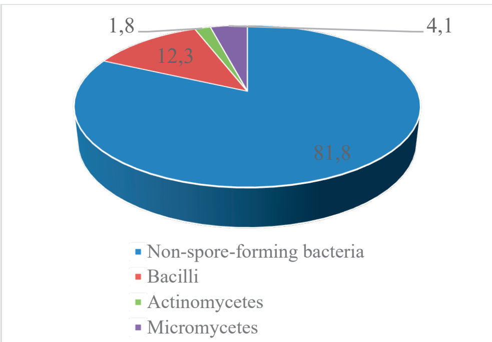

Figure 1. Percentage participation of microorganisms in the composition of the total microflora (control)

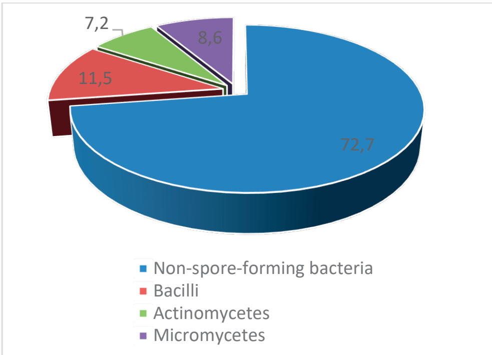

Figure 2. Percentage participation of microorganisms in the composition of the total microflora (*Mentha spicata* L.)

The percentage of non-spore-forming bacteria in the non-vegetated variant is higher, while in the variant with spearmint the amount of this group of microorganisms decreases at the expense of an increase in the amount of actinomycetes and micromycetes. The amount of spore-forming bacteria - bacilli - remains relatively constant in both variants. Non-spore-forming bacteria and bacilli are mainly involved in the initial stages, and actinomycetes and micromycetes in the final stages of decomposition of organic matter.

The catalase activity of the tested variants is presented in Figure 3.

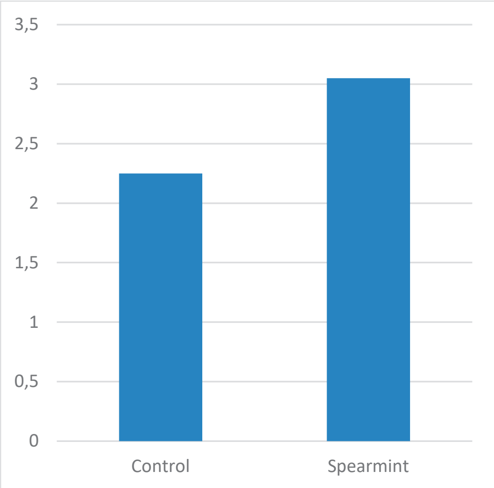

Figure 3. Catalase activity of soil microorganisms ($ml\ O_2/30\ min$)

Catalase is a respiratory enzyme that breaks down the toxic hydrogen peroxide that is released when proteins are broken down. The results show that the catalase activity increases 1.4 times in the variant with sedum compared to the variant without vegetation. Catalase values correlate with the amount of soil microorganisms. There is also catalase of plant origin.
The cellulase activity of the tested variants is presented in Figure 4.

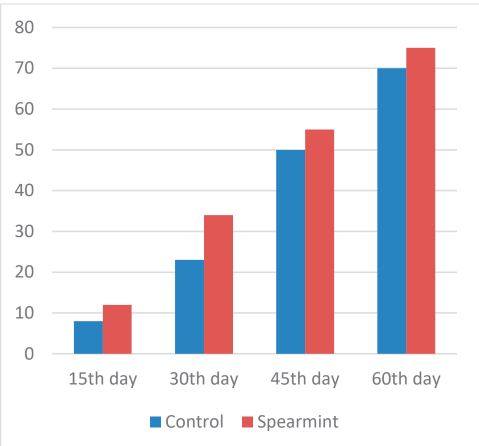

Figure 4. Cellulase activity of soil microorganisms (% degraded area)

Cellulase catalyzes the hydrolysis of cellulose, in which cellulose is initially broken down to cellobiose, which under the action of ß-glucosidase is broken down to glucose. The cellulase activity of the soil microorganisms was followed dynamically for a period of two months under laboratory conditions. The results showed higher cellulase enzyme values in the spearmint variant compared to the control. This trend correlates with the amount, composition and mineralization activity of soil microorganisms.
A number of factors are important for enzyme activity: soil type, soil humidity and temperature, content of nutritional elements, amount and composition of microflora, type of vegetation and others. Microbiological and enzymatic activity are proposed by a number of authors as sensitive soil indicators, including contamination with pathogenic microorganisms (Malcheva et al., 2021; Malcheva et al., 2022, Dilly et al., 2003; Nannipieri et al., 2000; Li et al., 2008; Perucci, 1992; Pascual et al., 1999; García-Gil et al., 2000; Ros et al., 2003; Crecchio et al., 2004; Bastida et al., 2008; Marcote et al. al., 2001; Malcheva, 2014a, b).
The results were negative for the presence of the tested pathogenic species Escherichia coli in both variants, which allowed to test for antimicrobial activity the prepared variants of plant extracts from roots, leaves, stems and whole plant of spearmint (Table 4). Essential oils are produced from various plant parts (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) as secondary metabolites (Palazzolo et al., 2013). Mentha essential oil is mainly produced from the leaves of the plant (Hiruma, 1993; Monte et al., 2001; Gobert et al., 2002; Lorenzo et al., 2002; Marchese et al., 2005; Sartoratto et al., 2004; Bertini et al., 2005; Bieski, 2005; Pľuchtová et al., 2018).

