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INFLUENCE OF GROWTH STAGE-SPECIFIC BIOSTIMULANT APPLICATION ON WHEAT GRAIN MORPHOLOGY

UTJECAJ PRIMJENE BIOSTIMULATORA U ODREĐENOM RAZVOJNOM STADIJU NA MORFOLOGIJU ZRNA PŠENICE

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ABSTRACT

Biostimulants are bioactive compounds that positively modulate plant metabolism, particularly enhancing grain yield. We evaluated, under controlled conditions, the impact of biostimulant application at three distinct growth stages—booting (BS), stem elongation (SES), and heading (HS)—both individually and in combination, using two winter wheat (*Triticum aestivum* L.) genotypes (Essekerka and Eminentia), to assess effects on grain performance. In Essekerka, 1000-kernel weight increased significantly following biostimulant application during HS alone and when applied during BS + SES + HS. In Eminentia, all treatments resulted in a significant increase in 1000-kernel weight compared to the control. Biostimulant treatments also enhanced grain area and width in both genotypes, although Essekerka showed significant area increases only in HS and BS + SES + HS treatments. Eminentia's grain length increased only in HS, compared to the control, while biostimulants tended to reduce grain circularity in both genotypes. Taken together, these findings suggest that applying biostimulants during multiple growth stages, particularly at heading, can effectively enhance overall grain yield, driven by improvements in 1000-kernel weight and grain dimensional traits.

Keywords: biostimulants, grain morphology, growth stage, wheat

SAŽETAK

Biostimulatori su bioaktivni spojevi koji pozitivno moduliraju metabolizam biljaka, posebno povećavajući urod zrna. U kontroliranim uvjetima proučavali smo utjecaj primjene biostimulatora u tri različita razvojna stadija – busanju (BS), izduživanju stabljike (SES) i klasanju (HS) - pojedinačno i u kombinaciji, koristeći dva genotipa ozime pšenice (*Triticum aestivum* L.) (Essekerka i Eminentia) kako bismo procijenili učinke na svojstva zrna. Kod Essekerke se masa 1000 zrna značajno povećala nakon primjene biostimulatora tijekom faza HS i tijekom BS+SES+HS. Kod Eminentie su svi tretmani rezultirali značajnim povećanjem mase 1000 zrna u usporedbi s kontrolom. Tretmani biostimulatorom također su povećali površinu i širinu zrna u oba genotipa, iako je Essekerka pokazala značajno povećanje površine samo u tretmanima HS i BS+SES+HS. Duljina zrna Eminentie povećala se samo u HS u usporedbi s kontrolom, dok su biostimulatori imali tendenciju smanjiti kružnost zrna kod oba genotipa. Zaključno, ovi rezultati upućuju na to da primjena biostimulatora tijekom različitih razvojnih faza, posebno u klasanju, može učinkovito povećati ukupni urod zrna, potaknuta poboljšanjem mase 1000 zrna i drugih morfoloških svojstava zrna.

Ključne riječi: biostimulatori, morfologija zrna, razvojni stadij, pšenica

1. INTRODUCTION

Biostimulants are organic or inorganic products containing bioactive substances and/or microorganisms that positively influence plant growth and productivity by enhancing nutrient absorption and assimilation efficiency, as well as tolerance to abiotic stresses (Franzoni et al., 2022). These products also play a critical role under drought conditions by improving water uptake and increasing nutrient use efficiency, which are essential for plant development and yield (Dara, 2021). Biostimulants are typically classified as organic, sustainable, or environmentally friendly inputs, as they promote crop performance without causing harmful side effects (Bulgari et al., 2014). This aligns with the objectives of the European Green Deal, which aims to reduce pesticide use (Ginter et al., 2022). Moreover, biostimulants can contribute to both environmental and economic sustainability, as many are derived from the valorization of organic waste from agri-food and industrial production chains (Xu and Geelen, 2018).

