IMPACT OF MICROBIOLOGICAL PREPARATION ON YIELD AND SEED COMPOSITION IN THE INDUSTRIAL HEMP VARIETY FUTURA 75 - PRELIMINARY RESULTS

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Abstract
Hemp cultivation in Slovenia currently takes place on just over a hundred hectares. The predominant varieties are those used for seed and seed-flower production (Futura 75 and Fedora 17), while smaller areas are dedicated to flower varieties. As hemp is promoted as a suitable crop for sustainable agriculture, we aimed to investigate whether we could influence the yield by using a microbiological preparation that is expected to enhance the utilization of nutrients from the soil, thereby reducing the need for conventional fertilization. During the 2022 growing season, we applied a commercial microbiological product containing a consortium of microbial species with the prevalence of the *Pseudomonas putida* strain to half of the test plots in the field trial. Compared to the control group, the group treated with microbiological product exhibited increased height, with indications of differences in root characteristics (larger main root mass, shallower roots with a larger main root diameter, and greater root ramification). However, there were no differences in flower, seed, and stem yields between the two groups. Furthermore, there were no variations in the analysis of basic seed nutrients, fatty acid composition, or CBD and THC content in the dried inflorescence. Given the prevailing drought conditions in 2022, it is essential to conduct additional research on tested microbiological preparation in order to draw credible conclusions and make recommendations for its further use in hemp production.
Key words: hemp, microbial preparations, nutritional composition, fatty acid composition, yield

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VPLIV UPORABE MIKROBIOLOŠKEGA PREPARATA NA PRIDELEK IN SESTAVO SEMEN INDUSTRIJSKE KONOPLJE SORTE FUTURA 75 - PRELIMINARNI REZULTATI

Izvleček
Pridelava konoplje v Sloveniji trenutno poteka le na nekaj več kot sto hektarjih. Prevladujejo sorte za pridelavo semena in kombinacije seme-socvetje (Futura 75 in Fedora 17) ter na manjših površinah sorte za socvetja, katerih sortiment se iz leta v leto spreminja. Ker je konoplja rastlina, ki se promovira kot primerna za trajnostno kmetijstvo, smo želeli preverili ali lahko vplivamo na pridelek z uporabo mikrobiološkega pripravka, ki bi naj povečal izkoristljivost hranil iz tal in tako zmanjšal potrebe po gnojenju. V poljskem poskusu smo v vegetacijski sezoni 2022 na polovici testnih ploskev aplicirali komercialni mikrobiološki preparat, ki vsebuje združbo različnih vrst mikrobov, s prevalenco seva *Pseudomonas putida sp.* V primerjavi s kontrolno skupino je skupina, tretirana s preparatom, zrasla višja, trend je nakazoval razlike v karakteristikah korenin (večja masa glavnih korenin, bolj plitke korenine z večjim premerom glavne korenine in večjo razvejanostjo in gostoto koreninske grude). Pridelek socvetja, semen in stebel se med skupinama ni razlikoval. Prav tako ni bilo razlik v sestavi osnovnih hranilnih snovi v semenu, maščobno-kislinski sestavi semena in vsebnosti CBD ter THC v biomasi socvetij. Zaradi izjemno neugodnih vremenskih razmer v letu 2022 (suše) bi bilo potrebno izvesti dodatne poskuse, da bi lahko podali kredibilne zaključke in priporočila za uporabo preizkušenega komercialnega mikrobiološkega preparata v pridelavi konoplje.
Ključne besede: konoplja, mikrobni preparat, maščobno kislinska sestava, hranilna vrednost, pridelek

1 INTRODUCTION

Hemp, a versatile and environmentally friendly crop, has been gaining significant attention for its potential in various industries, including textiles, food, construction, and wellness products (Adamovičs and Zēverte-Rivža, 2015). With its ability to grow in diverse climates and soils, hemp holds great promise for sustainable agriculture (Sunoj Valiaparambil Sebastian et al., 2023).

