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EFFECTIVENESS OF BIOLOGICAL TREATMENT OF ZUCCHINI SEEDS *BACILLUS* SPP. INOCULANT ON MORPHOLOGICAL CHARACTERISTICS OF SEEDLINGS

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Abstract

The challenges of food security and climate change require the development of sustainable methods in agriculture, such as biological seed treatment. This research examines the effect of inoculation of squash seeds (*Cucurbita pepo* L., genotype Fina) with a mixture of strains of *Bacillus* sp. on the morphological characteristics of seedlings. The experiment was conducted in the greenhouse of the Institute for Vegetable Crops Smederevska Palanka during April-May 2024. The seeds were inoculated before sowing, a control (seeds treated with distilled water) was also included. The properties of plant height, steam diameter, width and length of the first true leaf, mass of the above-ground part of the plant and root mass were analyzed. The results show a statistically highly significant effect of the treatment on plant height (17.3 cm in the treatment versus 12.8 cm in the control), while no statistical significance was established for the other traits, but higher values were measured in seedlings of inoculated seeds. Positive correlations were found between the plant height and the mass of the above-ground part of the plant ($r=0.684^{**}$), as well as between the mass of the above-ground part of the plant and the width of the first true leaf ($r=0.546^{*}$). These results indicate the potential of biological treatment to improve the growth of squash seedlings, which is a significant contribution to sustainable production with reduced use of chemical agents.

Key words: zucchini, biological seed treatment, Bacillus spp., inoculation, seedling growth

Introduction

Challenges in the production of health-safe food and sustainability are becoming more and more important in the modern world. Rapid world population growth, climate change and ecosystem degradation are putting pressure on agriculture to ensure sufficient and secure food supply. According to FAO (2020), millions of people suffer from hunger and malnutrition, and by 2050 the population will reach 9.7 billion, which requires an increase in food production by at least 60% (FAO, 2021). Continued climate change and increasing demand for food encourage the search for more effective seed treatment methods that would not only help maintain yields but also ensure their increase. Extreme weather events, the spread of pathogens and soil degradation reduce crop productivity, with fungal diseases accounting for 15–20% of losses. Pathogen resistance to fungicides is increasing (Fisher et al., 2018), which highlights the need for sustainable alternatives, including innovative seed treatment

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technologies such as biological methods. In particular, biological treatments stand out as an environmentally acceptable alternative to chemical methods, using microorganisms (bacteria, fungi and algae) to improve germination, stress resistance and reduce chemical dependence (Stojiljković et al., 2025). These treatments positively affect the microbiological activity of the soil and contribute to the circular economy (Spadaro et al., 2017; Fabiano et al., 2018, Bajagić et al., 2025). Bacteria such as *Bacillus subtilis* and *Trichoderma harzianum* improve biomass plant and germination even under unfavorable conditions (Umapathi et al., 2021; Neto et al., 2021). Methods such as biopriming and microbial consortia increase nutrient uptake and yields (Ahmed et al., 2023). Mycorrhizal fungi improve phosphorus availability (Kandasamy et al., 2020). Biostimulants from waste activate enzymes and hormones, reducing fertilizer use (Khasanov et al., 2020; Song et al., 2023).

Seed preparation and treatment are essential stages of agricultural production, which directly affect germination, plant health, productivity and crop quality. Different technologies - chemical, biological and physical - have specific advantages and disadvantages, and their effectiveness depends on the type of seed, environment and objectives. Hozzein et al. (2019) point out that high-quality seeds ensure better yields, but require measures against seed-borne diseases. Treatments can reduce oxidative stress and improve plant resistance, as shown by studies in wheat and melon (Iqbal et al., 2019; Rady et al., 2019). The genus *Bacillus* has been particularly studied for plant growth promotion, siderophore production, phosphate solubilization and resistance induction (Myresiotis et al., 2014; Kashyap et al., 2019).

