




Influence of different methods of application of effective microorganisms in nutrition of wheat on weight by 1000 grains, yield, and content of crude wheat proteins (*TRITICUM SP*)

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Abstract

The research was carried out in three vegetative seasons 2015/16, 2016/17, and 2018/19, on an experimental plot in Padinska Skela, Belgrade, Serbia. The supplement included four NPK treatments (kg ha⁻¹) and foliar treatment with effective microorganisms (EM 7 l ha⁻¹) T1-129: 60: 60; T2-129: 60: 60 + 1 EM; T3-106: 60: 60 + 2 EM; T4-83: 60: 60 + 3 EM. Four wheat genotypes were sown (Ratarica, Pobeda, Nogal, and Apache). Thousand grain weight and grain yield were determined. The thousand grain weight (TGW) was in the variety Pobeda 43.73 g, and the highest grain yield was in the variety Ratarica 7.02 t ha⁻¹. Both varieties are medium late, bread varieties. The treatments have different effects on the studied variables. The TGW was the highest in the T2 treatment of 40.81 g and the grain yield in T3 of 6.62 t ha⁻¹, which was at the level of statistical significance of $p > 0.01$. The crude protein content was highest in the treatment of T2 13.93% ($p > 0.01$).

Keywords Wheat nutrition · Genotypes · Mass of 1000 grains · Wheat grain · Proteins

Introduction

In addition to corn and rice, wheat is the most common grain in the human diet. In human nutrition, wheat participates with 53% in developed countries, and with 85% in less developed countries, of the total world production. (Pena 2007; Đurić et al. 2015). It is an important source of food in regions with rapid population growth, such as Africa, Asia, and Latin America (Donn et al. 2015). Climate change will

expand the area of cereal cultivation while reducing yields in some southern parts of Europe (Lotze-Campen 2011). Gregory and George (2011) report that a 67% increase in world wheat production will be needed to keep pace with world population growth by 2030, where 21% yields by cultivating new areas, while 12% refers to intensive production of existing areas. The production and excessive application of N fertilizers contribute to a number of environmental problems (Xia et al. 2017). In order to meet the requirements of sustainable production for the preservation of the environment in the production technology, the use of microbiological preparations is of increasing importance. Previous research has shown that wheat can form a good association with diazotrophs in the soil. Using inoculation of wheat seeds with associative diazotrophs (*Azotobacter chroococcum*, *Azospirillum lipoferum*, *Beijerinckia Derox*, and *Klebsiella planticola*), the grain yield can be increased by 11.64% as well as the protein content in the grain by 6.40% (Cvijanović et al. 2008). According to research by Andres et al. (2009), inoculation of wheat seeds with species from the genera *Azospirillum* and *Pseudomonas* can increase root biomass by 40% and grain yield by 16%. Inoculation of wheat seeds and intensification of plant germination increase the activity of the indigenous rhizosphere

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microbial population (Cvijanović et al. 2007), which synthesizes substances that promote growth, siderophores, and antibiotics, which reduce the dependence on nitrogen and phosphorus fertilizers (Ahemad and Kibret 2014). Roljević et al. (2018) in 3-year research on the application of different groups of microorganisms in the nutrition of wheat and alternative cereals in the organic system, found an increase in grain yield by 13.9%. Salmabi et al. (2018) found that the use of mixed culture as an inoculant of wheat seeds with active species *Azotobacter*, *Agrobacterium*, *Bacillus*, *Pseudomonas*, *Trichoderma*, *Azospirillum*, and *Rhizoglyphus irregularis* can significantly increase grain yield at the level of $r < 0.05$ in relation to control. In recent years, more and more research has focused on the use of biostimulators that include live microbiological cultures, microbial extracts, products of their metabolism (hormones, antibiotics, indoleacetic acid, lactic acid, etc.). Biostimulants can stimulate plant growth and yield even when used in small amounts, so they can be used complementarily. Recognizing the need to use biostimulants in agriculture, the European Council of the Biostimulant Industry (EBIC 2012) recognized the need to establish a legal framework for the regulation of these products. Biostimulants are defined in the draft EU regulation 2016 as nutritional materials that affect nutritional processes regardless of their own nutrient content (Joner et al. 2019).