Table 4. Antimicrobial activity against *Escherichia coli* of the studied extracts of spearmint

Variant	Sterile area, cm			
	Roots	Leaves	Stems	Whole plant
Decoct	0.9	0.2	0.2	0.2
Tincture	0.7	0.5	0.4	0.6
Medicinal wine	1.0	0.5	0.8	1.0
Medicinal vinegar	0.5	0.4	0.4	0.5
Medicinal oil	1.1	0.8	1.0	1.1

The sterile zone for extracts of *Mentha spicata* root decreases in the following order: medicinal oil > medicinal vinegar > decoction > tincture > medicinal wine. Compared to the root extracts, the sterile zone of the leaf extracts had lower values, indicating that the rhododendron roots had a more effective effect against *Escherichia coli* compared to their leaves. The sterile zone for spearmint leaf extracts decreases in the following order: medicinal oil > medicinal vinegar = tincture > medicinal wine > decoction. Compared with the root and leaf extracts, the sterile zone of the stem extracts was intermediate between the root and leaf results of the tincture, medicinal vinegar and medicinal oil, indicating that the coriander roots had a more effective effect and the leaves with weaker effect against *Escherichia coli* compared to its stems. In medicinal wine, the same effect against the pathogen was found for leaf and stem extracts. The sterile zone for coriander stem extracts decreases in the following order: medicinal oil > medicinal vinegar > tincture = medicinal wine > decoction. Compared to the rest of the whole plant extracts, the sterile zone is comparable to root extracts - the largest retention zone compared to leaf and stem extracts (except for decoction). It was found that the sterile zone in the decoction variant was greater only in the root extracts, in the leaf, stem and whole plant extracts it was the same and the lowest. The sterile zone for coriander whole plant extracts decreases in the following order: medicinal oil > medicinal vinegar > tincture > medicinal wine > decoction. In terms of antimicrobial activity, gram-negative bacteria (Escherichia coli and others) are more sensitive to coriander oil than gram-positive bacteria (Saygi et al., 2021; Silva et al., 2011). Similar results against *Escherichia coli* were obtained in the study of antibacterial activity of savory oil (Blažeković et al., 2010). A study of the same variant extracts, but from coriander, found higher soil biogenicity and antibacterial activity against E. coli in coriander compared to coriander (Naskova et al., 2023). Joden oil exhibits moderate (Shahbazi, 2015) and strong (Bardaweel et al., 2018)) antibacterial activity against *Escherichia coli*. The antimicrobial activity of spearmint oil is due to its chemical composition - oxidized monoterpenes, monoterpene hydrocarbons, phenolic compounds such as carvone and limonene (Bardaweel et al., 2018; Telci et al., 2010).

The additional conditions and factors - reducing the pH of the medium when using medicinal vinegar and medicinal wine, the inclusion of additional plants - grapes (wine), vinegar (apples, grapes), olives (solvent olive oil for the medicinal oil), the nature of the solvent (Kačániová et al., 2020) also influence the retention zone. In the variants with medicinal vinegar, a general sterile zone is formed around the wells, in the variants with medicinal wine - a bubble halo at a distance of 0.5 cm around each well - probably reactions from the created acidic environment. In the medicated oil variants, the sterile zone increases towards the interior, showing an enhanced effect of combining the root, leaf, stem, whole plant variants and combining the spearmint extracts with olive extract (olive oil). As the time of action of the extracts increases (24 h, 48 h, 72 h), the diameter of the sterile zone increases by about 0.1 cm. Essential oil from the plant *Satureja hortensis* L. manifested varied antibacterial activity against *Escherichia coli*, *Salmonella enteritidis*, and *Bacillus subtilis*, depending on the concentration of essential oil used as well as the type of bacteria (Blažeković et al., 2010).

Some of the options and results are presented in Figure 5.

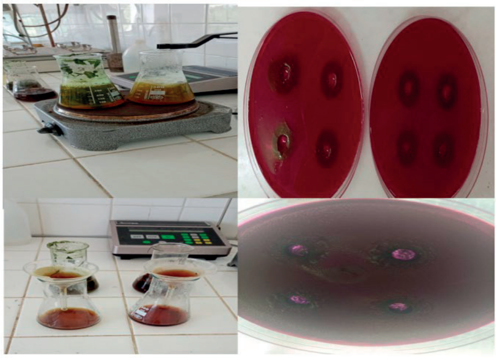

Figure 5. Photo material of some variants and results

CONCLUSIONS

The content of nitrogen, phosphorus and potassium slightly decreases in the variant with spearmint compared to the control, without vegetation, i.e. *Mentha spicata* does not have a major impact on the dynamics of macronutrients in the soil. The macronutrient values obtained were close at the beginning and end of the experiment.

The variants with spearmint increase the biogenicity of soil microorganisms, but in general the composition and percentage participation of the investigated groups of microorganisms is preserved. In all variants, the main share in the composition of the general microflora is occupied by non-spore-forming bacteria, followed by bacilli, and the least represented are actinomycetes and micromycetes.

The values of the enzymes catalase and cellulase correlate with the amount of microorganisms. Their activity is higher in the variant with spearmint compared to the variant without vegetation. A number of factors are important for enzyme activity: soil type, soil humidity and temperature, content of nutritional elements, amount and composition of microflora, type of vegetation and others.

Root and whole plant extracts showed higher antimicrobial activity against *Escherichia coli* compared to leaf and stem extracts. The strongest antimicrobial activity of the plant extracts was found in the medicated oil and medicated vinegar variants and the weakest in the "decoction" variants (except for the root extracts).

A possible reduction in the pH of the medium when using medicinal vinegar and medicinal wine, the inclusion of additional plants, the nature of the solvent, the concentration of the extract and its exposure time also affect the diameter of the retention zone.

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