Among organic non-microbial biostimulants are natural substances such as humic acids, protein hydrolysates, and seaweed extracts (Rouphael and Colla, 2018). A specific subset of these includes amino acid-based formulations obtained through chemical synthesis or via hydrolysis of plant and animal proteins using chemical or enzymatic methods (Popko et al., 2018). These biostimulants are typically applied as foliar sprays. In contrast, microbial-based biostimulants—such as plant growth-promoting rhizobacteria from genera including *Azospirillum*, *Azotobacter*, and *Rhizobium* spp., as well as mycorrhizal fungi—are usually applied to the soil or seeds (Fiorentino et al., 2018). Biostimulants can exert direct effects on plant metabolism and physiology or act indirectly by improving soil properties and microbial activity (Di Mola et al., 2019). According to EU Regulation 2019/1009, a plant biostimulant is defined as a product that stimulates plant nutritional processes independently of its nutrient content, with the sole purpose of enhancing one or more specific plant or rhizosphere characteristics (Maignan et al., 2022).

Wheat is a globally significant crop and a fundamental source of food and feed, contributing 15–25% of global protein intake and 20% of daily caloric intake (Bin Safdar et al., 2023). The use of biostimulants in wheat cultivation has shown promise in improving productivity in an environmentally sustainable manner (Sellami et al., 2025). Several studies have reported that the application of plant biostimulants in wheat can enhance biomass accumulation and, consequently, grain yield (Sirbu et al., 2022). For instance, the combined application of biostimulants and herbicides has been associated with grain yield increases of up to 14.7% compared to herbicide treatment alone (Kanatas et al., 2022). Additionally, when biostimulants were applied 12 and 26 days after sowing, grain yield increased by 8.2% (Al Majathoub, 2004).

Zulfiqar et al. (2020) demonstrated that biostimulants can promote plant growth through multiple mechanisms involving physiological, biochemical, and molecular pathways. In winter wheat, biologically active substances derived from *Paulownia tomentosa* were found to positively affect seed germination, tillering, grain weight, and overall yield (Turaeva et al., 2024). Similarly, the application of seaweed extracts in combination with humic acids was reported to enhance the 1000-kernel weight (Muhammad et al., 2013). However, other studies observed only marginal increases in grain weight when biostimulants were combined with various other substances (Matysiak et al., 2018). Posmyk and Szafrańska (2016) concluded that the significant increases in wheat grain yield may result from the synergistic effects of biostimulants and organic nutrition sources on cellular development, enzymatic regulation, and photosynthetic efficiency. Another reason lies in the fact that the determinants of grain size and weight can be divided into pre-anthesis and post-anthesis factors (Gasparis and Miłoszewski, 2023).

To date, numerous field-based studies have evaluated the effects of biostimulants on wheat growth and development (Sellami et al., 2025). Nevertheless, despite their potential benefits, limited research has been conducted on the influence of biostimulants on wheat grain morphology . The present study investigates the effectiveness of biostimulants applied at various developmental stages in enhancing wheat grain morphology and grain number.

2. MATERIALS AND METHODS

2.1. Experimental Layout

The experiment was conducted under controlled conditions in a greenhouse (Gis Impro d.o.o., Vrbovec, Croatia) using two winter wheat genotypes from the Agricultural Institute Osijek. The experiment included a total of five treatments:

1. Treatment 1 – Control – no biostimulant application.
2. Treatment 2 – application of the biostimulant *Fertiactyl Starter* (13% N, 5% P, 8% K, 4% C, GB, humic and fulvic acids) at the booting stage (GS 20).
3. Treatment 3 – application of the biostimulant *Fertiactyl Vital* (9% N, 5% P, 4% K, 0.05% B, 0.02% Cu, 0.02% Fe, 0.1% Mn, 0.01% Mo, 0.05% Zn, and Seactiv complex) at the stem elongation stage (GS 30).
4. Treatment 4 – application of *Fertileader Vital* at the heading stage (GS 59).
5. Treatment 5 – combined application of *Fertileader Starter* and *Vital* at three growth stages: booting, stem elongation, and heading.