Hemp production in Slovenia is decreasing. From approximately 500 hectares in 2015, the hemp cultivation area has decreased to 103.6 hectares in 2023 (Ministry for Agriculture, Forestry and Food of Slovenia - personal communication). French varieties continue to dominate, with Futura 75 and Fedora 17 being the most prevalent. These varieties are primarily grown for seed production (for oil) and for combined seed-flower production. There is a growing trend of small-scale cultivation, focusing on flower varieties such as Eletta Campana, Enectarol, and Midwest. Over the past three years, there has been a significant shift in the assortment. Some varieties that were highly represented in 2021 have practically disappeared in 2023, including Antal, Carmagnola Selected, and Tiborszallasi (Ministry for Agriculture, Forestry and Food of Slovenia - personal communication).

Most hemp production in Slovenia is conducted in an extensive and environmentally friendly manner, 18.42 ha also under organic certification (Ministry for Agriculture, Forestry and Food of Slovenia - personal communication). Hemp is frequently recommended as a crop suitable for resource conservation in agriculture and as a plant adapted to climate change. It is typically grown without the use of herbicides, can withstand high temperatures and elevated CO_2 levels in the atmosphere, and is relatively drought-resistant (Chandra et al., 2011; Tang et al., 2018; Van der Werf, 2004). However, to achieve optimal yields it requires high-quality soils rich in organic matter, nutrients and irrigation in periods of drought (Bajić et al., 2022).

To reduce the use of mineral fertilizers, promote the mineralization of organic fertilizers, and enhance nutrient availability, the use of microbial preparations is becoming increasingly popular in agriculture (Jacoby et al., 2017). Microbial fertilizers harness the power of beneficial microorganisms to improve soil health and enhance plant growth. These microorganisms include nitrogen-fixing bacteria, mycorrhizal fungi, and other beneficial microbes that form symbiotic relationships with plants. The beneficial *Pseudomonas spp.* are ubiquitous in soils and competitively colonize all compartments of the plant microbiome, including the rhizosphere, the surface of aerial organs (phyllosphere), and the inner plant tissues (endosphere). The host plants, in turn, benefit from growth- and health-promoting effects (Balthazar et al., 2022). By incorporating these beneficial microbes into their farming practices, hemp growers, especially those who do not have their own organic fertilizers, might not only boost crop yields but also contribute to a more sustainable and environmentally friendly agricultural future.

In this context, we decided to test whether a commercial microbiological product based on the microbial consortia prevalent in *Pseudomonas putida* strain improves the utilization of nutrients from the organic matter of clover that was incorporated into the soil before hemp sowing. This test aimed to assess the impact of microbial preparation on biomass yield, plant development, and the nutritional quality of hemp seeds.

2 MATERIALS AND METHODS

2.1 Field trial

A randomized block design (3 blocks x 2 treatments) to assess the impact of microbial preparation on hemp yield in a field experiment conducted at GERK PID 964486 (WGS 84 46°22'58,59" N 15°44'19,71" E) was employed during the 2022 growing season. The randomized block design was chosen to account for the expected spatial variability and to reduce the potential bias associated with soil heterogeneity. The chemical analysis of soil showed a pH of 6.09, 42.5 mg/100g P_2O_5, 31.5 mg/100 g K_2O and 4.13% of humus.

Cultivated field had a prior crop of black clover, which was ploughed under in spring before the field preparation for the hemp sowing. In this experiment, half of the designated plots received an application of a commercial microbiological consortia preparation (MICROB). The total number of microorganisms is declared as minimum of 4.10^9 CFU/cm^3. The consortia consist of various microbial specimens like *Azotobacter chroococcum, Azospirillum brasilense, Bacillus megaterium* and prevalent *Pseudomonas putida*. The general objective of this preparation is to expedite the decomposition of post-harvest residues, straw, and organic matter within the soil matrix, concomitant with enhancing the assimilation of soil-borne nutrients. Conversely, the remaining half of the plots remained untreated, serving as the control group for comparison (CONTROL). All applications were administered during the evening hours, with subsequent seedbed preparation and the actual sowing performed post-application. No ordinary fertilizers were used.

The chosen hemp variety for seed production was Futura 75. Sowing density was 35 kg/ha. Sowing was conducted on 12^{th} of June 2022 using a conventional grain seeder with inter-row spacing of 25 cm (every other row closed). Test plots measured 4x4 m each, samples were taken from one square meter in each plot.

