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The diameter of the bush in the first year of cultivation reached 25-30 cm, at a planting distance of 40 x 30 cm, the rows were not completely closed until autumn, which is unfavorable due to the increased possibility of weed development.

Triple treatment with biostimulators in the first year of thyme cultivation did not significantly affect the quality parameters such as the share of leaves in the dry herb and the content of essential oil in the dry leaf. Share of leaves in dried thyme herb is a parameter that is not subject to significant changes due to agricultural practices, it is stable and genetically determined. In the cultivation of thyme in mountainous areas, the minimum requirements of 50% of leaves in the dry herb is met in all treatments. The average content of essential oil in dry leaf is 2.62 ml / 100 grams of dry leaf, the minimum quality criteria are prescribed in the European Pharmacopoeia of 1.2 ml / 100g and in the German Pharmacopoeia (DAB) with 1.5 ml / 100g of dry leaf are significantly exceeded.

Thyme is a perennial plant that can reach ten years of age in nature, and it usually remains in cultivation for up to three years. The research of the effects of biostimulants on the growth and quality of thyme will continue in the same plantation for the next two years. Additional attention in the research will be focused on the parameters with established positive effects of biostimulants and the quality of dry drugs and essential oil.

Special thanks to the Center for Agriculture and Rural Development of Primorje – Gorski Kotar County in Stara Sušica for cooperation, providing areas for field experiment, purchasing of seedlings and support in conducting research, but particularly for the possibility of growing aromatic and medicinal plants in the hilly areas of Primorje – Gorski Kotar County.

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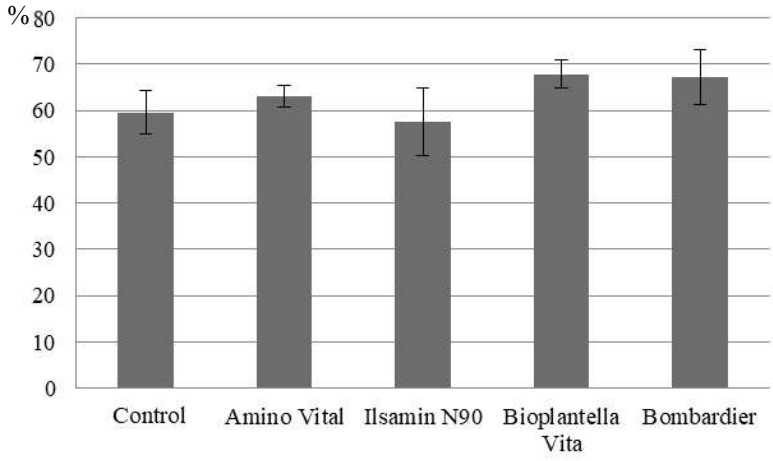
Figure 5. **Percentage of leaves in dry herb**



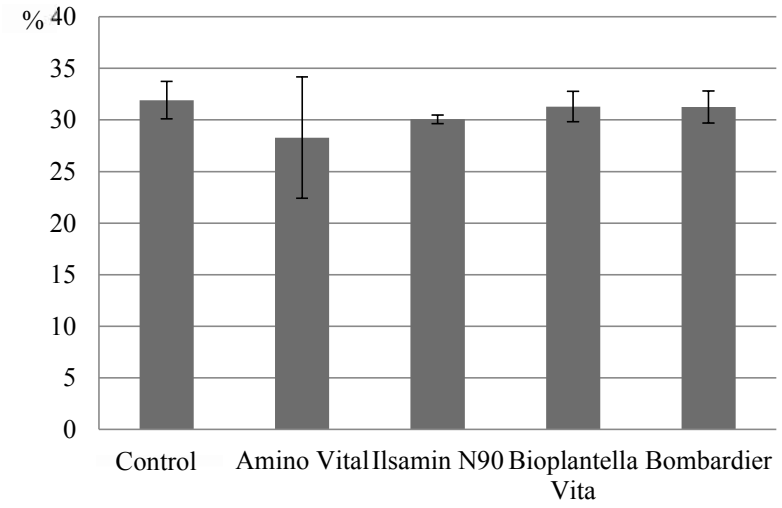
LSD Tukey 5% = 11.03; LSD Tukey 1% = 14.03

The highest amount of essential oil was determined in the treatment with Bombardier biostimulator (2.79 ml/100 g), 2.71 ml/100 g in the treatment with Ilsamin N90; Bioplantella Vita and Amino Vital contained 2.57 ml/100 grams of leaves, and the minimum amount of essential oil of 2.46 ml/100 g of leaves was determined in the control. All treatments in the experiment, including control significantly exceeded the minimum quality criteria of the thyme leaf drug of the German Pharmacopoeia (DAB 2020) with 1.5 and the European Pharmacopoeia (EuPh 10.0) with 1.2 ml/100 grams of dry leaf.