Within each treatment, genotypes were randomized according to a randomized block design with four replicates, each consisting of four plants grown in a 2.5 L pot filled with soil (pH-H_2O: 5.5–7.0; organic matter: 70.0–85.0%; N (½ vol.): 100–200 mg L^{-1}; P_2O_5 (½ vol.): 100–150 mg L^{-1}; K_2O (½ vol.): 200–400 mg L^{-1}). Nitrogen fertilization was applied at the booting stage (GS 20) using calcium ammonium nitrate (CAN, 27% N) per grain/plant. Two protective agents were applied to control diseases and pests. The first treatment was with the fungicide *Falcon Forte* (spiroxamine 224 g L^{-1}, tebuconazole 148 g L^{-1}, prothioconazole 53 g L^{-1}) at the stem elongation stage (GS 30), and the second, one week later, was with the insecticide *Vantex* (gamma-cyhalothrin 60 g L^{-1}) after aphid emergence (GS 31). During germination and booting, temperatures were maintained at 8–12°C at night (14 hours) and 10–14°C during the day (10 hours), with a maximum light intensity of 250 µmol m^{-2} s^{-1}. During stem elongation, day and night durations were equalized, with day temperatures maintained at 15–18°C and night temperatures at 11–14°C. Before heading, day length was extended to 14 hours, with temperatures maintained at 21–24°C during the day and 17–20°C at night, and a maximum light intensity of 750 µmol m^{-2} s^{-1}.

2.2. Grain morphology

After maturity, wheat spikes were collected from each treatment to analyze the morphological characteristics of the grain (1000-kernel weight, area, width, length, and circularity) using the MARVIN precision system for efficient seed analysis (MARViTECH GmbH, Wittenburg, Germany). Grains were distributed over the measurement area, and images were captured using a digital camera. The images were then analyzed using dedicated image processing software. The analysis procedure was validated using control images.

2.3. Statistical analysis

For grain morphology, four biological replicates were used to calculate the mean values for each treatment (control and biostimulants). Fisher's Least Significant Difference (LSD) test ($\alpha = 0.05$) was applied to assess whether the differences in performance between treatments were statistically significant for each variety individually (control treatment vs. biostimulant treatments) (StatSoft Inc., Tulsa, OK, USA). The results of the analyzed traits are presented as the mean value of four replicates ± standard deviation.

3. RESULTS AND DISCUSSION

Grain yield is the product of a few components, including the number of spikes per unit area, grain number per spike and grain weight, often expressed as 1000-kernel weight (Mandea and Săulescu, 2018). According to that, grain size and weight are also the two major determinants of grain yield. It was previously reported that the grain yield of different crops was substantially higher in the treatments with all biostimulants tested compared to the no-biostimulant treatment (Xiao et al., 2021; Gawęda et al., 2024). The traits that influence grain yield may vary under biostimulant treatment, depending on the genotype, environment, growth stage in which the biostimulant is applied, biostimulant type etc. Szczepanek et al. (2018) reported that wheat reaction was influenced by the biostimulant dosage and developmental stage of the plant during biostimulant application. Some researchers reported that the number of grains per spike does not significantly differ between the tested combinations of biostimulants. For example, the best results were obtained for the reference product—ear number per m^2 was up to 17% higher than in the control group (Popko et al., 2018).

3.1. Grain morphology under biostimulant treatments at different growth stages

The 1000-kernel weight increased in both genotypes when treated with biostimulants compared to the control (Figure 1A). In Essekerka, the 1000-kernel weight significantly increased by 24.31% and 16.01% at the heading stage (HS) and the combined booting + stem elongation + heading stages (BS+SES+HS), respectively, compared to the control. In Eminentia, a significant increase was observed in all biostimulant treatments, with increases of 21.44%, 27.84%, 26.38%, and 19.52% at BS, SES, HS, and BS+SES+HS, respectively, compared to the control. In another research, polyprenol foliar application during vegetation had a noticeable effect on the number of productive tillers, grains per spike, grain weight per spike, and 1000-kernel weight, which are primary prerequisites for high grain yield formation (Sellami et al., 2025). In the same research, foliar treatment of winter wheat with biostimulants revealed an upsurge in grain weight per spike, which also exceeded the control by 13.7% and 7.8%, as well as 1000-kernel weight by 12.2% and 6.2%, respectively. Similarly, wheat plants treated with Vigro produced larger grain size (higher 1000-kernel weight) (Al Majathoub, 2004). In the three-year research of Szczepanek et al. (2018), the yield structure elements responding to the extract from algae during different growth stages included the number of grains per spike and 1000-kernel weight. In the current research, grain area followed a similar trend, with significant increases of 15.60% and 11.68% at HS and BS+SES+HS in Essekerka, and significant increases in all biostimulant treatments in Eminentia compared to the control (Figure 1B). For example, several parameters were improved over the control as a direct result of the biostimulant treatments, i.e. number of tillers, grain number per spike, grain size and yield (Al Majathoub, 2004). The results from the current research considering changes in 1000-kernel weight and grain area were expected, as individual grain weight is mainly formed after anthesis, compared with grain number (Xie et al., 2015). However, it can be concluded that these were genotype-specific traits as one genotype significantly elevated 1000-kernel weight and grain area due to biostimulant application at HS and in the combined biostimulant application at BS+SES+HS, compared to other genotypes that had significant elevation in all applications.