Biostimulators with effective microorganisms (EM) are increasingly used in agricultural production. Effective microorganisms are mixed cultures consisting of photosynthetic bacteria, lactic acid bacteria, yeasts, actinomycetes, and fermentation fungi. In the system of low input production, effective microorganisms are gaining more and more importance and occupy a significant place in agriculture (Calvo et al. 2014). The use of different groups of microorganisms can alleviate plant stress from abiotic factors, which is very important in times of climate change. Effective microorganisms belong to a group of microorganisms known as PGPR, because they produce growth hormones known as auxins, gibberellins, cytokinins, ethylene, and abscisic acid. Most research is focused on the incorporation of effective microorganisms into the soil. When introduced into the soil, they enhance the activity of mycorrhiza, suppress pathogenic fungi and nematodes, mineralize organic matter, promote and enhance indigenous microbial flora (Kumar and Sai Gopal 2015; Cvijanović et al. 2017). Effective microorganisms can be applied by foliar sprays, stimulating the activity of beneficial microorganisms in the plant phyllosphere. Yue et al. (2002) obtained an increase in the rate of photosynthesis in functional leaves during mid-vegetation by foliar application of EM in soybeans. They can be used as inoculants to increase the diversity of microflora and have an influence on physical and chemical characteristics of soil, decompose organic matter, stimulate plant growth and yield, decrease effects of monoculture, protect plants from diseases, and

help remove physiological disorders in plants (Babalola 2010; Mayer et al. 2010; Schenck and Müller 2009).

Given that low input is the world's commonest method of production (low external input/low-input sustainable agriculture), the aim of this paper is to determine the importance of the use of effective microorganisms on grain yield and thousand grain weight.

Materials and methods

Design of experimental research

Experimental research was conducted during 2017–2019 in the experimental plots of the Institute of PKB Agroekonomik in Padinska Skela (ΨN 44° 56', λE 25° 28'). The area of the experimental plots was 576 m², and the individual plots 5 m². The plots are arranged according to the plan of divided plots in four repetitions. The pre-crop in each year of research was maize.

Factor A

The production of field crops largely depends on the meteorological conditions characteristic of different types of grain during the year, and especially during the vegetation period, so as a factor, they cannot be omitted in research of this type.

Factor B

Four varieties of wheat of good technological quality were sown (November 22, 2016; October 25, 2017; November 16, 2018): Ratarica—medium late winter bread wheat variety type B₁–A₂ selection of the PKB Agroekonomik Institute, Pobeda—selection of the Institute of Field Farming and Vegetables Novi Sad, medium late bread variety type A₁–A₂ with pronounced high adaptability to environmental conditions; Nogal—Nogal yunta quattro—selection of French early wheat variety type A₂. Apache—selection Limagrain France, medium late variety type B₁. All were sown at 400 plants m⁻².

Factor C

For wheat nutrition as a basic fertilizer, a complex NPK fertilizer 15:15:15 was used in the amount of 400 kg ha⁻¹, which was applied during the basic tillage in the fall. In the spring, UREA 46 N% and supplementary foliar feeding with microbiological preparation EM Aktiv (6 l ha⁻¹) were used in wheat fertilization, which contains a mixture of a large group of effective microorganisms. (EM): *Lactobacillus plantarum*, *Lactobacillus casei*, *Streptococcus lactis*, *Rhodopseudomonas palustris*, *Rhodobacter*

sphaeroides, *Saccharomyces carevisiae*, *Streptomyces albus*, *Streptomyces griseus*, *Aspergillus oryzae*, *Mucor hiemalis* *Bacillus subtilis*, *Bacillus megaterium*, *Azotobacter sp.* The formed treatments are shown in Table 1.

At the end of vegetation, TGW and yield at 13% moisture were measured, as well as the content of crude protein determined by the micro-Kjeldahl method (Bremner and Mulvanej 1982).

To evaluate the data, we used descriptive statistics and analysis of variance (ANOVA) in program DSAASTAT. Three-way ANOVA was used to test effects of genotype, treatment and growing season. All results were calculated at a significance level LSD of 0.01 and 0.05.