In his research, Juričić (2019) found a significant increase in the content of bioactive compounds (phenols, nonflavonoids and flavonoids) in treatment with biostimulators Humistar, Phyl green and Delphan Plus, while the treatment with AminoVital lowered the content of phenolic compounds, nonflavonoids and flavonoids compared to control and other treatments. Thyme essential oils contain considerable amount of phenolic compounds and it is to be expected that biostimulators will have a significant effect in the second year of cultivation. Naghdi Badi et. al. (2015) confirm the statistically significant effect of amino acid-based biostimulators in 10 and 20% methanol solution on the essential oil content and thymol and carvacrol in *Thymus vulgaris* L. essential oil.

4. CONCLUSION

Based on the analysis of the results in the study of the effects of different biostimulators on the growth and quality of thyme *Thymus vulgaris* L. grown in the mountainous regions of Gorski Kotar, the following can be concluded: in the first year of thyme cultivation, after three treatments with biostimulators, 14 days apart, the positive effect of Amino Vital and Ilsamin N90 was statistically confirmed to affect the hight of thyme shrubs.



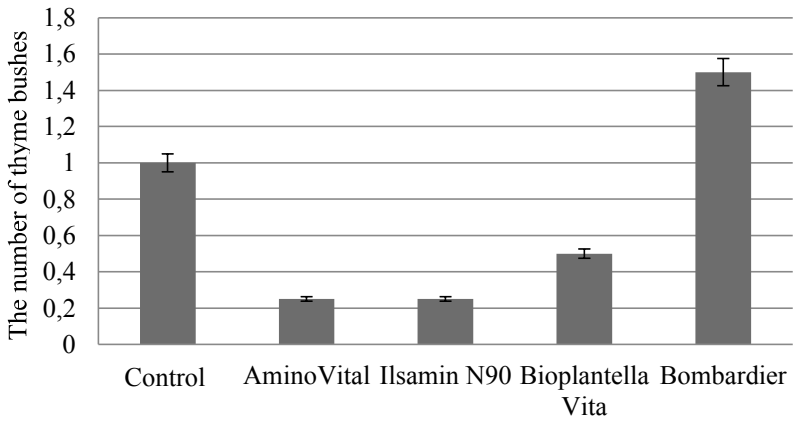
LSD Tukey 5% = 6.3675; LSD Tukey 1% = 8.1005

Of the initial total mass (100%), an average of 30% remains after drying fresh thyme herb (Figure 4). No statistically significant differences in fresh to dry weight ratios were found between the tested treatments in the first year of cultivation and after triple application of the biostimulator.

Naghdi Badi et al. (2015) found a significant increase in dry matter of stems, leaves and young shoots as the effect of the use of biostimulators based on amino acids called Kadostim, Fosnutren, Humiforte and Aminolforte in 10% and 20% methanol solution. Juričić (2019) confirmed the increase in dry matter content in nettle. The dry matter content of nettles was on average 24.78% in the control and 25.19% in the treatment with the biostimulator AminoVital in the herb from the first harvest. In the second harvest, the dry matter content of the herb was increased to 32.99% in the control and 29.86% in the treatment with AminoVital. It is expected that in the second year of thyme cultivation the dry matter content will also increase, especially in the herb from the second harvest of the same year, which is initiated by the growth of thyme in summer, during warm, dry and intensely sunny months.

A significant indicator of the quality of dry herb is the share of leaves in dry herb. The lowest percentage of leaves in the dry herb was found in the treatment with the biostimulator Ilsamin N90 and averaged 57.6 %, the highest in the treatment of Bioplantella Vita with 67.9 % (Figure 5). The percentage of leaves in the control averaged 59.6 %. The content of dry matter has a tendency of increasing in treatments with Amino Vital, Bioplantella Vita and Bombardier. One of the main goals in thyme breeding is to improve the share of leaves to more than 50% in dried thyme herb. All treatments, including control in the experiment met the quality criterion of at least 50 % leaf content in dried thyme herb.