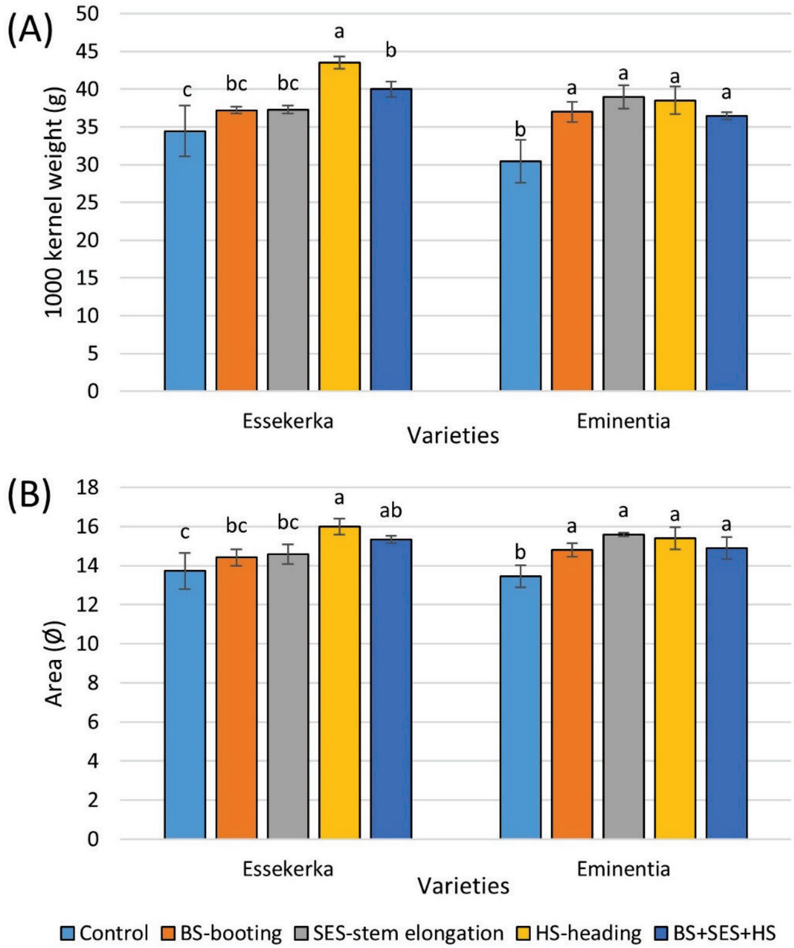

Figure 1 1000 kernel weight (A) and grain area (B) of two wheat genotypes under control treatment without biostimulant application and biostimulant application at three distinct growth stages—booting (BS), stem elongation (SES), and heading (HS)—both individually and in combination. Different letters mean different statistical significance ($p < 0.05$) between treatments. Bars represent mean values of four replicates ± SD.

Slika 1. Masa 1000 zrna (A) i površina zrna (B) dva genotipa pšenice u kontroli bez primjene biostimulatora i primjenom biostimulatora u tri razvojna stadija—busanje (BS), izduživanje stabljike (SES) i klasanje (HS)—pojedinačno i u kombinaciji. Različita slova označavaju različitu statističku značajnost ($p < 0,05$) između tretmana. Stupci predstavljaju srednje vrijednosti četiri ponavljanja ± SD.