2.2 Data Collection

Data collection involved monitoring crop growth parameters, pests, diseases and weeds throughout the growing season. The sampling was carried out on 26^{th} of September 2022 after 98 days from sowing. We collected the plants manually from 1 m^2 in each block and measured plant height, seed, stem, main root and inflorescence yield, diameter of stems and main root, CBD content, basic nutrient content and fatty acid composition of seeds. Weeds were identified and their mass was determined. The seed yield in manual sampling was much higher than in harvest with combine harvester. Ten days after the samples were taken, the whole field was mechanically harvested for seed, namely. The yield of mechanically harvested seed was only 30 % of the manually harvested (calculated per hectare). This loss can be contributed to mechanical disturbances (seeds falling down) and bird consumption.

2.3 Fatty acid analysis of hemp seeds

Fatty acid analysis of hemp seeds was performed in laboratory of Department of Animal Science, Biotechnical Faculty, University of Ljubljana. Hemp seeds were ground using laboratory homogenizer Grindomix (Retsch). The fatty acid composition of seeds was analysed using a gas chromatographic method after the *in-situ* transesterification of lipids, as described previously by Park and Goins (1994). Briefly, fatty acids seed samples (approximately 0.5 g) were transmethylated using 0.5 M NaOH in methanol followed by 14% BF_3 in methanol. Fatty acid methyl esters were extracted using hexane. For separation of fatty acid methyl esters, an Agilent 6890 gas chromatograph equipped with an DB-FATWAX UI (30 m × 0.32 mm i.d. × 0.25 µm, Agilent, Santa Clara, CA) and flame-ionization detector was used. Instrument was calibrated using Nu-Check-Prep (Elysian, MN) GLC reference standards 411, 85, 68A and 674. Total fatty acids in seeds were calculated according to Ministry of Agriculture, Fisheries and Food (1998) by multiplying fatty acid proportion with lipid content in samples.

2.4 Proximate analysis of hemp seeds

The relative amounts of protein (AOAC Official method 954.01), lipid (AOAC Official method 954.01), water (AOAC Official Method 934.01), and ash (AOAC Official method 942.05) in seed samples were estimated by proximate analysis in the laboratory of Department of Animal Science, Biotechnical Faculty, University of Ljubljana. Carbohydrate content in seeds was calculated by difference (100 – water – lipids – proteins – ash).

2.5 Cannabinoid analysis of inflorescence

Cannabinoid content was measured by Spectral Fingerprints laboratories (SFP d.o.o., Medenska cesta 4a, 1000 Ljubljana, Slovenia) with gas chromatography with flame ionization detector (GC-FID full spectrum_v1.0).

2.6 Statistical Analysis

The two-way t-test was performed using SAS STAT software to compare the means of the MICROB and CONTROL group. The analysis included the calculation of the t-statistic, degrees of freedom, and the corresponding p-value. Prior to conducting the t-test, several assumptions were checked, including normality of the data distribution, homogeneity of variances, and independence of observations. Normality was assessed using the Shapiro-Wilk test. The data of fatty acid composition was transformed by arcus sinus of square root to ensure normal distribution.

3 RESULTS AND DISCUSSION

The average daily temperatures throughout the hemp growing season ranged between 10 and 29°C, with three heatwaves (June, July and August) during which the average daily temperature did not drop below 20°C. The longest heatwave period lasted from mid-July to mid-August (Table 1). In comparison, the thirty-year (1981–2010) average daily temperatures for the mentioned months were 18.6°C, 20.4°C and 19.6°C, respectively (ARSO, 2023). Simultaneously, in 2022 there were extended periods without precipitation, with the longest one occurring from mid-July to early August and the last one in the first half of October (ARSO, 2023). The total amount of participation in 2022 at the nearby meteorological station was 742.4 mm (Table 1), which is 190 mm less than the thirty-year average. The hemp crop visually appeared to be in better condition than the adjacent corn crops, which showed significant damage due to drought.

Table 1: Average and maximum temperatures, sunshine hours, and precipitation levels at the meteorological station at the airport »Edvarda Rusjana Maribor« (lon=15.6818, lat=46.4797, height=264m) (ARSO, 25.10.2023).