Figure 3. **Loss of thyme bushes per plot**



LSD Tukey 5% = 1.49; LSD Tukey 1% = 1.67

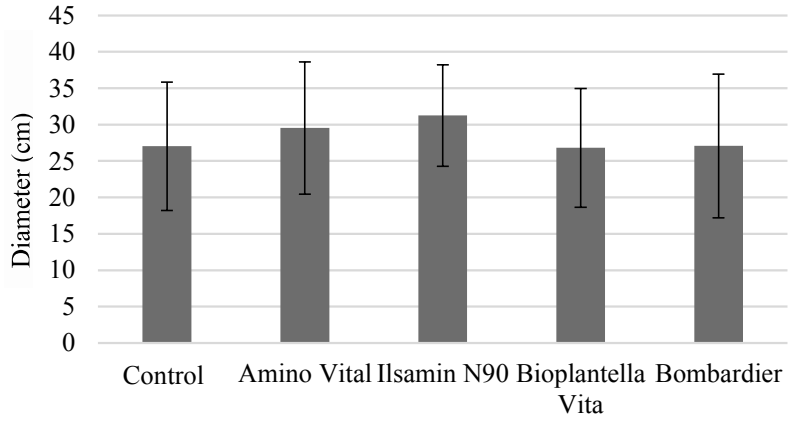
3.2. The effect of biostimulant application on quality parameters of thyme

The quality of aromatic and medicinal plants is defined by the dry matter content, the percentage of leaves in the dry herb and the content of bioactive compounds.

The ratio of fresh to dry mass is an indicator of the loss in mass of the plant of thyme in the drying process. Herbs with a higher water content, i.e., a lower dry matter content, will have a less favorable ratio of fresh to dry mass after drying. Drying of fresh thyme herb is carried out at temperatures of 35-45 ° C to preserve the essential oil to an average moisture content of 10%.

Figure 4. **Fresh to dry mass ratio**

Figure 2. **The effect of biostimulators on thyme bush diameter**



LSD Tukey 5% = 5.948; LSD Tukey 1% = 7.1385

According to research by Juričić (2019), the use of biostimulators Humisar, Phylgreen, AminoVital and Delphan Plus on nettle showed that biostimulants primarily affect net bioactive substances, not on morphometric characteristics of the plant. The highest values in mass, height, number of nodules and number of leaves of a representative shoot were achieved in the treatment of Amino Vital. A statistically significant increase in plant diameter was found to be result of application of the biostimulator Radifarm in the production of pansy seedlings *Viola tricolor* var. *hortensis* DC. Radifarm has been applied in the rhizosphere zone of the seedling, and in its composition contains amino acids, a complex of vitamins, nitrogen, potassium, and iron. A statistically significant increase compared to the control was found in the number of leaves and the number of formed flowers (Zeljković et al. 2021).

The number of dried and dead shrubs in the control plot averaged 1 shrub per 10 m^2 (1000 shrubs per ha), while the highest shrub loss was determined from 1.5 shrubs per 10 m^2 (1500 / ha) in Bombardier treatment (Figure 3). However, the loss of shrubs in the plantation must be attributed to the destruction caused by wild animals, deer, and stags, and after replanting the plantation was fenced with an electric fence, the so-called electric shepherd. In the other treatments with biostimulators, reduced loss of shrubs through drying was noted. Similar results on shrub loss after planting and after the first overwintering of thyme plantations were observed in the cultivation of seven different varieties of perennial winter thyme in a location with milder winter conditions in a location with a maritime influence in northern Europe (Dudaš et al. 2002).

positively affected not only high of the plant, but also other measured parameters of growth.

Kwiatkowski et al. (2020) studied the effect of application of biostimulants in various concentrations. Growth stimulant Bio-algeen, fertilizer Herbagreen Basic, and Effective Microorganisms in the form of EF Farming spray was used on thyme shrubs. Their results also confirmed positive effects on shrub growth and productivity.

In this research, Bioalgeen - a biostimulator based on algae extracts had a statistically confirmed positive effect after double foliar application on growth and yield of thyme shrubs. Matching results were accomplished in research conducted on butter salad, where Bioalgeen had a significant positive effect on growth, total yield, and reduction of non-marketable yield. Additionally, the positive effect was also measured in the content of total dry matter and vitamin C (Dudaš et al. 2016).