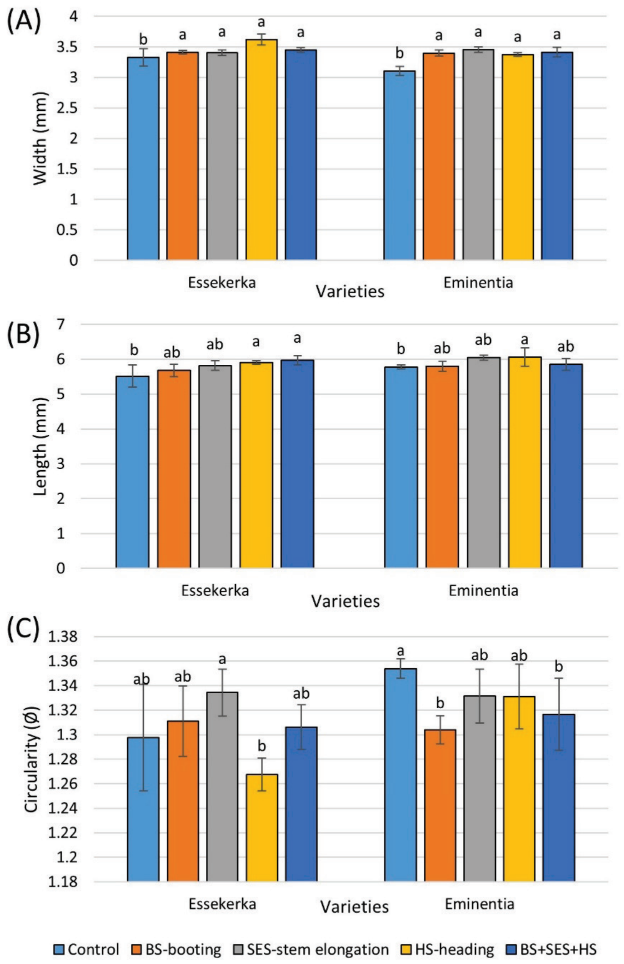

Figure 2 Grain width (A), grain length (B), and grain circularity (C) of two wheat genotypes under control treatment without biostimulant application and biostimulant application at three distinct growth stages—booting (BS), stem elongation (SES), and heading (HS)—both individually and in combination. Different letters mean different statistical significance ($p < 0.05$) between treatments. Bars represent mean values of four replicates ± SD.

Slika 2. Širina zrna (A), duljina zrna (B) i kružnost zrna (C) dva genotipa pšenice u kontrolnom tretmanu bez primjene biostimulatora i primjenom biostimulatora u tri različita razvojna stadija—busanje (BS), izduživanje stabljike (SES) i klasanje (HS)—pojedinačno i u kombinaciji. Različita slova označavaju različitu statističku značajnost ($p < 0,05$) između tretmana. Stupci predstavljaju srednje vrijednosti četiri ponavljanja ± SD.

The size and shape of wheat grains remain under consideration of researchers aiming to improve the grain yield potential. Grain width significantly increased in both genotypes under all biostimulant treatments (Figure 2A). The highest increase in Essekerka was observed at HS (8.59%), while in Eminentia, it occurred at SES (11.35%). Previous research shows that grain weight has a relatively higher correlation with grain width, compared with the well-assessed index of projected grain area (Haghshenas et al., 2022). However, in the current research, grain area and grain width showed the same trend of increase in both genotypes under biostimulant applications at different growth stages.

Grain length significantly increased in Essekerka at HS and BS+SES+HS by 6.67% and 8.14%, respectively, and in Eminentia only at HS (4.63%) (Figure 2B). It was obvious that this was a similar pattern of increase for 1000-kernel weight. This can be related to the positive regulator of grain size 1 (*TaPGS1*) that can increase grain length and grain weight (Guo et al., 2022). From the current research, it can be seen that grain length is also a genotype-specific trait.