Results

Climate conditions (air temperature and precipitation)

Differences between average air temperature and sum precipitation wheat vegetation (Fig. 1).

In the autumn of October–November 2017, the temperatures were optimal as well as the amount of precipitation. The warmest winter month was December 6.5 °C, with a water deficit, of only 10.8 mm recorded. There was lower than average precipitation of 34.4 mm in April, but with the humidity that had been retained since March and a mean temperature of 13.9 °C, good conditions were created for further crop development. The month of June had precipitation and a higher temperatures, which significantly affected

Table 1 Factor C divided into four treatments

Treatments	Before sowing NPK (kg ha ⁻¹)	In supplementation UREA kg ha ⁻¹)	EM Aktiv	Total nutrients N: P ₂ O ₅ : K ₂ O
T 1	400	150	Control	129: 60: 60
T 2	400	150	Stage: tillering begins	129: 60: 60
T 3	400	100	Stage: tillering begins and stage: flowering	106: 60: 60
T 4	400	50	Stage: full tillering begins, stage: flowering and stage: grain fill	83: 60: 60

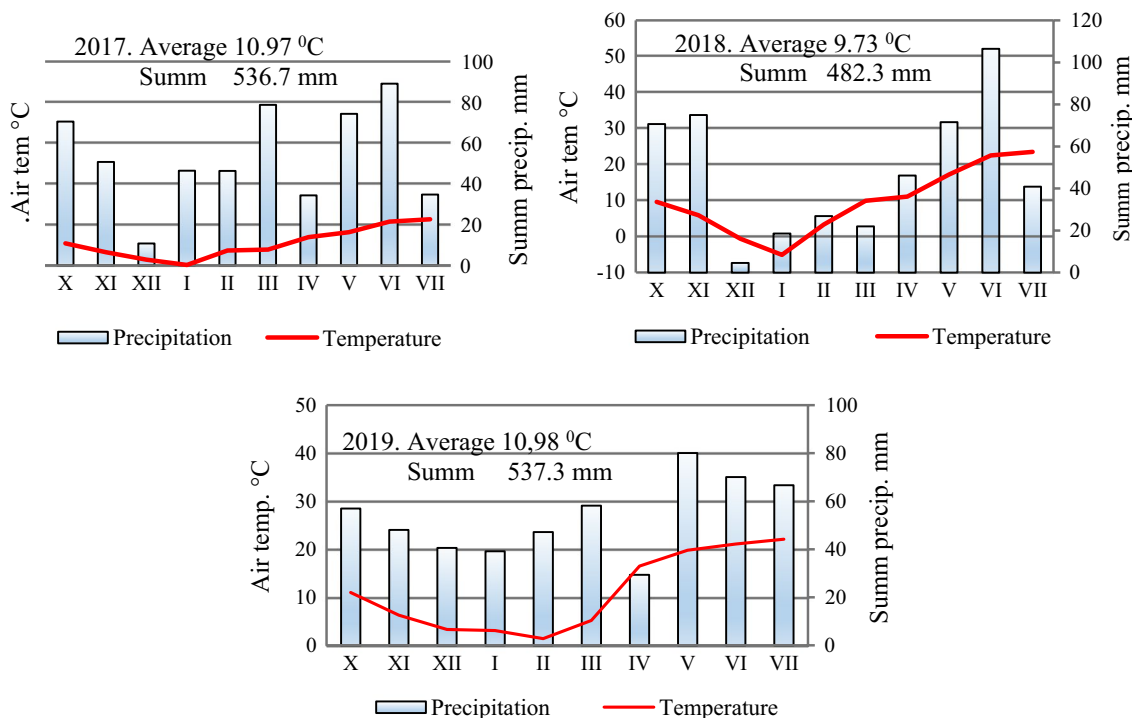


Fig. 1 Average air temperature (°C) and sum precipitation (mm) in wheat vegetation 2017–2019 year