Zucchini (*Cucurbita* pepo L.), native to Central America, is an important vegetable crop, but traditional cultivation methods have problems such as uneven growth and low productivity (Martínez-Valdivieso, 2015; Farid et al., 2022). These problems affect the yield and quality of squash and increase production costs. For this reason, the research of seedling conditions in order to improve seedling quality has become an important topic in current agricultural research (Uddain et al., 2019). In their research, Moravčević et al., (2016) came to theconclusion that for the production of early vegetable seedlings with a protected root system should be used. Continuous feeding of seedlings has a positive effect on the photosynthetic apparatus and the development of the root system. This measure shortens the planting period and increases the economy of production (Moravčević et al., 2020). The novelty of the work is the evaluation of the specific inoculant *Bacillus* sp. on squash seeds, which contributes to the development of sustainable methods to improve nutrient absorption and seedling growth, in line with EU sustainable agriculture policies.

The aim of the work is to investigate the effectiveness of the inoculant in improving the growth and development of zucchini seedlings, with a focus on certain morphological characteristics and the correlation between them.

Materials and methods

The experiment was carried out in the greenhouse of the Institute for Vegetable Crops Smederevska Palanka (coordinates: 44°22' N, 20°47' E, altitude 125 m) during April-May 2024. The Fina zucchini genotype was used, created at the Institute for Vegetable Crops Smederevska Palanka. The experiment was arranged in a completely randomized design with three replications. Each experimental unit consisted of 15 plants (15 pots). Two treatments were applied:

1. Control – untreated seeds, soaked in distilled water only (treatment 1).
2. Inoculation with a mixture bacterial preparation of *Bacillus* sp. the commercial name *Bacillomix semennops*, concentration 2.3 × 10^9 CFU/ml; pH 6.5–7.0; light yellow odorless liquid (treatment 2). Zucchini seeds were treated with a 3% solution of the preparation.

Inoculation procedure

Seeds were placed in sealed plastic bags, covered with the appropriate volume of inoculant (or distilled water in the control), and hydrated for 1 h at 18 ± 2 °C with occasional gentle shaking. After hydration, seeds were spread on sterile filter paper, air-dried for 24 h at room temperature in the dark, and subsequently sown. This procedure ensures adhesion of bacteria to the seed coat without affecting germination capacity (Harman et al., 2021).

Sowing of treated and untreated seeds was done in plastic pots (with commercial substrate (Pindstrup Mosebrug A/S, Denmark). Seedling analyzes were performed in the laboratory when plants had 2–3 true leaves (4 weeks after sowing). During the vegetation period, standard agrotechnical measures were applied in the production of zucchini seedlings.

Analysis of morphological traits

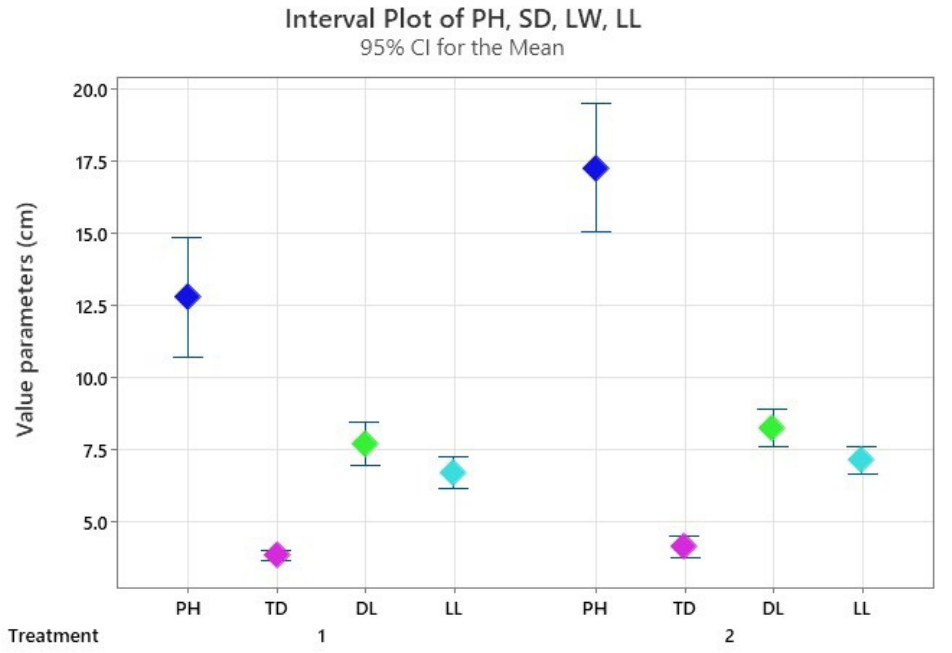
The mass of the above-ground part of the plant and the root mass were measured using a technical scale (IoT-Line Precision Balance 572-31, Kern & Sohn GmbH, Germany); plant height, steam diameter, width and length of the first true leaf with a digital caliper (Wiha Digi-Max W29422, Germany).