Grain circularity refers to roundness, where more circular grains often have better milling efficiency. Grain circularity showed no significant differences compared to the control in Essekerka (Figure 2C). In contrast, a significant decrease in circularity was observed in Eminentia at BS and BS+SES+HS, compared to the control, suggesting a slight morphological alteration. In addition, PCA clearly separated the varieties, with Essekerka tending more towards traits related to grain weight and size, while Eminentia was more associated with circularity. However, research by Marinciu et al. (2021) showed that 1000-kernel weight was positively correlated with grain area, width, length and circularity. In the current research, 1000-kernel weight was in positive relation with grain width while grain circularity was on the opposite side thus showing negative relation (Figure 3). In addition to grain width, 1000-kernel weight also showed a positive correlation with grain length and area, suggesting that larger grains contribute to higher grain mass. Previous conclusions about the relation of grain weight and width are reported by Alamery and Al-Badri (2023) who showed that visual markers of grain weight can be found in the wheat grain width. This might imply that broader seeds contribute significantly to overall grain weight mass, likely due to increased endosperm volume. Further, in the current research, based on the principal components analysis (PCA) that was used to determine relations among grain morphological traits, it was observed that 1000-kernel weight and grain width had negative relation with grain circularity. This is in accordance with previous research that reported on the relation between grain quality indicators and grain circularity (Ishen et al., 2024). Overall, that confirms the well-known thesis that grain yield and related traits are in negative correlation with grain quality. However, the genetic basis of the grain size of wheat is largely unexplored (Gasparis and Miłoszewski, 2023). The current research highlights the potential of biostimulants in wheat breeding programs, emphasizing the importance of enhancing grain morphology traits for improving grain yield.

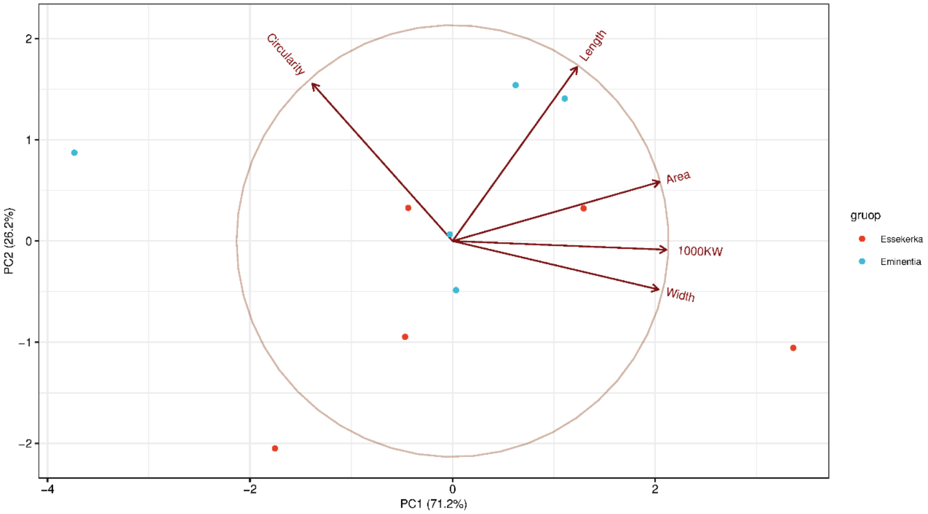

Figure 3 Principal component analysis of five morphological traits related to grain morphology

Slika 3. Analiza glavnih komponenti pet morfoloških svojstava povezanih s morfologijom zrna

4. CONCLUSION

1. Biostimulant application significantly increased grain morphology traits, ranging from 2.3% (stem elongation stage, SES) to 27.8% (SES).
2. In Eminentia, biostimulants applied at all growth stages (individually or combined) significantly increased 1000-kernel weight, grain area, and grain width; in Essekerka, grain width increased significantly.
3. In Essekerka, application at the heading stage (HS) alone or combined in the booting stage (BS) + SES + HS significantly increased 1000-kernel weight and grain area.

4. Some grain morphology traits were genotype-specific under biostimulant application.

5. Further studies with more wheat genotypes under diverse conditions, including field trials, are recommended.

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