the grain development processes and significantly determined the grain yield. The mean summer air temperatures were within optimal limits with a relatively good precipitation distribution. The warmest month was June at 22.6 °C. In 2018, December–February had frost temperatures from – 0.6 °C (December), to – 5.1 °C (January), without snow. Such climatic conditions lead to a decrease in the synthesis and consumption of sugar during the night, whereby their amount in the leaves and the nodules is reduced, which reduces the resistance to stress. During the period of grain filling and ripening, the average air temperature (June 22.5 °C and July 23.5 °C) was higher than in 2017 and 2019. The highest amount of precipitation was 106.5 mm in June, with showers that affected the lodging of crops. The winter of 2019 (December–February) was quite mild with uniform average temperatures from 1.4 to 3.3 °C, without icy days. In this period, a total of 127.0 mm of precipitation was recorded with a good distribution of 39.2 to 47.2 mm. The highest amount of precipitation was 80.1 mm in May, which created better conditions for grain development. The temperature rise continued in June 21.1 °C and in July 22.1 °C quite uniform and characteristic for that period of the year. The amount of precipitation was less, which accelerated the ripening of the grain. The average air temperature and the amount of precipitation were quite uniform in 2017, but with a different distribution that significantly affected the differences in grain yield.

Thousand grain weight (TGW)

The TGW depends on the weight of the grains themselves as well as the biological characteristics of the genotype and to a greater or lesser extent on the environmental conditions. Analysis of TGW mass variance and yield based on LSD test showed that the main sources of variation (year, variety and fertilization) as well as their interactions have a statistically significant impact.

TGW is a productive trait that significantly affects the level of yield. The mean TGW of all examined parameters was 40.57 g. TGW was the highest in 2019 at 44.34 g because this year in the period from April to July there was the highest amount of precipitation. Agrometeorological conditions in wheat vegetative growth had a statistically highly significant effect on differences in TGW the weight of 1000 grains. The lowest TGW was in 2018 (36.64 g), which is 10.58% less than in 2017 and 20.68% less than in 2019. These results were influenced by the uneven amount of precipitation in 2018, because 106.5 mm of water sediment fell in June, which affected the lodging of plants and water retention on the surface. Although TGW is defined by the genetic material, it also depends on agroecological conditions, applied cropping practices and ranges over a wide interval. Varietal specificity had a highly significant effect on

TGW differences. Ratarica and Pobeda varieties had the best results. Variety Ratarica (41.82 g) showed higher TGW than cultivar Pobeda (43.73 g) by 4.63%, cultivar Nogal (37.30 g) by 17.23% and cultivar Apache (39.43 g) by 10.90%. All identified differences were at the level of high statistical significance ($p > 0.01$) (Table 2).

The application of foliar treatment with EM (factor C) significantly affected TGW at lower amounts of mineral nitrogen. On average for all years, the treatment T2 (40.81 g) and T3 (40.74 g) had the greatest impact. There was no statistical significance between the treatment T2 and T3, but in relation to the T1 (40.33 g) control and T4 (39.14 g) there was a significant difference ($p > 0.01$). TGW at T2 was greater than 1.19% (T1) to 4.29% (T4). T3 treatment increased TGW by 1.01% compared to T1 and by 4.08% compared to the T4 treatment.

Grain yield

Wheat is a plant that is grown in the open field, and the yield is a very quantitative character, so environmental factors can affect the parameters concerned with yields. Agrometeorological conditions had an impact on differences in yield levels over years that were not statistically significant. In 2018, the lowest yield of 6.22 t ha⁻¹ was measured, i.e. the yield was 0.260 t ha⁻¹ (4.18%) lower than in 2017 (6.48 t ha⁻¹), and 0.510 t ha⁻¹ (8.19%) lower than in 2019 (6.73 t ha⁻¹). This difference was influenced by a lower amount of precipitation in 2018 (482.3 mm). As temperature is a significant factor in achieving the yield, it is likely that the lower temperature in the vegetation in 2018 (9.73 °C) affected the achieved result. The obtained results are comparable with the results that obtained lower grain yields in the dry year than in the colder and wetter year.

Genotype (factor C) highly significantly determined grain yield. The cultivar Ratarica had the highest yield on average 7.02 t ha⁻¹. Differences in yield level in relation to other varieties are statistically very significant. In relation to the variety Apache (6.52 t ha⁻¹) and Pobeda (6.50 t ha⁻¹) the difference was 7.66%, and in relation to the variety Nogal 19.59%. Wheat fertilization is an agrotechnical measure that affects the production of morphological and generative properties of soil. Previous research on the application of biofertilizers in combination with minerals has given good results.