Statistical data processing

The data were statistically processed using one-factor analysis of variance (ANOVA) in the IBM SPSS Statistics 26.0 program, at the significance levels $p<0.05$ and $p<0.01$. Comparison of means was tested by LSD (Least Significant Difference Test). In the program Minitab (Trial version) a visual representation of the data (Interval Plot) was made. Each point on the graph (Interval Plot) represents the average value of the examined parameter for a certain combination of factors. Lines extending above and below each point represent the 95% confidence interval for the mean value. It tells about the certainty (95%) that the actual average value of the given parameter for that combination of factors is within that interval. The wider the interval, the greater the variability of those combined factors. *Pearson* correlation analysis was also performed for the relationships between the studied traits. The results are presented in tables and graphs.

Resuts and discussion

In order to have a successful production, it is a necessary that the zucchini seedlings are healthy and vital. The use of microbial inoculants-biofertilizers reduces the use of expensive nitrogen fertilizers, enables the plant to absorb phosphorus more easily, influences the dynamics and direction of microbiological processes that indirectly affect the maintenance and increase of soil fertility (Milošević et al., 2001), thereby increasing soil biogenicity and producing an ecological and highly valuable product (Šević et al., 2025). Glamočlija et al., (2022) state that biopreparations based on bacteria and fungi can be an alternative or supplement to mineral nutrients in the production of cultivated crops. Seed preparation and treatment are important stages of agricultural production, which directly affect seed germination, plant health, productivity and overall crop quality. Seed treatment is the process of preparing seeds for sowing to ensure better germination, growth percentage and disease resistance. Hozzein et al., 2019 state that the use of high-quality seeds is one of the main conditions for obtaining high yields. Studies show that seed treatments can reduce oxidative stress and improve plant resistance (Rady et al., 2019). Pre-sowing seed treatments with beneficial microorganisms have relatively low application costs, as they require one treatment, and the active ingredients are applied in low doses. Seed treatments do not cause any changes within the seed, and the active ingredients provide an advantage to the crop during germination and seedling growth.



