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INFLUENCE OF SIMULTANEOUS TREATMENT OF SEEDS WITH ZnONPs AND *Bacillus subtilis* ON THE BIOLOGICAL QUALITY PARAMETERS OF RED CABBAGE SEEDLINGS

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ABSTRACT

Minimizing the negative environmental impact of agrochemicals necessitates new strategies to achieve sustainable food production. Consequently, zinc oxide nanoparticles (ZnONPs) and plant growth-stimulating bacteria (*Bacillus subtilis*) have been proposed as a method to enhance the growth parameters. The effect of ZnONPs at a concentration range of 0.2–1.4 mg cm^{-3} on the planktonic growth of *B. subtilis* bacteria, production of the hormone indole-3-acetic acid, biofilm formation, the ability to biodegrade complex compounds such as Evans Blue, and an increase in oxidative stress was assessed. Concentrations of 0.2 and 0.4 mg cm^{-3} ZnONPs were used to further test the simultaneous effects of ZnONPs and *B. subtilis* on red cabbage growth. Moreover, the influence of the simultaneous use of ZnONPs and *B. subtilis* on seed germination, physiological characteristics, and the content of minerals in red cabbage seedlings grown in the soil was examined. The simultaneous use of ZnONPs and *B. subtilis* bacteria improves the number of germinated seeds, the length of red cabbage seedlings, and the content of photosynthetic pigments and antioxidants compared with the control or single treatment of seeds with only *B. subtilis* or ZnONPs. The simultaneous use of *B. subtilis* and zinc oxide nanoparticles resulted in a higher content of zinc and sodium in red cabbage seedlings, while the content of macronutrients such as Mg and K, and micronutrients such as Fe, Mn, and Co was lower or close to the control value. The combination of *B. subtilis* + 0.2 mg ZnONPs turned out to be better than *B. subtilis* + 0.4 mg ZnONPs, as it produced the highest number of germinated seeds, greater plant and root length, and a higher content of chlorophylls, phenolic compounds, and antioxidants. The results indicate that ZnONPs enhance the role of *B. subtilis* as plant growth-promoting bacteria.

Key words: zinc oxide nanoparticles, PGPR, physiological characteristics, seed germination

INTRODUCTION

Seedling quality is among the most important factors influencing vegetable cultivation and yield. Red cabbage (*Brassica oleracea* var. *capitata* L.), of the family Cruciferae, is commonly cultivated in the temperate climate zone. As a vegetable, red cabbage is valued for its wealth of nutrients. It is a good source of minerals, especially potassium, vitamins K, C, A and E, and phenolic compounds, including anthocyanins,

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glucosinolates, quercetin, and dietary fiber [Adelanwa et al. 2015]. It is less popular than white cabbage and is cultivated on a smaller scale. It has requirements similar to white cabbage, including fertile soil rich in humus, and is sensitive to drought. It is grown from seedlings, and production is significantly influenced by the application of micronutrients, deficiencies of which adversely affect its quality, including color. A distinguishing trait of red cabbage is its higher requirement for potassium, zinc and boron [Adelanwa et al. 2015].

One way to improve the early stages of plant growth and increase crop production is to use plant-growth-promoting rhizobacteria (PGPR) at the seed germination stage. The growth-promoting activity of PGPR can be explained in various ways: they increase nutrient availability for plants, produce growth regulators, induce resistance to phytopathogens, and improve soil structure [Boddupalli et al. 2017, Basu et al. 2021, Mahapatra et al. 2022]. Many bacterial species are included in PGPR. The best-represented genus is *Pseudomonas*, along with *Bacillus*, *Enterobacter* and *Erwinia*. Many studies have confirmed the potential of *Bacillus* species (e.g. *B. cereus*, *B. subtilis*, *B. coagulans*, *B. laterosporus*, *B. megaterium*, *B. mycoides*, *B. pasteurii*, and *B. sphaericus*) to support plant growth, reduce or combat plant pathogens, mitigate the effects of stress, colonize the roots of plants, and regulate physiological and molecular processes in plants [Tsavkelova et al. 2006, Ma et al. 2017, Khan et al. 2022]. The beneficial effects of various *Bacillus* species, including *B. subtilis*, on plants may be manifested as a supply of minerals to the plant through the reduction of atmospheric nitrogen to available forms for plants, increased growth and nitrogen uptake by plants, and acceleration of nitrogen transformation in the soil [White et al. 2019, Qin et al. 2022]. *Bacillus subtilis* is a PGPR species commercially used in bioprotection. It activates induced systemic resistance in plants and stimulates plant development. The presence of *B. subtilis* bacteria in the rhizosphere increases the uptake of nutrients by plants. By secreting organic acids into the soil (including gluconic, ellagic, acetic, succinic and propionic acid), these bacteria dissolve phosphates, increase soil fertility, effectively release phosphorus from compounds unavailable for plants [Basu et al. 2021, Izydorczyk et al. 2022] and support phosphorus uptake and plant growth. Phytohormones such as auxins, gibberellins, cytokinins and ethylene produced by *B. subtilis* stimulate plant growth [Pereira et al. 2020]. Owing to its ability to produce siderophores, *B. subtilis* mediates the supply of iron ions to plants [Khan et al. 2022]. Numerous studies have shown that inoculation of plants with *Bacillus subtilis* mitigates stress caused by abiotic factors and helps to increase the yields of certain crop plants. In biotic and abiotic stress conditions, *B. subtilis* can induce the systemic resistance of plants and increase their tolerance for adverse environmental factors [Hashem et al. 2019]. As microorganisms promoting plant growth, *Bacillus* bacteria are involved in the biological control of fungi and bacteria pathogenic to plants, such as *Penicillium chrysogenum*, *Clavibacter michiganensis*, *Fusarium oxysporum* and *Rhizoctonia solani*, causing their death or limiting their development [Saberi-Rise and Moradi-Pour 2020]. They synthesize antibiotics and compounds with fungicidal and antiviral properties, thus benefitting plant growth [Mardanova et al. 2016].