The combined application of EM and mineral nitrogen in T3 influenced the achievement of the highest grain yield (6.62 t ha⁻¹), (Table 3).

The application of effective microorganisms twice in vegetation with 106 kg N ha⁻¹ had the greatest influence on the examined parameters. The highest TGW was recorded in the variety Pobeda 48.68 g in 2017. The highest yield was the variety Ratarica 7.47 t ha⁻¹ in 2019. The use of effective

Table 2 Influence of examined variables on TGW (g)

Year (A)	Genotype (B)	Treatment (C)				\bar{X}_{AB}	\bar{X}_A
		T1	T2	T3	T4		
2016/17	Ratarica	42.00	41.00	42.00	42.00	41.75	40.63
	Pobeda	42.00	43.00	41.00	43.00	42.25	
	Nogal	39.50	39.50	38.00	38.50	38.88	
	Apache	39.50	39.50	39.50	40.88	39.63	
\bar{X}_{AC}		40.75	40.75	40.13	41.10		
2017/18	Ratarica	37.63	36.35	47.30	48.58	37.60	36.74
	Pobeda	40.05	41.25	48.68	39.33	40.77	
	Nogal	33.65	32.10	39.62	43.08	32.89	
	Apache	34.70	35.83	41.18	44.14	35.69	
\bar{X}_{AC}		36.51	36.38	44.20	43.78		
2018/19	Ratarica	45.33	38.00	38.43	39.18	46.10	44.34
	Pobeda	47.48	39.13	42.60	31.93	48.18	
	Nogal	39.15	31.93	33.90	35.53	40.12	
	Apache	42.93	36.16	36.70	36.16	42.97	
\bar{X}_{AC}		43.72	36.31	37.91	35.70	\bar{X}_B	
\bar{X}_{BC}	Ratarica	41.65	41.17	42.58	43.58	41.82	
	Pobeda	43.18	44.08	44.09	36.58	43.73	
	Nogal	37.43	37.99	37.17	36.58	37.30	
	Apache	39.04	40.01	39.13	39.83	39.43	
\bar{X}_C		40.33	40.81	40.74	39.14		
Average 2016–2019						40.57	
	A**	B**	AB**	C**	AC**	BC ^{ns}	ABC ^{ns}
F test	0.772	0.000	0.000	0.000	0.000	0.232	0.765
LSD 0.01	1.30	2.06	2.91	0.92	1.30	1.83	2.59
LSD 0.05	1.14	1.54	2.18	0.69	0.98	1.39	1.97

microorganisms as a supplement can be successful in different agroecological conditions.

Based on the analysis of world trends, the increase in wheat yield during the twenty-first century was the result of intensification of production. The application of biostimulators with EM can improve the development of production technology for the production of bread wheat genotypes in healthy food and environmental protection.

Crude protein content

In addition to the yield, the quality of wheat is of great importance for human health and well-being. According to many previous studies, agroecological factors and genotype have a significant influence on protein content.

In 3-year studies, the examined variables (years, genotype, and treatments) and genotype/treatment interaction had a highly significant effect on protein content. The highest protein content was determined in 2018, 13.67%, because the most rain fell in the period May–July. The variety Pobeda had the highest protein content of 13.69%. On average, T3

treatment had a statistically highly significant effect on the protein content of 13.93% (Table 4).

Cluster analysis

Similarities and differences between the four examined four varieties, for three traits, by years, were determined by applying a hierarchical cluster analysis and are shown in Fig. 2.

Figure 2 shows that there are two clusters (I, II), of which cluster I includes the varieties Pobeda and Ratarica. Cluster II includes the varieties Nogal and Apache.

Based on such grouping, it is concluded that the varieties Pobeda and Ratarica have relatively more similar characteristics in the years of the field experiment in 2015, 2016 and 2017, compared to the varieties Nogal and Apache, which stood out in the second cluster in 2015, 2016 and 2017 years. Which means that these two clusters have a different genetic structure, i.e., that the varieties in these two clusters reacted differently to the applied amounts of fertilizers, which is most likely due to their genetics (Serbian and French).