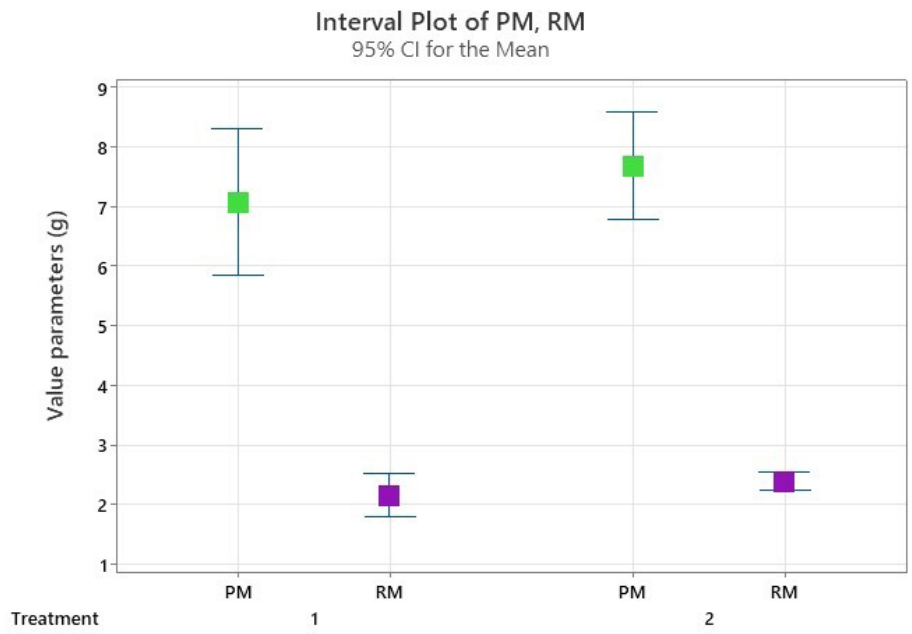
Legend: Treatment: 1- without microbiological inoculant; 2 – with microbiological inoculant; PH – plant height; SD - steam diameter; LW – width of the first true leaf; LL - length of the first true leaf

Figure 1. Interval plot with 95% confidence interval for the average values of plant height, steam diameter, width and length of the first true leaf (cm) in zucchini seedlings

Grafikon 1. Intervalni plot sa 95% intervalom poverenja za prosečne vrednosti visina biljke, prečnik stabla, prečnik i dužina prvog pravog lista (cm) kod rasada tikvice

Graph 1 shows the results of traits related to the morphology of zucchini plants and presents the average values of plant height, stem diameter, width and length of the first true leaf in the control treatment and the treatment where seed inoculation with the *Bacillus* mixture was applied. A well-developed seedling has a plant height of 15-25 cm. The analysis of variance determined the influence and significance of the factors on the examined properties (Table 1). The applied treatment had a statistically highly significant effect on this trait and the plant height was measured at 17.3 cm, while in the control treatment the height was 12.8 cm, which indicates the effect of the treatment on improving the growth and quality of seedlings. The results show that seed inoculation with a mixture of *Bacillus* spp. significantly affects some morphological characteristics of zucchini seedlings. The results are in agreement with the results of earlier studies that emphasize the role of *Bacillus spp*. in promoting growth through hormone production and improved nutrient absorption (Lagunas et al., 2001; Luna Martinez et al., 2013).

The statistical analysis of the data showed that the influence of the observed factor on the investigated properties mass of the above-ground part of the plant and root mass did not show a statistically significant difference in the obtained values (Table 1). The average values (Graph 2) in the treatment with inoculated seeds and the control treatment were approximate and the mass of the above-ground part of the plant was 7.7 g, that is, 7.1 g in the control. The root mass in the treatment



Legend: Treatment: 1- without microbiological inoculant; 2 – with microbiological inoculant; PM – mass of the above-ground part of the plant; RM – root mass

Figure 2. Interval plot with 95% confidence interval for the average value of mass of the above-ground part of the plant and root mass (g) in zucchini seedlings

Grafikon 2. Intervalni plot sa 95% intervalom poverenja za prosečnu vrednost mase nadzemnog dela biljke i mase korena (g) kod rasada tikvice

with applied seed inoculation was 2.4 g, while the measured value in the control was 2.2 g. Savvas et al. (2024) suggest in their work that zucchini roots can be effectively colonized by the tested mixture of microorganisms *Bacillus* sp. when applied via seed inoculation. That study showed that the tested mixture of strains of *Bacillus* sp. has biostimulant activity, because its application through seed preparation had a positive effect on the vegetative growth of zucchini seedlings.