The diameter of thyme bushes in the first year of cultivation ranged between 25 and 30 cm. After three consecutive biostimulator treatments, the diameter of the bush tended to increase in treatments with Amino Vital and Ilsamin N90 (Figure 2.). The effect of triple treatment with biostimulators, however, did not lead to a statistically significant increase in bush diameter.

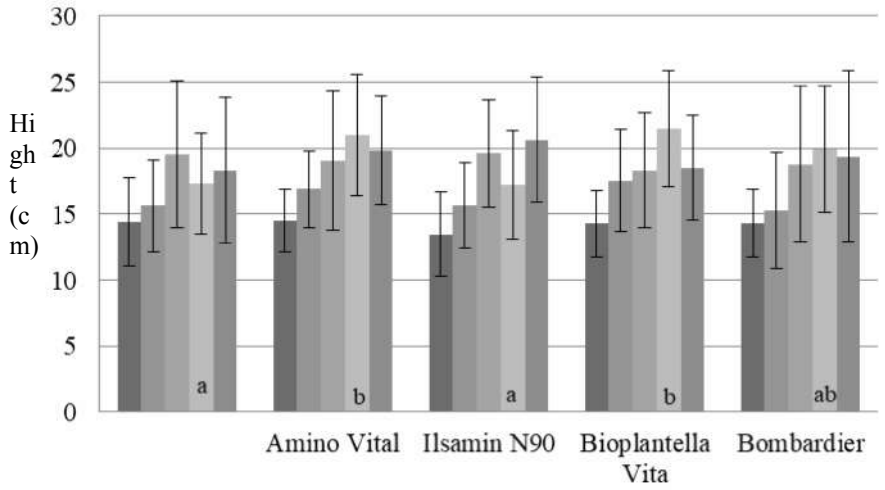
Juričić (2019) obtained similar results and found that the lowest number of nettle shoots per plant was achieved by using the AminoVital biostimulator. He also found significant differences in the number of nods among treatments, but only in the second harvest. The diameter of the bush depends directly on the growth of shoots and the number of nods, i.e., on the activation of lateral buds branching. In the process of harvesting of nettle, the tops of the bush with apical meristems are removed, which inhibit the activation of lateral buds and the development of lateral branches. Therefore, differences in the number of nodules and lateral branches after the first harvest are expected.

The diameter of the thyme bush will increase in the second year of cultivation due to the development of more shoots and due to the removal of apical buds after harvest. Consequently, lateral buds will activate and the develop branches of secondary, tertiary, and higher order.

over 40 cm (Prasanth et al., 2014), but the height of the bush depends on the variety (Dudaš and Böhme, 2013), technology and growing conditions (Dudaš et. al. 2002).

Statistical analysis of the results showed that in the first three measurements there was no statistically significant difference in plant height among the examined treatments. In the fourth measurement, conducted on September 28, 2021, after the third treatment with biostimulators, a significant difference in plant growth was confirmed in the treatment with biostimulators Amino Vital and Bioplantella Vita in relation to control and treatment Ilsamin N90 (Figure 1). No significant differences in bush height were found between control, Ilsamin90 and Bombardier application.

Figure 1. **The effect of biostimulators on the height of thyme bush**



27.07. (LSD Tukey 5% = 2.877, 1% = 4.698); **09.08.** (LSD Tukey 5% = 4.467, 1% = 5.235); **29.08.** (LSD Tukey 5% = 3.487, 1% = 4.179); **28.09.** (LSD Tukey 5% = 3.007, 1% = 3.609); **12.11.** (LSD Tukey 5% = 3.458, 1% = 4.15)

Naghdi Badi et. al. (2015) confirm positive effect on growth of thyme shrubs in parameters such as plant height, number of branches, number of leaves, as well as dry matter of stems, leaves and young shoots as a result of application of biostimulators based on amino acids. A significant increase in thymol and carvacrol was also found to be a result of using biostimulants in 20% methanol solution. The positive effect of biostimulators on vegetative growth, root development and even yields has been also confirmed in other plants. Additionally, biostimulators based on seaweed extracts and enzyme hydrolyzed animal protein (Pepton) have a statistically significant positive effect on root development, vegetative growth, flowering and yields of cherry tomato (Polo and Mata 2018). Polo and Mata (2018) also showed how both kind of biostimulants

Block design of the experiment was as follows: every treatment was carried out in four repetitions on randomly distributed plots of 10 m^2. Every individual plot consisted of five rows with planting distance of 40x30 cm. There were 17 seedlings of thyme planted in every row. Every seedling contained 3 individual plants.