Contemporary agriculture makes use of innovative products, including nanotechnology. Intensive work on using nanomaterials in vegetable cultivation is also being carried out. Nano-zinc oxide, owing to its antimicrobial properties, is used in plant protection products to inhibit the growth of plant pathogens [Mirzaei and Darroudi 2017, Rajput et al. 2021]. It is also used in agriculture as a growth regulator, fertilizer, pesticide, and component of seed dressings [Prajapati et al. 2018, Rajput et al. 2021]. Zinc oxide nanoparticles (ZnONPs) are a foliar and soil fertilizer component which accelerate plant growth and development. The effects of ZnONPs on plants depend on their concentration [Shaymurat et al. 2012, Xiang et al. 2015, Sarkhosh et al. 2022]. Low concentrations of ZnONPs can stimulate seed germination and plant growth, partly by supplying zinc ions as a micronutrient, and they exert an antimicrobial effect [Rajput et al. 2021]. High concentrations can be toxic for plants and other elements of the soil environment [Raskar and Shankar 2014, Plaksenkova et al. 2020]. Despite literature data on ZnONPs as an antimicrobial agent, little is known of their impact on the environment of soil microbes, especially those promoting the growth of bacteria.

Many studies have been conducted to determine the beneficial effects of PGPR on plant growth and yield, as well as on the growth-stimulating effects

of nanoparticles, but information on the simultaneous impact of PGPR and ZnONPs on the early stages of seedling development is scarce. The present study investigated the potential of the simultaneous effect of ZnONPs and PGPR on the germination and growth of red cabbage seedlings. First, experiments were carried out to examine the response of the PGPR species *B. subtilis* to various concentrations of ZnONPs. Various methods were used to assess the toxicity of ZnONPs for this microorganism, including assessment of the planktonic growth rate, free radical generation, changes in the activity of selected enzymes, the ability to form a biofilm, and the ability to transform complex compounds such as the azo dye Evans blue. The aim of the next set of experiments was to test the simultaneous effect of *B. subtilis* and ZnONPs on selected growth parameters of red cabbage in its early stages of development. The toxicity of selected concentrations of ZnONPs for germination of red cabbage seeds was assessed. Biometric tests were used to evaluate the growth of the cabbage seedlings, and the content of selected substances was measured in the seedlings, i.e. certain elements, photosynthetic pigments, antioxidants, and phenolic compounds, as these substances influence the physiology of the plant and are also of importance for human health.

MATERIAL AND METHODS

Nanoparticles

Zinc oxide nanoparticles (ZnONPs) were purchased from Sigma Aldrich (catalogue no. 721077). The product has a declared particle size of <100 nm measured by dynamic light scattering (DLS) and a zeta potential of +46.1 ±1.5 mV [Wang et al. 2013].

Microorganism

The strain *B. subtilis* PCM 2224 was obtained from the Polish Collection of Microorganisms (PCM). The strain was kept deep-frozen (−80 °C, in 20% glycerol). The inoculum was cultured in 24 h nutrient broth.

Testing of the toxicity of various concentrations of ZnONPs for *B. subtilis*

A bacterial suspension with 5×10^4 CFU cm^{-3} in nutrient broth was prepared from the 24 h inoculum. Selected parameters characterizing the growth of *B. subtilis* in the presence of ZnONPs at concentrations of 0.2–1.4 mg cm^{-3} were determined. The effect of different concentrations of ZnONPs on the planktonic growth of bacteria in a liquid culture was tested by measuring the optical density of the culture after 24 h [Krzepiłko et al. 2023]. The MIC (minimum inhibitory concentration, i.e. the minimum concentration of an antimicrobial agent which inhibits the growth of microorganisms, in mg cm^{-3}) was determined by the microdilution method in liquid nutrient broth, measuring the optical density (OD) of the culture [Chavan and Nadanathangam 2019]. The MBC (minimum bactericidal concentration, i.e. the minimum concentration of ZnONPs at which 99.9% of bacteria die, in mg cm^{-3}) was determined by plating onto solid nutrient agar [Chavan and Nadanathangam 2019].

The effect of various concentrations of ZnONPs on biofilm formation by *B. subtilis* was tested by spectrophotometry in Bioscreen wells using crystal violet [Ma et al. 2017]. After 48 h of growth of *B. subtilis* in the control culture in nutrient broth and in the culture with ZnONPs, the supernatant was decanted, and the bacterial biofilm formed in the wells was washed with physiological saline, dried, and stained with 400 µL of 0.1% crystal violet for 20 min. The wells were then washed and dried, and the dye bound to the biofilm was dissolved in 400 µL of 30% acetic acid. The absorbance of crystal violet measured at 600 nm is proportional to the amount of biofilm formed [Naher et al. 2014]. The results were presented as a percentage of the absorbance of the control sample.

The level of oxidative stress induced by the various concentrations of ZnONPs in *B. subtilis* cells was measured by spectrophotometry using the nitro-blue tetrazolium (NBT) method. Superoxide anions cause NBT to produce formazan in an alkaline environment [Paździoch-Czochra 2013]. The absorbance of formazan is proportional to the amount of superoxide anion radicals. A reaction mixture containing 0.2 cm^{-3} of a 24 h culture of *B. subtilis*, 0.05 cm^{-3} of 1 M NaOH, 0.1 cm^{-3} of 5 mM NBT solution, and 2.65 cm^{-3} distilled water was incubated for 30 min, and the absorbance was measured at 560 nm.

The ability of *B. subtilis* to transform complex compounds was tested using Evans blue dye (EB) – $C_{34}H_{24}N_6Na_4O_{14}S_4$, molecular weight 960.81 g mol. Preliminary tests showed that Evans blue at a concentration of 0.1 mg cm^{-3} does not inhibit the growth of *B. subtilis*, which was confirmed by measuring the

OD of a culture growing in the presence of the dye. Incubation of the growth medium with 0.1 mg cm^{-3} Evans blue and various concentrations of ZnONPs for 48 h also did not lead to its decolorization.