2022	Ave. T [°C]	Ave. max T [°C]	Abs. max T [°C]	Sunlight duration [h]	Precipitation [mm]
Jan	0.8	6.6	14.2	137	32.3
Feb	4.2	10.8	16.2	164.3	12.3
Mar	4.4	12.1	22.7	229.4	5.6
Apr	9.5	15.2	22.9	213.7	87.7
May	17.3	23.1	29.7	236.3	69.2
Jun	21.8	27.2	33.7	296.7	73.3
Jul	22.6	29.2	35.7	312	53.7
Aug	21.7	28.4	35.2	240.5	78.8
Sep	15.3	20.8	28.4	154.3	162.7
Oct	13.1	20	23	186.7	17.2
Nov	6.7	11.2	23.9	84.2	55.6
Dec	2.1	5.3	16.5	61.3	94
Year	11.6	17.5	35.7	2316.4	742.4

Hemp plants have grown significantly higher in the MICROB group (Table 2). Also, the yield of main roots showed a trend to be higher in the MICROB group (Table 3). The main roots in MICROB group tend to be shorter, with higher percentage of high ramification (28.3 % vs 19.7 % in CONTROL, data not shown) and wider main root diameter (Table 2). On the contrary, the results show numerically higher yield of aboveground biomass in the CONTROL group, however with no statistical significance (Table 3). This is probably connected with higher density of plants in this group. The density of plants was very variable, pointing out either problems with seeding or germination. *Pseudomonas putida* is a plant growth promoting rhizobacteria (PGPR) that establishes commensal relationships with plants. The interaction involves a series of functions encoded by core genes which favour nutrient mobilization, prevention of pathogen development and efficient niche colonization (Molina et al., 2020). PGPRs were capable to improve growth and development, secondary metabolite accumulation and antioxidant capacity or Finola variety under glasshouse conditions (Pagnani et al., 2018). *Pseudomonas* sp. inoculation also increased the photosynthetic rate at the vegetative and reproductive stages, and the harvest index of a hemp (Lyu et al., 2019). Inoculation with other microorganisms, for example *Trichoderma harzianum,* positively affected root density, inflorescence number, fresh weight moisture and compactness of inflorescence and cannabidiol (CBD) content in Fedora 17 and Felina varieties (Kakabouki et al., 2021). Slow-release potassium fertiliser with added soil microorganisms increased stalk diameter, height, shoot dry weight and the contents of tetrahydrocannabinol (THC) and similar cannabinoids (De Prato et al., 2022). The problem in microbial effect on hemp yield might be in the periods of drought, which could negatively affect the microbial activity. *Pseudomonas putida* has very low tolerance to air desiccation in comparison to other bacteria. Desiccated state has a decreased cell size, retracted cytoplasm and damaged membranes which can be repaired after prolonged rehydration or rehydration in the presence of plant root exudates (Pazos-Rojas et al., 2019). On the other hand, *Azotobacter chroococcum* strains, also present in the used microbial preparation, exhibited high tolerance to drought stresses and could alleviate the negative effects exerted by abiotic stress in tomato plants (Viscardi et al., 2016).

The conclusion of this preliminary trial is that in the following field trials, the microbial identification and activity should be measured alongside other parameters to confirm the effect of microbial preparations on plant production.

Table 2: Measurements of crop density and hemp plant height on microbially treated and untreated field areas (mean ± std err)

	Plant density (1000 plants/ha)	Plant height (cm)	ϕ of stem (mm)	ϕ of central root (mm)	Root length (mm)
CONTROL	550.0 ±23.0	129.0±3.2	6.05±0.14	5.96±0.35	201.10±3.00
MICROB	463.3±117.2	144.8±3.9	7.15±1.00	7.11±0.57	189.20±5.81
P-value	0.538	0.037	0.385	0.052	0.166

Table 3: Yield (kg dry matter per hectare) of hemp biomass on microbially treated and untreated field areas (mean ± std err)

	Yield of inflorescence (kg DM/ha)	Yield of seeds (kg DM/ha)	Yield of stems (kg DM/ha)	Yield of roots (kg DM/ha)
CONTROL	2000.6±450.4	1765±141.6	3600±236.8	235.0±20.7
MICROB	1598.7±361.0	1487±70.5	3492±419.8	396.3±49.0
P-value	0.297	0.179	0.836	0.064