Hoeing and mulching with sawdust, as well as monitoring and analysis of parameters of agro-ecological conditions were carried out in the plantation. The application of biostimulators was performed three times in intervals of 14 days with the recommended dose of 30 ml/10 liters of water per 100 m^2. Morphometric parameters of thyme growth were monitored: height and diameter of shrubs, changes in the number of shrubs per plot, and thyme quality parameters (% share of leaves and essential oil content in dried thyme leaves).

In the first year of thyme cultivation, the planned harvest in the initial stages of flowering was not carried out due to the late onset of flowering of the young plantation in late September and early October. The absence of harvest was due to insufficient time for regeneration of shrubs after harvest and the danger of freezing during the winter. Partial sampling of the above-ground part of the herb was performed in order to control the quality in the first year of thyme cultivation in the mountainous areas of Gorski Kotar.

The collected data was analyzed with the statistical software IBM SPSS Statistics 23, the statistical analysis included ANOVA, Multivariate Analysis of Variance and Tukey test at the significance threshold of p 0.05 and p 0.01.

3. RESULTS AND DISCUSSION

Initially, biostimulators were used only in organic agricultural production, and over time they were integrated into various cultivation systems (Rouphael and Colla 2020). Correspondingly, the demand for biostimulators and the supply of various biostimulators on the market is increasing, and thus more frequent and intensive scientific research on the effects of individual biostimulators on the growth and yield of various crops is necessary. The effect of the application of selected biostimulators on the growth and quality of herb winter, perennial thyme type "Deutcher Winter", an old standard variety of thyme from free pollination is presented in the following text.

3.1. Effect of biostimulators on thyme morphometric parameters

The growth of thyme shrubs under the influence of four different biostimulators in this study was monitored by measuring the height of the bush in five times during vegetation. The first three measurements were performed in the period immediately before treatment with biostimulators. In the first year, the shrubs under treatment reached a height of up to 20 cm on average, while thyme shrubs in control remained lower than 20 cm in height.

Similar results in thyme shrubs growth to a height of approximately 20 cm in the first year of cultivation were achieved in a study of the cultivation of Italian and French thyme in Istria near Pula (Dudaš et al., 2010). Other researches show that thyme rarely grows

Soil analysis was performed before planting. The soil is slightly alkaline, pH 7.8. The results of soil analysis showed a good supply of phosphorus (poor supply of 0-10 mg, optimal 11-20 mg P_2O_5 / 100 g soil), optimal supply of potassium (optimal 11-20 mg K_2O / 100 g soil), high supply of magnesium and total calcium, which is reflected in EC value of 0.23 ds/m. The disintegration of the aggregate is slow, good stability of the aggregate has been determined. Prior to planting, soil preparation and meliorative fertilization with organic pelleted fertilizer was carried out (Bioplantella, 16 kg/per 280m^2). Organic agriculture has been designated as a production system for the cultivation of thyme in this research.

Thyme was planted manually on 11th of June 2021. Approximatly 2000 seedling grown from biological (DEMETER) seeds from free pollination variety were used for forming the plantation. Two-year-old seedlings (Bingenheimer Saatgut A.G.) were grown in containers in standard peat substrate Klasman Steckmedium. Before planting, the seedlings were initially shortened to a height of 6-7 cm. Morphological differences between individual seedlings were observed during pruning.

Field experiment was carried out on 280 m^2 of total area. The research was formed as five blocks experiment – Control (no treatment) and four blocks treated with one of next biostimulators: Amino Vital, Ilsamin N90, Bioplantella Vita and Bombardier. Selected biostimulators belong to the group of biostimulators based on hydrolyzed proteins and amino acids. Table 1 resumes all of the used biostimulators. The deviation from the decelerated content was stated only for biostimulator Bombardier. Among all used biostimulators, only Bombardier has humus extract i.e., it contains fulvo acid (23.1 %)