Table 3 Influence of examined variables on wheat grain yield (t ha^{-1})

Year (A)	Genotype (B)	Treatment (C)				\bar{X} AB	\bar{X} A
		T1	T2	T3	T4		
2016/17	Ratarica	6.63	7.07	7.38	6.85	6.98	6.48
	Pobeda	6.46	6.17	6.62	7.13	6.59	
	Nogal	6.29	6.52	6.64	6.85	6.57	
	Apache	5.54	6.08	6.10	5.44	5.79	
\bar{X} AC		6.23	6.46	6.69	6.57		
2017/18	Ratarica	6.63	7.13	6.78	6.82	6.84	6.22
	Pobeda	6.28	5.65	6.18	6.88	6.25	
	Nogal	5.59	6.08	5.25	5.44	5.59	
	Apache	5.88	6.19	6.10	6.67	6.21	
\bar{X} AC		6.09	6.26	6.08	6.45		
2018/19	Ratarica	7.24	7.06	7.47	7.18	7.23	6.73
	Pobeda	6.28	6.43	7.01	6.91	6.66	
	Nogal	6.67	6.45	6.73	7.26	6.78	
	Apache	6.04	6.12	7.14	5.63	6.23	
\bar{X} AC		6.56	6.51	7.09	6.75	\bar{X} B	
\bar{X} BC	Ratarica	6.83	7.09	7.21	6.95	7.02	
	Pobeda	6.34	6.08	6.60	6.98	6.50	
	Nogal	5.72	6.09	6.16	5.50	5.87	
	Apache	6.28	6.38	6.49	6.93	6.52	
\bar{X} C		6.29	6.41	6.62	6.59		
Average 2017–2019						6.48	
	A**	B**	AB*	C**	AC ^{ns}	BC ^{ns}	ABC ^{ns}
F test	0.979	0.000	0.028	0.000	0.527	0.110	0.186
LSD 0.01	0.91	0.40	0.57	0.26	0.36	0.51	0.72
LSD 0.05	0.80	0.30	0.42	0.19	0.27	0.39	0.55

Discussion

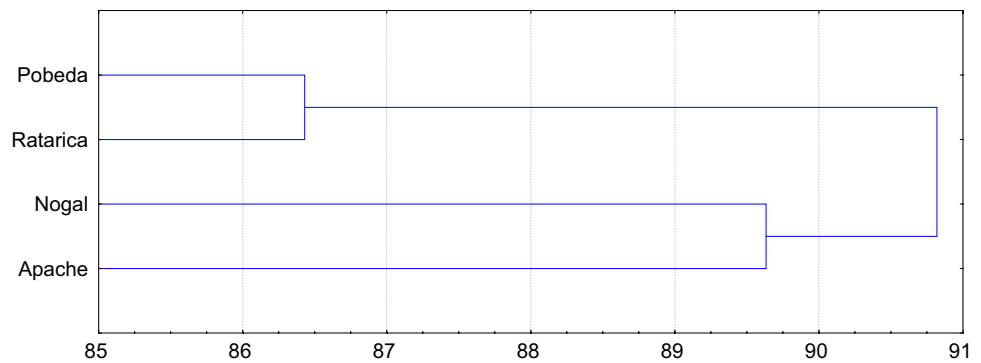
Due to the great importance of commercial wheat, research should focus on determining the compatible relationship of wheat genotype and group of microorganisms that can be applied with foliar sprays to achieve economically viable yield. In addition to the genotype, the grain yield of winter wheat is greatly influenced by the fertilization system, which is one of the key factors that affect the level of yield and its quality, but it should be harmonized with climatic and soil conditions and variety requirements (Djuric et al. 2018).