The capacity for biotransformation was tested by culturing *B. subtilis* for 48 h on nutrient broth supplemented with Evans blue at a concentration of 0.1 mg cm^{-3} and ZnONPs at concentrations of 0.2–1.4 mg cm^{-3}. The cultures were then centrifuged, and the absorbance of the supernatant was measured at λ= 606 nm. The growth medium with 0.1 mg cm^{-3} Evans blue dye, without bacteria, was used to establish the initial absorbance (A0). The degree of decolorization was determined according to the following formula:

$$\text{Decolorization (\%)} = [(A0 - A1)/A0] \times 100$$

where: A0 – absorbance of a control sample of dye, A1 – absorbance of the sample following incubation with bacteria [Xia et al. 2019].

The effect of various concentrations of ZnONPs on the ability of *B. subtilis* to produce indole-3-acetic acid (IAA). *B. subtilis* bacteria were cultured in a nutrient broth enriched with tryptophan (100 µg cm^{-3}) and with ZnONPs at 37 °C for 48 h. The cultures were centrifuged, and 0.2 cm^{-3} phosphoric acid and 4 cm^{-3} Salkowski's reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$) were added to the supernatant (2 cm^{-3}), which was then incubated for 1 h. The absorbance was measured at λ = 530 nm [Ahmed and Hasnain 2010].

Preparation of *B. subtilis* bacteria for seed inoculation. Bacteria were cultured for 24 h on nutrient broth. The culture was then diluted with distilled water so that the concentration of bacterial cells was 1×10^7 CFU cm^{-3}, and the bacterial suspension was added to a 0.1 g sample of seeds.

Cabbage seeds

Red cabbage seeds of the Koda cultivar were purchased from W. Legutko Przedsiębiorstwo Hodowlano-Nasienne Sp. z o.o.

Substrate. The experiments were carried out on Aura soil substrate for sowing seeds and transplanting seedlings (produced by Agaris, Poland), containing peat in various degrees of decomposition, perlite, sand, chalk, and multi-component fertilizer, with pH 5.5–6.5 and salinity (NaCl) below 1.2 g L^{-1} (manufacturer's information). The soil substrate was sterilized in an autoclave (121 °C, 30 min).

Growing red cabbage seedlings in soil with zinc oxide nanoparticles (ZnONPs)

Preparation of seeds for sowing. Before each experiment, the seeds were washed for 10 min in 3% hydrogen peroxide and then rinsed thoroughly with distilled water [Xiang et al. 2015]. Seed samples of 0.1 g were placed in test tubes. The following samples were prepared:

Control – 0.1 g of seeds in distilled water,
BS – 0.1 g of seeds inoculated with *B. subtilis*,
0.2 mg ZnONPs – 0.1 g of seeds soaked in 1 cm^3 of a suspension of zinc oxide nanoparticles at a concentration of 0.2 mg,
0.4 mg ZnONPs – 0.1 g of seeds soaked in 1 cm^3 of a suspension of zinc oxide nanoparticles at a concentration of 0.4 mg,
BS + 0.2 mg ZnONPs – 0.1 g of seeds soaked in 1 cm^3 of a suspension of *B. subtilis* bacteria and zinc oxide nanoparticles at a concentration of 0.2 mg,
BS + 0.4 mg ZnONPs – 0.1 g of seeds soaked in 1 cm^3 of a suspension of *B. subtilis* bacteria and zinc oxide nanoparticles at a concentration of 0.4 mg.

Sowing seeds and growing plants. After 24 h, the contents of the tubes with the seeds prepared as described above were transferred to pots with 70 g of soil, and the seeds were then covered uniformly with 30 g of soil. Throughout the experiment, the moisture level of the substrate was measured with a Basetech BT-235PT soil moisture meter, the plants were watered with distilled water, and moisture was maintained at 60–70%. The plants were grown in constant conditions – a 12 h/12 h light/dark cycle and a temperature of 21 °C. After 21 days, the plants were gently removed from the soil and washed thoroughly, and the length of the roots and the entire plants was measured. This plant material was analyzed for mineral composition, the content of photosynthetic pigments and the content of antioxidants, including phenolic compounds.

Laboratory tests of plant material

Determination of mineral composition. The content of calcium (Ca), potassium (K), magnesium (Mg), zinc (Zn), iron (Fe), copper (Cu) and manga-

nese (Mn) in the plant material was determined by the ASA method according to PN-EN ISO 6869:2002.

Preparation of extracts

Aqueous, methanolic (70% v/v) and acetone (80% v/v) extracts of the plants were prepared. Red cabbage seedlings and the extraction solution in a 1:10 weight ratio were homogenized and then centrifuged for 5 min at 5000 rpm. The supernatant was frozen and used for subsequent analysis [Krzepiłko et al. 2016].

Measurement of the content of photosynthetic pigments

The chlorophyll concentration in the red cabbage seedlings was measured by spectrophotometry in an acetone extract [Ni et al. 2009]. The absorbance was measured at 645 nm, 663 nm and 470 nm using a Shimadzu UV-1280 spectrophotometer. The content of chlorophyll and carotenoids was expressed in mg 100 g^{-1} fresh weight (FW).

Measurement of the content of phenolic compounds

The assay was prepared using a methanol extract. The total content of phenolic compounds was measured by spectrophotometry using Folin's reagent [Lamuela-Raventós 2017]. The absorbance was measured after 1 h at 765 nm [Singleton et al. 1999]. The content of phenolic compounds was expressed in gallic acid equivalents per 100 g fresh weight of seedlings.

Total antioxidant capacity

The total antioxidant capacity (TAC) was determined by two common methods: with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical [Reet al. 1999] and with DPPH (2,2-diphenyl-1-picrylhydrazyl) radical [Brand-Williams et al. 1995]. The antioxidants contained in the material analyzed reacted with the radical, resulting in a change in the color of the solution. In the ABTS method, antioxidant capacity was determined in an aqueous extract from red cabbage seedlings. The absorbance of the solution was measured at 415 nm 30 min after the reagents were mixed. In the DPPH method, TAC was determined in a methanol extract from red cabbage seedlings. The absorbance of the solution was measured at 515 nm 60 min after the reagents were mixed. In both methods, the total antioxidant capacity was expressed as Trolox equivalents per 100 g fresh weight of cabbage seedlings.