Table 1. **Biostimulators used in the field experiment**

Biostimulators	**AminoVital**	**Ilsamin N90**	**Bio Plantella Vita**	**Bombardier**
Percentage of free amino acids	25 %	>10 %	-	13 %
Percentage of nitrogen (N)	8 %	8,9 %	-	8,1 %
Manufacturer's description of biostimulator	Plant strengthening agent containing 50% of amino acids and peptides in dry matter, what matches for 8% of total nitrogen	The formulation is based on a set of free amino acids as a result of enzymatic hydrolase	100% natural multivitamin biostimulator with an ideal combination of organic substances, proteins, amino acids, peptides and vitamins	Biostimulator rich in amino acids, micro and macro nutrients and fulvic acid (humic extract)

Source: declared manufacturer's specifications

thyme is divided into two groups; winter perennial and summer, Mediterranean type of thyme. The summer, Mediterranean type of thyme is a perennial shrub in subtropical regions, while in the continental part it is annual due to insufficient resistance to low temperatures.

During history, a diverse terminology has been created for this *Thymus vulgaris* L. in Croatian language; true thyme, grandmother's thyme, wild basil, etc. Morphologically, thyme is classified as a semi-shrub that rarely grows over 40 cm in height. The leaves are tiny differing in shape, color and hair shape between species and subspecies (Prasanth et. al. 2014). It originates from the western Mediterranean region, so it is adapted to a temperate to dry climate with plenty of sun and without excessive precipitation. Thyme is a thermophilic and photophilous plant, varieties of perennial winter thyme are grown in continental and mountainous areas.
Numerous studies on the thyme assortment have shown that today's varieties of winter perennial thyme consist mostly of free pollination varieties, but hybrid high-yielding thyme varieties adapted for cultivation in hilly areas have also been developed (Dudaš and Böhme 2013).

Thyme is recognized for its rich and distinct phytochemical profile. Its phenolic profile mainly consists of flavonoids (zeaxantin, lutein, pigenin, naringenin, luteolin and thymonin) and specific phenolic component named thymol (Dauqan and Abdullah 2017). Additionally, thyme is abundant in the content of other medicinal components, volatile oils, vitamins. Therefore, thyme is characterized by antimicrobic, antioxidative, anticancerogenic, antifungal and antiviral effects.

Due to its high content of volatile aromatic components, thyme has exceptional repellent traits and is consequently rarely attacked by phytopathogens and pests. Its resistance makes thyme perfect for organic production.

As previously stated, the use of biostimulators is in accordance with principles of organic production. Furthermore, the outcomes of biostimulators use on spices, aromatic and medicinal plants has not been sufficiently studied. The aim of this research is to study the effects of biostimulator use in thyme cultivation, through analyzing its impacts on overwintering and frost hardiness, the height of shrubs, the yield of fresh, dry herb and leaf drugs, the percentage of leaves in the herb, dry matter content, essential oil content and total yield of essential oil.

2. MATERIALS AND METHODS

The effects of use of biostimulators on yield and quality of thyme has been conducted in mountain region of Gorski Kotar in cooperation with Center for Agriculture and Rural Development in Stara Sušica. Perennial winter thyme of the "Deutscher Winter" variety has been planted in the plantation. The soil type in Stara Sušica is brown soil on clasts. This terrestrial soil is characterized by automorphic method of wetting, i.e., saturating only by rainwater, with no longer retention of excess water in the soil profile, nor the occurrence of excessive moisture (Husnjak and Bensa 2018). These soil conditions are ideal for thyme cultivation.

Seaweed extracts, containing many active mineral and organic compounds, contribute to plant growth and can improve tolerance to stressful conditions. Hydrolyzed proteins and amino acids indirectly improve enzyme activity, regulate growth regulators and biochemical processes. Additionally, they are a significant source of organic nitrogen and can boost defenses against abiotic stresses. Inorganic compounds have a similar effect, which with their cations and anions of phosphite, phosphate, bicarbonate, silicate, sulfate, and nitrate have a direct effect on plant metabolism.

Biostimulators with microorganisms (bacteria, yeasts, filamentous fungi, and microalgae) isolated from soil, plants, water, or composted fertilizers increase tolerance to stressful conditions, modify the hormonal status of the plant, as well as the hormone production and promote the uptake and fixation of some nutrients (Torre et. al. 2015). Amino acid-based biostimulators are dominant on the Croatian market (Gluhić 2020). The typical composition of these biostimulators consists of free amino acids, micro and macronutrients, sugars, vitamins, cytokinin, auxins, abscisic acids, and betadine. In accordance with their composition, biostimulators are classified as organic fertilizers and soil improvers and are accordingly regulated by the Fertilizers and Soil Improvers Act (NN 163/03, 40/07, 81/13, 14/14, 32/19).