We also sent the samples to the lab for informative cannabinoid testing, although the hemp in this case was grown for the seed production and cannabinoids were of no commercial interest. This is why we just made a joint sample from all three plots in each treatment/group. The percentages of CBD and Δ9-THC in both tested groups were identical, 0.82 % and 0.03 % of dried inflorescence mass, respectively. Thus, the CBD yield per hectare was 16.40 kg in CONTROL and 13.03 kg in MICROB group. Statistical analysis was not performed as we took only one average sample from all three plots from each group. The CBD content of our samples was higher if we compare it to the results of Burgel et al. (2020), where Futura 75 reached 0.15 % of CBD in inflorescence, yielding together 3,9 kg of CBD per hectare. The CBD content can be affected by several factors (weather conditions, daily light, fertilisation, time of harvest, stress etc.) thus CBD content of Futura 75 variety can vary from 0.14 %

to 2.2 % in the time of harvest (Folina et al., 2020; Hillig & Mahlberg, 2004; Pavlovic et al., 2019). From other cannabinoids and terpenes measured only cannabichromene (CBC) was detected in small percentage (0.04 % in both samples), all others were below detection level.

Table 4: The proximate analysis of hemp seeds (g per kg of dry matter (mean ± std err))

(g/kg DM)	CONTROL	MICROB	P-value
Crude fibre	275.87±4.73	279.15±3.97	0.446
Crude ash	58.46±0.97	60.95±0.68	0.112
Crude fat	302.73±4.74	296.66±2.36	0.336
Crude protein	265.0.5±3.39	266.37±0.94	0.741
N-free extract	98.73±2.59	96.88±5.20	0.771

Proximate analysis showed no differences between the nutrient seed composition of two test groups (Table 4). The approximate hemp crude fat, protein and fibre content was around 30 %, 27 % and 28 %, respectively. The fatty acid profile of the seeds also showed a typical Futura 75 profile with no differences between microbially treated or untreated group (Table 5). The oil extracted from hemp seeds is highly nutritious because it is rich in polyunsaturated fatty acids (mostly linoleic acid, α-linolenic acid, oleic acid, and some rare fatty acids like γ-linolenic acid and stearidonic acid), some of which are essential and must be acquired from diet (Alonso-Esteban et al., 2023; Leizer et al., 2000). There are some studies which demonstrate that the use of *Pseudomonas* spp. inoculants increased seed yield, oil yield and/or composition in desirable fatty acids in other oilseed crops including sesame, sunflower, flax, soybean, canola, and corn gromwell (Balthazar et al., 2022). To our knowledge, this was not yet demonstrated for hemp. Additionally, more research is still needed to investigate the largely unknown mechanisms resulting in these alterations of seed oil content and composition by microbes.

Table 5: Fatty acid profile of hemp seed (g per 100 g of total fatty acids (mean ± std err))

FA (g/100 g)*	CONTROL	MICROB	P-value
SFA	9.46±0.15	9.43±0.07	0,872
MUFA	10.95±0.23	10.8±0.16	0,637
PUFA	79.58±0.38	79.75±0.23	0,719
n6 PUFA	56.63±0.26	56.68±0.16	0,871
n3 PUFA	20.38±0.42	20.53±0.22	0,777
n6/n3 ratio	2.78±0.07	2.76±0.03	0,835
γ linolenic acid	2.56±0.23	2.54±0.04	0,951
Stearidonic acid	0.96±0.09	0.96±0.002	0,966

*For assessing normality of the data, the data was transformed by arc sinus square root transformation. The data presented in the table is means as gram per 100 grams of fat.

4 CONCLUSION

Unfortunately, based on preliminary one year experiment we cannot confirm any effective influence of used microbial consortia commercial preparation on measured parameters except plant height and a trend in changes of some root parameters. There are many possible reasons for such outcome, however none of them was confirmed. We did not test the viability of microbes in the preparation, neither did we have the possibility to measure the microbial populations in the soil during the vegetation season. Due to the drought conditions, various microbes in the consortia could be differently affected due to their different susceptibility to dry conditions. The microbes are not necessarily destroyed by drought, but can become temporary less active (depending on the species). However, there is still scientific evidence that even in drought stress conditions, plants benefit from microbial consortia applications to soil. We can conclude that the effectiveness of various new microbial commercial preparations should be regularly evaluated in field trials as outdoor field experiments are lacking and these preparations will gain more and more popularity in the future.

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