RESULTS

Characteristics of *Bacillus subtilis* following incubation with ZnONPs

The first experiments were aimed at testing how *B. subtilis* responds to various concentrations of ZnONPs. *B. subtilis* showed a high level of resistance to the bactericidal effect of ZnONPs, as indicated by the high MIC above 1.6 mg cm^{-3} and MBC of 1.8 mg cm^{-3}. The addition of nanoparticles at concentrations of 0.2–1.4 mg cm^{-3} inhibited the planktonic growth of the cells (Table 1). A higher concentration of nanoparticles was negatively correlated with the optical density of the culture. At the highest concentration of 1.4 mg cm^{-3} ZnONPs, the optical density of the *B. subtilis* culture was only 26% of the control value.

The addition of ZnONPs to the *B. subtilis* bacterial culture caused oxidative stress in the cells, as confirmed in the NBT test. A small amount of superoxide anion radical was produced in the control sample, as indicated by the low absorbance of formazan. Even the lowest concentration of 0.2 mg cm^{-3} ZnONPs increased the production of superoxide anion by 30% relative to the control. *B. subtilis* cells produced the highest levels of this ROS at concentrations of 1.2 mg cm^{-3} and 1.4 mg cm^{-3} ZnONPs (496% and 479% of the control value, respectively).

The crystal violet assay showed that the *B. subtilis* cells in the control sample produced a biofilm (Table 1). The addition of ZnONPs at concentrations of 0.2–0.6 mg cm^{-3} increased the amount of biofilm produced by *B. subtilis*. At concentrations of 0.8 mg cm^{-3} ZnONPs and higher, the amount of biofilm produced was similar to the control.

The ability of *B. subtilis* to produce IAA plays an important role in stimulating plant growth. The amount of IAA in the growth medium was tested using Salkowski's reagent. The cells produced the largest amounts of IAA in the control conditions, i.e., 20 µg cm^{-3}. The amount of this hormone decreased in the samples treated with ZnONPs, but even at the highest concentrations of nanoparticles, IAA did not decrease by more than 23% of the control level.

The ability of *B. subtilis* to transform various organic compounds was tested based on the decolorization of the azo dye Evans blue. *B. subtilis* was capable of such transformations as after 48 h approximately 42% of the initial dye in the control sample had been

Table 1. Effect of ZnONPs on growth, biofilm formation and metabolite production by cells of *Bacillus* sp. expressed as a percentage of the control value

ZnONPs [mg cm^{-3}]	Planktonic growth [OD]	Biofilm formation [% of control]	IAA production [% of control]	Superoxide anion production [Formazan absorbance]	Evans blue decolorization [% of decolorization]
Control	1.396 a	100 a	100 a	0.070 a	41.66 a
0.2	1.218 b	145.83 b	88.14 b	0.100 b	40.65 a
0.4	1.025 c	181.69 c	81.92 c	0.179 bc	38.23 a
0.6	0.943 d	149.51 b	79.40 c	0.239 cde	38.05 a
0.8	0.673 e	102.68 a	79.66 c	0.215 cd	36.50 b
1.0	0,655 e	103.60 a	81.02 c	0.284 cdf	34.81b
1.2	0.520 f	102.97 a	77.97 c	0.347 f	33.12 b
1.4	0.363 g	98.59 a	77.34 c	0.335 ef	30.02 c
Correlation coefficient	–0.99056	0.969245	–0.46647	–0.802817	–0.98406

Values sharing the same letter in a column are not significantly different.
OD – optical density, IAA – indole-3-acetic acid

Table 2. Influence of ZnONPs and *B. subtilis* on the growth parameters of red cabbage

Sample	Percentage [%] of seeds germinating in days					Sprout length in 21 days [cm]	Root length in 21 days [cm]
	5	7	9	12	14		
Control	18.67 a	25.33 a	77.33 a	82.67 a	86.67 a	8.8 a	2.83 a
BS	25.33 b	33.33 a	85.33 b	86.67 a	90.67 a	9.8 b	3.1 b
0.2 mg ZnONPs	25.33 b	45.33 b	77.33 a	86.67 a	93.33 b	9.53 b	2.7 a
0.4 mg ZnONPs	37.33 c	46.67 b	78.67 a	86.67 a	93.33 b	9.47 b	2.53 a
BS + 0.2 mg ZnONPs	30.67 b	45.33 b	82.67 b	90.67 a	98.67 b	11.43 cd	3.47 b
BS + 0.4 mg ZnONPs	32.00 b	41.33 b	70.67 a	88.00 a	90.67 a	10.63 cd	3.27 b

Values sharing the same letter in a column are not significantly different. BS – *Bacillus subtilis*

decomposed (Table 1). Decolorization by *B. subtilis* cells in the presence of ZnONPs was less intensive than in the control, but even at the highest concentration of 1.4 mg cm^{-3} ZnONPs, 30% of the initial dye was decomposed. This assay was aimed at determining the concentrations of ZnONPs that would least disturb the metabolism of *B. subtilis* cells.

Concentrations of 0.2 and 0.4 mg cm^{-3} ZnONPs were used for further testing of the simultaneous effects of ZnONPs and *Bacillus subtilis*. Assessment of the growth parameters of *B. subtilis* showed that these concentrations had the least toxic effects.

Growth of red cabbage seedlings in soil in the presence of *Bacillus subtilis* and ZnONPs

The effect of the nanoparticles and *B. subtilis* bacteria, separately or in combination, on the germination and growth of red cabbage seedlings was evaluated. In the experiment in soil, concentrations of 0.2 and 0.4 mg ZnONPs per 0.1 g of seeds were used. Treatment of seeds with zinc oxide nanoparticles alone or inoculation with *B. subtilis* alone improved the germination rate in comparison with the control (Table 2). Among all treatments, the highest germination rate (99%) was obtained after 14 days in the BS + 0.2 mg ZnONPs treatment.