Due to the high cost of fertilizers and the awareness of supporting sustainable agriculture and reduced pollution, in which research is growing, one of the possibilities would be the use of effective microorganisms. Considering that Serbia is in the process of joining the EU, in terms of research, it monitors fertilization measures, while at the state level, measures have not yet been prescribed. The level of grain yield, and the protein content is significantly affected by inoculation of wheat seeds with the genus *Azotobacter* diazotrophs (Singh et al. 2007) and *Azospirillum* (Sharma et al. 2007). It can be concluded that in different agroecological

conditions, the application of effective microorganisms as a supplement can replace 23–46 kg N ha^{-1} . Bacterization of wheat with diazotrophs does not always show the expected results, and the complexity of the relationship between wheat and bacteria is the reason for that (Nemeth 2006).

In all examined variables, the best results were in the cultivar Ratarica and Pobeda. The application of effective microorganisms is a cheap and simple technique that is used and that does not pollute and falls into a sustainable context. By including effective microorganisms in wheat production, the quality and quantity of different wheat genotypes can be significantly improved. Grain yield was already 5.24% higher than at T1 (6.29 t ha^{-1}), 3.27% from T2 (6.41 t ha^{-1}) and 1.53% from T4 treatment (6.59 t ha^{-1}). Similar results were obtained by Teixeira Filho et al. (2017) where only wheat was inoculated with *Azospirillum brasilense* species and found that a higher yield was achieved with less than 139 kg N ha^{-1} in wheat supplementation. In two-year studies of the effect of water efficiency (WUE), biological and chemical fertilizers in different moisture regimes, the obtained results show an increase in wheat grain yield in treatments with a combination of biofertilizers *Azotobacter*

Fig. 2 Dendrogram of productive traits in 4 wheat varieties



and Phosphobacteria, of 61.28% and 16.13%, compared to the control (Moradgholi et al. 2021). The same authors state that *Azotobacter* and phosphobacteria biofertilizers, alone or in combination, increased the grain yield, biological yield, harvest index, water use efficiency and protein percentage in the wheat cultivar Arg. Mohammadi et al. (2011) found that the variety and medium/variety interaction for yield was highly significant 24 wheat genotypes, with an estimated heritability of 56.9%.

In different agrometeorological conditions, the application of effective microorganisms several times in the growth period through the leaves can alleviate the stress of plants and achieve economically significant yields. Mladenov et al. (2014) found that the decrease in wheat yield as a result of rain (107.6 l/m^2) in the phase of full maturity occurs due to grain shedding or activation of physiological processes in the grain, as well as plant traits (position of classes at full maturity, grain size, propensity to shed). The same authors on the basis of the linear regression concluded each l/m^2 in rain during the grain filling stage reduces the yields of 10 kg ha^{-1} (0.12%) and 10 l/m^2 reduces the yield of 100 kg ha^{-1} (1.2%). In two years of research by Đurić et al. (2017) on the influence of agrometeorological conditions, it was found that in the vegetative season with more precipitation than 176 mm, and average temperatures lower by $3.1 \text{ }^\circ\text{C}$, the TGW had higher values. Mohammadi et al. (2019) concluded that the characteristics of genotypes of wheat have a great influence than years as a factor. According to their research, the results show that efficiency of photosystem II and thousand-kernel weight were positively associated with grain yield, while heading date, plant height and canopy temperature were negatively associated with grain yield under drought conditions.

Zonuz et al. (2014) concluded that the use of biofertilizers and nitrogen fertilizers in certain combinations, in the phase of germination and flowering of wheat, leads to the full development of plants, increased number of pods, high grain mass. Xiaohou et al. (2001) conducted research in China to determine the effects of foliar application of effective microorganisms on yield and its quality in the production of tea, cabbage and sweet corn and found a yield

increase of 25%, 14%, and 12.5%. Lim et al. (1999) studied the effects of different EM applications on rice and maize yields. The yield of both species increased by 7.2–7.4% in seed treatment, 7.1–7.4% in seedling treatment.

In addition, they significantly affect the protein content. Evers (2000) found that the shape and size of grains also affect the protein content, and indirectly the baking quality of the final products.

During the treatment with 106 kg N ha^{-1} and two foliar treatments with effective microorganisms, a highly significantly higher protein content of 13.93% was determined. Monaghan et al. (2001) found that advanced wheat cultivation technologies, including different modern genotypes, can increase grain protein concentration and grain yields. According to Smutná and Tokatlidis (2010), genotypes with good yielding performance and protein concentration should be selected.

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