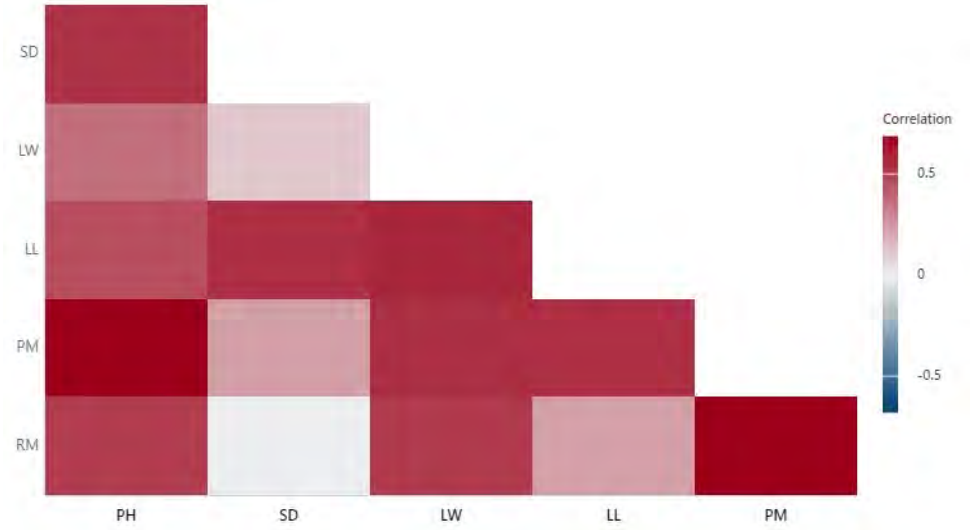
Analyzing the results for steam diameter (0.39 cm in the control vs. 0.41 cm in the treatment), width (7.7 cm vs. 8.2 cm) and length of the first real leaf (6.7 cm vs. 7.1 cm), as well as mass of the above-ground part of the plant (7.1 g vs. 7.7 g) and root mass (2.2 g vs. 2.4 g), no statistically significant difference was found, but higher values of the analyzed trait in inoculated seed plants. *Bacillus subtilis* is a rhizobacterium attributed to the synthesis of phytohormones, such as indoleacetic acid, abscisic acid, gibberellins and cytokinins, which favor root growth and the increase in the number of root hairs (Araujo 2008; Araujo, 2010). In the research results of Neto et al. (2021) and Umapathi et al., (2021) found that by inoculating seeds with *Bacillus subtilis* and *Trichoderma harzianum*, higher values of root biomass were obtained even under unfavorable conditions, while in the results of our research, higher values were determined in the treatment with seed inoculation, but without statistical significance. These results indicate that the effect of the inoculant may be selective, perhaps due to specific experimental conditions or interactions with the substrate, which requires further research in different environments (Ahmed et al., 2023; Song et al., 2023).

Table 1. Analysis of variance for the observed factor and the significance of its influence on morphological traits of seedling

Tabela 1. Analiza varijanse za posmatrani faktor i značaj njegovog uticaja na morfološke osobine rasada

	Parameter	***MS***	***F test***	***Sig.***	**LSD p<0.05**	**LSD p<0.01**
PH	Between Groups	100.352	11.221**	0.004	2.809	3.849
	Total	261.332				
SD	Between Groups	0.002	0.975ns	0.337	0.043	5.829
	Total	0.039				
LW	Between Groups	1.404	1.478 ns	0.240	0.916	1.254
	Total	18.505				
LL	Between Groups	0.968	1.813 ns	0.195	0.686	0.941
	Total	10.578				
PM	Between Groups	1.842	0.800 ns	0.383	1.426	1.954
	Total	43.315				
RM	Between Groups	0.276	1.815 ns	0.195	0.367	0.502
	Total	3.015				

PH – plant height; SD - steam diameter; LW – width of the first true leaf; LL - length of the first true leaf; PM – mass of the above-ground part of the plant; RM – root mass. MS – Mean Square; F test – F statistics; Sig. – significant: $p < 0.01$very significant (**); $p < 0.05$ significant (*); $p < 0.05$ no significant (ns); LSD test (Least Significant Difference Test)



Legend: PH – plant height; TD - tree diameter; DL – diameter of the first true leaf; LL - length of the first true leaf; PM – mass of the above-ground part of the plant; RM – root mass