Table 3. Mineral contents in red cabbage plants

Sample	Mineral content [mg 100 g^{-1} DW]							
	Na	K	Mg	Fe	Mn	Cu	Zn	Co
Control	209 a	6010 a	548 a	27.4 a	11.0 a	1.38 a	4.61 a	0.012 a
BS	341 b	6170 a	580 a	42.4 b	10.5 a	2.89 b	5.03 a	0.015 a
0.2 mg ZnONPs	345 b	4660 b	494 b	35.2 a	8.03 a	1.27 a	9.58 b	0.010 a
0.4 mg ZnONPs	321 b	4240 b	471 b	25.9 a	10.3 a	2.33 a	15.1 c	0.010 a
BS + 0.2 mg ZnONPs	259 a	5620 a	534 a	22.7 a	9.73 a	1.92 a	10.6 b	0.007 a
BS + 0.4 mg ZnONPs	283 b	5910 a	578 a	27.8 a	9.44 a	1.32	5.26 a	0.009 a

Values sharing the same letter in a column are not significantly different. BS – *Bacillus subtilis*

Table 4. Influence of ZnONPs and *B. subtilis* on the content of photosynthetic pigments in red cabbage seedlings

Sample	Chlorophyll [mg 100 g^{-1} FW]			Carotenoid [mg 100 g^{-1} FW]
	a	b	a + b	
Control	23.30a	20.80 a	44.1 a	11.58 a
BS	23.36 a	20.35 a	43.71 a	11.01 a
0.2 mg ZnONPs	23.47 a	20.41 a	43.89 a	11.63 a
0.4 mg ZnONPs	22.50 a	22.15 b	44.65 a	10.28 a
BS + 0.2 mg ZnONPs	24.39 b	25.38 c	49.78 b	10.8 a
BS + 0.4 mg ZnONPs	24.38b	27.58 c	51.95 b	11.62 a

Values sharing the same letter in a column are not significantly different. BS – *Bacillus subtilis*

At this time, about 26% of the seeds had failed to germinate in the control group, about 7% in the ZnONP sample, and 10% in the BS sample (Table 2).

After 21 days of growth in soil, well-developed red cabbage plants were obtained. They were about 9 cm long in the control sample, with roots about 3 cm long. ZnONPs applied alone at both concentrations stimulated the growth of the whole plants, which were about 8% longer than in the control treatment. The plants which grew from seeds that were only inoculated with *B. subtilis* were 11% longer than the control plants. However, the effect of inoculation of seeds with *B. subtilis* did not differ statistically significantly from the effect of ZnONPs alone. Simultaneous application of zinc oxide nanoparticles and bacteria statistically significantly increased the total plant length. Seedlings treated with *B. subtilis* and 0.2 mg ZnONPs were nearly 30% longer than the control seedlings. Inoculation of seeds with *B. subtilis* positively influenced the length of the roots, which were 10% longer than the roots of the control seedlings. Seeds treated with ZnONPs alone produced shorter roots than the control seeds, but the differences were not statistically significant. Plants treated with both *B. subtilis* and ZnONPs had the longest roots, and these differences were statistically significant compared with the control. In the BS + 0.2 mg ZnONPs treatment, the roots were about 22% longer than in the control treatment.

Mineral composition of red cabbage seedlings

The content of macronutrients, i.e. sodium (Na), potassium (K) and magnesium (Mg), and micronutrients, i.e. iron (Fe), zinc (Zn), copper (Cu) and cobalt (Co), was determined in the young red cabbage plants (Table 3). The highest concentrations were noted for potassium. In the samples only inoculated with BS, the content of all minerals except for manganese increased in the red cabbage seedlings relative to the control. The addition of zinc oxide nanoparticles in-

Table 5. Influence of ZnONPs and *B. subtilis* on the content of phenolic compounds and total antioxidant capacity (TAC) in red cabbage seedlings

Sample	Total phenols [mg GA 100 g FW]	TAC (ABTS) [mM Trolox 100 g FW]	TAC (DPPH) [mM Trolox 100 g FW]
Control	213.9 a	0.795 a	0.224 a
BS	227.75 b	2.259 c	0.216 a
0.2 mg ZnONPs	209.43 a	4.51 e	0.233 a
0.4 mg ZnONPs	191.81 a	1.83 b	0.229 a
BS + 0.2 mg ZnONPs	231.46 b	5.97 f	0.458 b
BS + 0.4 mg ZnONPs	208.86 a	3.07 d	0.346 b

Values sharing the same letter in a column are not significantly different. BS – *Bacillus subtilis*

creased the content of zinc and sodium in the seedlings compared with the control, while higher iron concentrations were noted only at 0.2 mg ZnONPs and higher copper content at 0.4 mg ZnONPs. In contrast, the content of magnesium, potassium, manganese, and cobalt was lower in the samples treated with ZnONPs alone than in the control plants. Simultaneous treatment with *B. subtilis* and zinc oxide nanoparticles resulted in higher zinc and sodium content in the red cabbage seedlings, while the content of macronutrients Mg and K and micronutrients Fe, Mn and Co was lower than or similar to the control value. All BS + ZnONPs samples had lower content of the minerals tested, except for zinc, in comparison to the BS treatment.

The chlorophyll content

The contents of chlorophyll *a*, *b* and *a* + *b* in the BS treatment and the treatments with 0.2 mg ZnONPs alone were similar to those noted for the control sample (Table 4). In the treatment with 0.4 mg ZnONPs alone, only the content of chlorophyll b increased (by about 6% above the control value). A statistically significant increase in the content of all chlorophyll types was noted in the plants treated with both *B. subtilis* and ZnONPs at both concentrations, especially in the case of chlorophyll b (by 22% in the BS + 0.2 mg ZnONPs treatment and by 33% in the BS + 0.4 mg ZnONPs treatment). The content of carotenoid pigments did not change statistically significantly in any experimental treatments.

The content of phenolic compounds

The content of phenolic compounds in the samples ranged from 192 to 232 mg g FW (Table 5). The addition of zinc oxide nanoparticles reduced the content of phenolic compounds compared with the control values, but the differences were not statistically significant. The content of phenolic compounds in the plants from the BS and BS + 0.2 mg cm^{-3} treatments differed statistically significantly from the control and the remaining treatments.