Biostimulators differ from fertilizers because they do not have the main nutritional role. They also differ from pesticides because they condition the plant without protection from pests. According to the Agriculture Act (NN 118/18, 42/20, 127/20, 52/2) biostimulators can be used in organic agriculture since they are obtained naturally. Nowadays we are witnessing the global change in climate and growing conditions bringing new challenges in plant production. At the same time, there is a permanent growth in demand for organically produced goods, particularly organically produced raw material used in pharmaceutical and cosmetical industry. To satisfy this high demand for organic products there is a need for application of new plant conditioning and strengthening agents in order to increase resilience, improve phytosanitary conditions and reduce pesticide use.

According to the Central Bureau of Statistics in the Republic of Croatia (2020), there is 863,000 hectares of used agricultural land. 60.7% of that area is under cereals (524,000 hectares), industrial crops are grown on 20.5% or 177,000 hectares and fodder on 11.9% or 103,000 hectares.

There are 35 different types of spices, aromatic and medicinal plants grown in Croatia grown on 5 – 8 thousand hectares, with positive trend in total area of production. In 2021, the total area under spices, aromatic and medicinal plants was 7,376.70 hectares (Agencija za plaćanja u poljoprivredi 2015-2021). In year 2021, the most common plant species in cultivation were chamomile (6,359.92 ha), immortelle (589.18 ha), hybrid lavender (225.32 ha) and fennel (93.29 ha). Thyme is cultivated on the area of 1.83 ha (2016) to 2.83 ha (2021) (Agencija za plaćanja u poljoprivredi 2016, 2021).

According to the taxonomic classification, thyme is classified in the genus Thymus. Genus Thymus, together with 210 other genera, forms the botanical family *Laminaceae*, one of the most important spice families. The genus *Thymus* contains 18 wild species and one cultivated species (*Thymus vulgaris* L.). Cultivated thyme has more than 300 subspecies, varieties, hybrids, and ecotypes (Mustafa et. al. 2020). The assortment of

EFFECTS OF BIOSTIMULANTS ON *THYMUS VULGARIS* L. 'DEUTCHER WINTER' CULTIVATED IN MOUNTAIN REGION OF CROATIA

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Abstract
Thyme is a perennial shrub whose cultivars enable differentiate cultivation in temperate continental, hilly or Mediterranean areas, and is used as a spice, medicinal or aromatic plant for the production of essential oils. A three-year study of the effects of biostimulants on thyme growth started by establishing a field experiment in June 2021. Biostimulants influences biochemical processes of the plant to enable a fully realization of the genetic potential of productivity due to changes in hormonal status, activation of metabolic processes, increasing dietary efficiency, stimulating growth, development and strengthening the ability to withstand abiotic and biotic stresses. The aim of using different biostimulants as Amino Vital, Ilsamin N90, BioPlantella Vita and Bombardier in field experiment is to strengthen plant resistance and to minimize the use of synthetic chemicals in the cultivation of high quality medicinal drugs. The parameters included in present research overwintering and frost hardiness, the height of shrubs, the yield of fresh, dry herb and leaf drugs, the percentage of leaves in the herb, dry matter content, essential oil content and total yield of essential oil (lha^{-1}). In the first year of cultivation, three foliar treatments with biostimulants were carried out and the initial effects of biostimulants were observed on the growth of shrubs, the height and diameter of shrubs.
Keywords: biostimulators, growth, morphometrical parameters, quality, thyme, yield

1. INTRODUCTION

Biostimulators are substances mostly based on natural material, used in ultrasmall and small doses in order to modify physiological and biochemical processes in plants (Yakhin et al. 2016). The main goal of treatment with biostimulators is to fully realize the genetic potential of plant productivity due to changes in hormonal status, activation of metabolic processes, increase nutrition efficiency, stimulating growth, development and strengthening the ability to withstand abiotic and biotic stresses. According to Torre, Battaglica and Caradonia (2015) biostimulators can be classified into several types according to their basic content; humic extracts, seaweed extracts, amino acids and hydrolyzed proteins, inorganic salts and microorganisms. Humic extracts contain humic acids, fulvic acid and humin formed by humification of organic matter, and their effect is manifested as increasing the efficiency of fertilizers, reducing soil compaction, and overall improving the content of biomass.