*Figure 3. Pearson's correlation coefficient between the studied morphological traits in zucchini seedlings. *. Correlation is statistically significant at the 0.05 level; **. Correlation is statistically very significant at the 0.01 level*

*Grafikon 3. Pirsonov koeficijent korelacije između proučavanih morfoloških osobina kod rasada tikvice. *. Korelacija je statistički značajna na nivou 0,05; **. Korelacija je statistički vrlo značajna na nivou 0,01*

Correlation analyzes indicate a positive medium-strong relationship between plant height and the mass of the above-ground part of the plant ($r=0.684^{**}$), as well as between the mass of the above-ground part and the width of the first true leaf ($r=0.546^{*}$), which confirms that the improvement of one trait supports the others. A weak negative correlation between the mass of the above-ground part of the plant and the steam diameter (r=-0.023) indicates possible compromises in development, but without significance. These results support the wider application of biological treatments aimed at reducing chemical dependency and improving sustainability, in line with EU policies (Khasanov et al., 2020; Paulikienė et al., 2025).

Conclusion

Biological treatment of squash seeds with an inoculant based on *Bacillus* spp. shows a positive influence on the growth of seedlings, especially on the height of the plant, while statistical significance was not determined for other traits, but higher values were determined for plants in the treatment with seed inoculation (the mass of the above-ground part of the plant was 7.7 g, i.e. 7.1 g in the control). Seed inoculation with a mixture based on *Bacillus* spp represents a sustainable alternative to chemical treatments, contributing to better productivity and ecological balance. Further research is recommended for adaptation to different production conditions and wider application in agriculture.

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Author contributions

Conceptualization, J.S. and I.T.; Methodology, J.S. and B.Š. Software, B.Š.: Data curation, D.J. Formal analysis, V.R.; Investigation, B.G; J.S. and I.T.; Resources, D.J. and B.G.; Project administration, V.R. and D.T.; Supervision, V.R. and D.T.; Visualization, J.S.; I.T. and B.Š.; Validation, J.S., B.Š. and D.J; Funding acquisition, B.G.; Writing — original draft, J.S.; B.Š. and I.T.; Writing — review & editing, V.R.; D.T. and D.J.

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EFIKASNOST BIOLOŠKOG TRETMANA SEMENA TIKVICE *BACILLUS* SPP. INOKULANTOM NA KLIJAVOST I MORFOLOŠKE OSOBINE RASADA

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Sažetak

Izazovi za bezbednost hrane i klimatske promene zahtevaju razvoj održivih metoda u poljoprivredi, poput biološkog tretmana semena. Ovo istraživanje ispituje uticaj inokulacije semena tikvice (*Cucurbita pepo* L., genotip Fina) smešom sojeva *Bacillus* sp. na morfološke osobine rasada. Eksperiment je sproveden u stakleniku Instituta za povrtarstvo Smederevska Palanka tokom aprila-maja 2024. godine. Seme je inokulisano pre setve, uključena je i kontrola (bez tretmana). Analizirane su osobine visina biljke, prečnik stabla, širina i dužina prvog pravog lista, masa nadzemnog dela biljke i masa korena. Rezultati pokazuju statistički visoko značajan uticaj tretmana na visinu biljke (17,3 cm u tretmanu naspram 12,8 cm u kontroli), dok kod ostalih osobina nije utvrđena statistička začajnost, ali su izmerene veće vrednosti kod biljaka rasada inokulisanog semena. Utvrđene su pozitivne korelacije između visine biljke i mase nadzemnog dela biljke ($r=0,684^{**}$), kao i između mase nadzemnog dela biljke i širine prvog pravog lista ($r=0,546^{*}$). Ovi rezultati ukazuju na potencijal biološkog tretmana na rast irazvoj rasada tikvice, što je značajan doprinos održivoj proizvodnji sa smanjenom upotrebom hemijskih sredstava.

Ključne reči: tikvica, biološki tretman semena, *Bacillus* spp., inokulacija, porast rasada

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