Total antioxidant capacity

Total antioxidant capacity was measured using the ABTS method in the aqueous extract from whole plants. The TAC value was lower in the control than in the remaining treatments. Inoculation of the seeds with *B. subtilis* and/or the addition of zinc oxide nanoparticles significantly increased the total antioxidant capacity of the extract. The data from all treatments differed statistically significantly (Table 5). The inoculation of seeds with *B. subtilis* increased TAC in the plants by approximately 2.8-fold in comparison to the control plants. Treatment with 0.2 mg ZnONPs was more beneficial than the concentration of 0.4 mg ZnONPs (5.7 and 2.7 times the control values, respectively). The simultaneous effect of zinc oxide nanoparticles and inoculation with B. subtilis strongly influenced antioxidant capacity. The aqueous extract from the red cabbage plants in the BS + 0.2 mg ZnONPs treatment had the highest antioxidant capacity (about 7.6 times the control value).

The determination of total antioxidant capacity using the DPPH method was carried out using methanol extracts. The measurements showed that the control plants and the plants treated with ZnONPs alone or *B. subtilis* alone had similar antioxidant capacity

(Table 5). However, the plants treated simultaneously with BS and zinc oxide nanoparticles had the highest total antioxidant capacity, and these differences were statistically significant. The TAC value was 204% of the control value in the BS + 0.2 mg ZnONPs treatment and 154% in the BS + 0.4 ZnONPs treatment.

DISCUSSION

The intensive use of chemical fertilizers and pesticides generates serious challenges for the sustainable development of agriculture. New strategies are needed to reduce the reliance on chemical fertilizers and pesticides, enhance soil biodiversity, and protect agricultural ecosystems. One novel solution may be the simultaneous use of beneficial soil bacteria (PGPR) and nanoparticles. However, implementation of these strategies requires adjustment of the concentration of ZnONPs to both the specific traits of the bacterial species and the requirements of the crop plant. Excessive concentrations of ZnONPs can be toxic for both PGPR and plants [Liu et al. 2022]. The present study investigated the effects of ZnO nanoparticles on *Bacillus subtilis* as a PGPR, as well as the use of *B. subtilis* and ZnONPs on the early growth stages of red cabbage. *Bacillus* is one of the most commonly studied genera of bacteria due to its ability to promote plant growth in economically important crops [Turan et al. 2014, Kang et al. 2019b]. However, its stimulatory effect on the rhizosphere of plants may be limited by the presence of toxic substances in the environment. Zinc oxide nanoparticles show antimicrobial activity against various bacteria in a manner dependent on their concentration and characteristics such as size, shape and charge, as well as stabilizing agents [Mirzaei et al. 2017, Talebian et al. 2013, Mahamuni-Badiger et al. 2019]. Other *Bacillus* species, such as *B. cereus* [Krzepiłko et al. 2023], *B. thuringiensis* and *B. megaterium* [Matyszczuk and Krzepiłko 2022], have reduced growth capacity in the presence of zinc oxide nanoparticles and show reactions characteristic of oxidative stress. *In vitro* tests on *B. subtilis* confirmed that ZnONPs exert a concentration-dependent antimicrobial effect (Table 1). The test of the effect of commercial zinc oxide nanoparticles against *B. subtilis* showed MIC values of >1.6 mg cm^{-3} and MBC > 1.8 mg cm^{-3}, which were the same as the values previously reported for *B. cereus* [Krzepiłko et al. 2023], *B. thuringiensis* and *B. megaterium* [Matyszczuk and Krzepiłko 2022]. Other authors have reported varied MIC and MBC values for ZnONPs for bacteria, i.e., MICs ranging from 80 g cm^{-3} to more than 3000 g cm^{-3} and MBCs from 150 g cm^{-3} to more than 3000 g cm^{-3} [Ahmed et al. 2010, Azam et al. 2012, Chavan et al. 2019].

The antibacterial mechanism of action of ZnONPs involves the generation of reactive oxygen species which damage cells and induce oxidative stress [Ahmed et al. 2010, Awasthi et al. 2017, Canaparo et al. 2020]. The tests on *B. subtilis* also confirmed that oxidative stress takes place in the bacteria cells in the presence of ZnONPs (Table 1). Oxidative stress may disturb processes essential for cell division and metabolism in *B. subtilis*. ZnONPs caused a concentration-dependent reduction in planktonic growth, IAA production, and biotransformation of an azo dye (Table 1). Inhibition of the planktonic growth of cells and biofilm formation by high concentrations of ZnONPs significantly limits colonization of the rhizosphere of plants by *B. subtilis*. With regard to the use of *B. subtilis* as a factor supporting plant growth, the reduction in the IAA concentration caused by ZnONPs should also be considered unfavorable (Table 1). Various *Bacillus* species are capable of producing IAA in a range from 16 to 55 µg cm^{-3} IAA [Tsavkelova 2006, Felici et al. 2008]. The *B. subtilis* strain tested in the current experiment produced 20 µg cm^{-3} IAA in the control treatment (Table 1). Production of IAA supports seed germination and plant growth and development [Cabra Cendales et al. 2017, Saberi-Rise and Moradi-Pour 2020]. Nanoparticles of various metals can reduce IAA secretion by rhizosphere bacteria such as *Pseudomonas aeruginosa*, *P. fluorescens*, *A. chroococcum*, *B. amyloliquefaciens*, and *P. chlororaphis* [Dimkpa et al. 2012, Boddupalli et al. 2017, Haris and Ahmad 2017].

The biofilm produced by rhizosphere bacteria on roots plays an important role in interactions supporting plant growth [Su et al. 2020]. However, metal nanoparticles can inhibit biofilm formation by bacteria [Habash et al. 2017, Qayyum et al. 2017, Lewis Oscar et al. 2015, Ghasemian et al. 2015]. The current experiment showed that concentrations of 0.2–0.6 mg cm^{-3} ZnONPs increase biofilm formation by *Bacillus subtilis* (Table 1), which may be beneficial when both fac-

tors, i.e., nanoparticles and bacteria, are applied to stimulate plant growth. Bacteria producing a biofilm on plant roots increase the sorption of organic and inorganic substances from the soil, which can stimulate plant growth [Mahapatra et al. 2022]. From among the concentrations of ZnONPs tested in the study, concentrations of 0.2 mg cm^{-3} and 0.4 mg cm^{-3} were chosen for further analysis because they had the least negative impact on the *Bacillus subtilis* strain. Considering the goal of the experiment, i.e., to create conditions stimulating seedling growth, it should be noted that these concentrations of zinc oxide nanoparticles, despite inducing minor toxic effects in *B. subtilis* (such as symptoms of oxidative stress, slight inhibition of planktonic growth and a reduction in IAA synthesis), also increase the amount of biofilm produced by bacterial cells, which may facilitate the adherence of bacteria to plant roots.

Inoculation of red cabbage seeds with a suspension of *B. subtilis* cells improved the germination and growth parameters of the seedlings in comparison with the control (Table 2). An increase was noted in the seed germination rate, the length of the roots and whole seedlings, and the content of minerals in the red cabbage plants inoculated with *B. subtilis* bacteria alone (Tables 2 and 3). Similar observations of the effects of bacteria promoting the growth of cabbage, cucumber, maize, tomato, and other plants have been described by other authors [Cabra Cendales et al. 2017, Turan et al. 2014, Pérez-García et al. 2023, Pereira et al. 2020]. These positive effects are ascribed to the metabolism of PGPR, production of growth hormones, and protection against environmental stressors [Basu et al. 2021]. The *B. subtilis* bacteria used in the present study exhibit characteristics of PGPR, as they improve the growth of seedlings (Table 2), increase their content of minerals (Table 3) and antioxidants, including phenolic compounds important for improving the resistance of plants (Table 5), produce the hormone IAA, and are capable of biofilm formation and transforming high-molecular-weight compounds (Table 1). *B. subtilis* strains are able to release phosphorus from inorganic sources and produce organic acids influencing soil pH [Cabra Cendales et al. 2017]. *Bacillus megaterium* and *B. subtilis* have also been shown to increase the content of macro- and micronutrients in cabbage seedlings [Turan et al. 2014]. Chinese cabbage plants had a higher content of nutrients owing to inoculation with *B. subtilis* [Kang et al. 2019a].

The effect of zinc oxide nanoparticles was then tested at two concentrations, 0.2 mg and 0.4 mg, on the germination and growth of red cabbage seedlings in soil. In response to treatment with zinc oxide nanoparticles alone, seedling length was significantly increased compared with the control seedlings (Table 2). Similar studies carried out by other authors [Awasthi et al. 2017, Elhaj-Baddar and Unrine 2018, Solanki and Laura 2018, Sarkhosh et al. 2022] have also shown positive effects of zinc oxide nanoparticles on stem length in young plants.

Lower concentrations of ZnONPs usually increase the germination and growth parameters of seedlings, whereas higher concentrations negatively affect growth parameters. ZnONPs at concentrations of both 0.2 mg and 0.4 mg improved germination of red cabbage seeds after 14 days in soil (Table 2).

A positive effect of ZnONPs on the germination rate of seeds has been confirmed for tomato and wheat [Amooaghaie et al. 2017], peanut [Prasad et al. 2012], wheat [Prajapati et al. 2018], and pepper *Capsicum annuum* L. [Garcia-López et al. 2018].

However, the effect of ZnONPs on seed germination is varied. ZnONPs applied at the same concentrations have shown varied effects in different plant species. Among radish, rapeseed, ryegrass, lettuce, maize, and cucumber, only the germination of maize was inhibited by a high concentration (2 mg cm^{-3}) of nanoparticles [Lin and Xing 2007]. A concentration of ZnONPs that increased the growth parameters of mung bean inhibited the growth of chickpea seedlings [Mahajan et al. 2011]. Another experiment comparing the effect of ZnONPs on the germination of cucumber, alfalfa and tomato seeds obtained a positive effect only on the germination parameters of cucumber [De la Rosa et al. 2013]. Plants of the *Brassicaceae* family are capable of hyperaccumulation of zinc [Belouchrani et al. 2016]. However, even closely related species may show different tolerance for the presence of ZnONPs. *Brassica juncea* mustard plants were shown to be more tolerant of ZnONPs than cabbage or rapeseed plants [Feigl et al. 2013, Raza et al. 2022]. Zinc oxide nanoparticles can have inhibitory or toxic effects on plants depending on the concentration [Shaymurat et al. 2012, Xiang et al. 2015, Raskar and Shankar 2014,

Plaksenkova et al. 2020, Rajput et al. 2021, Sarkhosh et al. 2022]. ZnONPs have been shown to induce oxidative stress in plant tissues, resulting in inhibition of growth, especially of the roots, and in genotoxic effects in the cells of various organs of onion plants [Shaymurat et al. 2012, Sun et al. 2019, Plaksenkova et al. 2020]. This finding is supported by observations of the roots of red cabbage seedlings. The roots of the cabbage plants treated with ZnONPs alone were shorter than in the control plants (Table 2). Other authors have described the toxicity of ZnONPs manifested as a decrease in biomass and the content of photosynthetic pigments in plants such as barley, green pea, mung bean, chickpea, tomato, and wheat [Mahajan et al. 2011, Dimkpa et al. 2012, Mukherjee et al. 2014, Kouhi et al. 2015, Chen et al. 2018, Faizan et al. 2018, Wang et al. 2018, Rajput et al. 2023]. The experiments on red cabbage did not show a significant effect of ZnONPs applied alone on the content of chlorophyll *a* or chlorophyll *a* + *b* (Table 4). However, the use of ZnONPs alone can improve the growth and yield of red cabbage plants by increasing the uptake and accumulation of zinc (Table 3). This can make plants more resistant to abiotic stressors like drought and salinity. Depending on its concentration, zinc can influence the uptake and accumulation of micro- and macronutrients by plants. In the present study, the addition of zinc oxide nanoparticles also increased the sodium content in the seedlings, while the contents of magnesium, potassium, manganese and cobalt were lower than in the control (Table 3). In Chinese cabbage plants, zinc became toxic when its concentration in the shoots exceeded 2.5 mmol g^{-1} dry weight [Stuiver et al. 2014]. The accumulation of zinc in red cabbage seedlings exposed to ZnONPs did not exceed 1.51 mg g^{-1} DW, which corresponds to approximately 0.023 mmol g^{-1} DW (Table 3). These values are much lower than the zinc phytotoxicity thresholds given for plants grown in soil [Long et al. 2003]. In addition, increased zinc content in red cabbage may increase its nutritional value, making it more beneficial for human consumption. Zinc deficiency due to inadequate food intake is a global nutritional problem, especially in developing countries. Therefore, fortification of plants with zinc is a priority in many studies [Javaid et al. 2020, Umar et al. 2021]. Moreover, antioxidant content measured by the ABTS method increased in the red cabbage plants growing in the presence of ZnONPs (Table 5), which improves cabbage health-promoting properties. The concentrations of phenolic compounds and antioxidants measured by the DPPH method in the treatments with ZnONPs alone did not differ significantly from the control treatments.

The simultaneous application of zinc oxide nanoparticles and *B. subtilis* bacteria was shown to improve the growth parameters of red cabbage seedlings compared with the control and to the treatment of seeds with BS or ZnONPs alone (Table 2). The BS + 0.2 mg ZnONPs treatment proved better than the BS + 0.4 mg ZnONPs treatment, as it resulted in the highest number of germinated seeds, significantly longer whole plants and roots (Table 2), and higher content of chlorophylls (Table 4), phenolic compounds and antioxidants (Table 5).

Interactions between nanoparticles and PGPR can enhance the beneficial characteristics of these bacteria for plants, such as biofilm formation (Table 1), induce the secretion of growth-promoting substances (Table 1) and the production of secondary metabolites by the plants (Tables 4 and 5), and improve the uptake of certain minerals (Table 3). Other authors also stress the beneficial interactions between metal nanoparticles and PGPR. Copper oxide nanoparticles (CuONPs) increase IAA synthesis in *Pseudomonas chlororaphis* bacteria, which are included among PGPR. Zinc oxide nanoparticles (ZnONPs) increase the production of siderophores. It is likely that the release of ions from nanoparticles increases in the presence of metabolites of these bacteria [Dimkpa et al. 2012]. Simultaneous application of the PGPR *Providencia vermicola* and ZnONPs mitigates symptoms of stress in Chinese okra *Luffa acutangula* caused by arsenic. The protective role of bacteria and nanoparticles was manifested as more intensive plant growth, increased content of photosynthetic pigments and metabolites such as proline, sugars, protein, and IAA, and reduced content of As in the leaves [Tanveer et al. 2022].

Tomato plants growing in the presence of ZnONPs and one PGPR (*Bacillus subtilis*, *Lactobacillus casei* or *Bacillus pumilus*) were characterized by better tolerance for salinity, faster growth and development, and a reduced level of DNA methylation [Hosseinpour et al. 2020]. Zinc oxide nanoparticles applied to plants are able to mitigate various abiotic stresses. Simultaneous treatment with *Bacillus fortis* IAGS 223 and ZnONPs

was shown to mitigate symptoms of phytotoxicity induced by Cd (75 mg kg^{-1}) in muskmelon *Cucumis melo* plants [Shah et al. 2021].

ZnO nanoparticles and PGPR have been shown to increase the number of root nodules in soybeans, which positively influenced the height and yield of the plants [Seyed and Khoramdel 2016]. Rye plants treated with $ZnSO_4$NPs and *Pseudomonas* spp. also had increased contents of zinc, nitrogen, phosphorus, and potassium, which translated to increased yield and higher nutrient content in the grain [Gudadhe et al. 2018].

The molecular activity of plant cells is also important in bacteria–NPs–plant interactions. Smaller-size ZnONPs have been shown to react directly with root tissues because they can move through the symplast, e.g. through the plasmodesmata, whereas larger-size NPs accumulate in the apoplastic space [Jha and Pudake 2016]. Plant roots can utilize components of bacterial cells in the microbivory process [White et al. 2019]. Plants can utilize nanoparticles as a source of minerals which protect against environmental stress, stimulate metabolic processes in their tissues, and increase the availability of root secretions for microorganisms. A thorough understanding of the interactions taking place in the rhizosphere of plants growing in the presence of nanoparticles offers the chance to find conditions in which all elements of the system, i.e. the plant, microorganisms, and nanoparticles, interact to achieve stimulation of plant growth and optimal conditions for the development of PGPR without posing a threat to the environment.

CONCLUSIONS

The application of zinc oxide nanoparticles in combination with *B. subtilis* bacteria can enhance the beneficial effects of these bacteria on plants, improving parameters of plant growth and development. The results highlight critical interactions among ZnONPs, plant growth-promoting bacteria (PGPB), and plants, revealing how these elements enhance plant growth.

Low concentrations of ZnONPs (0.2–0.4 mg cm^{-3}) promote bacterial biofilm formation, improve nutrient uptake, and stimulate plant growth. In contrast, high concentrations of these NPs (> 1.6 mg cm^{-3}) induce oxidative stress, inhibit bacterial growth, and hinder plant development. Seeds treated with *B. subtilis* and low concentrations of ZnONPs exhibited increased germination rates, longer roots and seedlings, higher chlorophyll content, elevated antioxidant production, and improved uptake of essential minerals like zinc, which is critical for addressing global zinc deficiency. ZnONPs stimulate *B. subtilis* to form biofilms, enhancing bacterial attachment to plant roots and facilitating nutrient absorption. While high concentrations of ZnONPs suppress the bacterial production of indole-3-acetic acid (IAA), low concentrations maintain sufficient IAA levels to support plant growth. ZnONPs also induce the production of reactive oxygen species (ROS), which, at controlled levels, enhance plant defences but can cause cellular damage at higher concentrations. This research supports sustainable farming practices by reducing chemical inputs and leveraging natural plant-microbe-nanoparticle interactions. However, a deeper understanding of the interactions among nanoparticles, PGPB, and plants is essential, particularly regarding the optimal nanoparticle concentrations for specific bacteria, plant species, and cultivation conditions. In addition to experiments in controlled laboratory conditions, studies in field conditions are needed in order to better understand and confirm the results. Only in this way will it be possible to effectively use zinc oxide nanoparticles to optimize plant growth while limiting the potential environmental and health risks.

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