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BACTERIOSTATIC AND ANTIOXIDANT PROPERTIES OF PAULOWNIA LEAF EXTRACTS (*Paulownia* spp.) AS NATURAL PRODUCTS IN CROP PROTECTION

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ABSTRACT

Paulownia leaf extracts were tested for their bacteriostatic and antioxidant properties against six pathogenic bacteria in vegetable and fruit crops. Paulownia leaf extracts were most effective against the *Clavibacter michiganensis* ssp. *michiganensis* and *Xanthomonas hortorum* pv. *carotae*. Paulownia extracts were less effective against *Agrobacterium tumefaciens*, *Pseudomonas syringae* pv. *lachrymans* and *Pseudomonas syringae* pv. *tomato*. Only *Erwinia carotovora* was resistant to the tested plant extracts. The type of extraction solvent significantly impacts the antibacterial activity and the flavonoid and polyphenol contents of plant extracts. Acetone and alcoholic extracts had a higher content of flavonoid and polyphenolic compounds than water extracts, which resulted in their better bacteriostatic properties. The growth inhibition zones of the tested bacteria and the contents of flavonoids and polyphenols were significantly correlated. However, the bacteriostatic properties and antioxidant activity were not significantly correlated.

Keywords: plant extracts, bacterial plant pathogens, agar well diffusion method, flavonoids, polyphenols, paulownia

INTRODUCTION

Paulownia is a deciduous tree native to central and western China [Zhu et al. 1986]. This plant is also cultivated in Southeast Asia, North and Central America, Western and Southern Europe and Australia [Yadav et al. 2013]. The genus *Paulownia* includes nine fast-growing species [Zhu et al. 1986, Yadav et al. 2013]. The most popular species are *Paulownia tomentosa*, *P. fortunei* and *P. elongata* [Yadav et al. 2013, Morote et al. 2023]. Recently, the 'Oxytree' (*P. fortunei* × *P. elongata*), 'Cotevisa 2' (*P. elongata* × *P. fortunei*) and 'Shan Tong' (*P. tomentosa* × *P. fortunei*) hybrids have also been widely cultivated in the world [Sedlar et al. 2020, Kadlec et al. 2021].

Paulownias are adaptable, extremely fast-growing, multipurpose trees [Zhu et al. 1986, Yadav et al. 2013]. They are widely used, including in biomass production [Zuazo et al. 2013, García-Morote et al. 2014], the wood industry [Woods 2008, Barbu et al. 2022], paper

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production [Rai et al. 2000, López et al. 2012], phytoremediation and reclamation of degraded soils [Doumett et al. 2008, Tzvetkova et al. 2015], as well as the pharmaceutical and cosmetics industries [Schneiderová and Šmetkal 2015, Guo et al. 2023]. Paulownia leaves are also used as green fertilizer and animal feed [Al-Sagheer et al. 2019, Huang et al. 2022]. Moreover, the flowers of paulownia are melliferous [Woods 2008].

Currently, horticulture faces main challenges related to the increase in the production of high-quality food, the reduction of cultivated areas and the reduction of chemical fertilizers and pesticides [Godlewska et al. 2021]. Furthermore, the problem of plant diseases has become increasingly important in recent years. For example, crown gall is a common disease in fruit tree crops like apples, pears, peaches, and cherries [Mańka and Grzywacz 2023]. However, in Poland, plant protection is based mainly on preventive methods. Therefore, plant extracts may prove beneficial due to their potential biological functions. However, there is little information on the effectiveness of paulownia extracts in horticulture. More than 130 physiologically active components have been isolated in paulownia plants, such as flavonoids, lignans, phenolic glycosides, terpenoids, glycerides and phenolic acids [Schneiderová and Šmetkal 2015, Hawrył et al. 2020, Guo et al. 2023, Sławińska et al. 2023]. These compounds have anti-inflammatory, antimicrobial, antioxidant, cytotoxic and anti-cancer properties. Therefore, paulownia leaves, flowers, wood, fruit and bark have high potential in traditional Chinese medicine.

Many studies have reported that plant extracts are effective against microorganisms. Paulownia extracts exhibit antibacterial activity against human pathogens, including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* [e.g. Popova and Baykov 2013, Ferdosi et al. 2021, İnci et al. 2021, Škovranová et al. 2024]. Paulownia leaf extracts show confirmed bacteriostatic effect against pathogenic bacteria in animal fodder, i.e., *Bacillus cereus*, *Staphylococcus aureus* and *Yersinia enterocolitica* [Dżugan et al. 2021]. However, there is still no reliable information about their properties against plant pathogenic bacteria. Since the number of registered chemical plant protection products in the European Union is limited, there has been a growing interest that seeks bacteriostatic effects in natural and ecological products. Paulownia extracts may have this potential, although existing literature on this subject is limited. Therefore, this study aimed to evaluate the bacteriostatic and antioxidant properties of paulownia leaf extracts against pathogens that cause diseases in vegetable and fruit crops.

MATERIALS AND METHODS

Plant material and extract preparation

The fully expanded leaves of *P. tomentosa* × *P. fortunei* hybrids ('9503 UR', '9501 UR', 'SH 7 UR') and *P. tomentosa* genotypes ('LuP 4/20A', 'WEG 9 PEG') were obtained from trees growing in the field paulownia collection established in Central Poland (Mazovia; 51°43'51.4" N 21°45'54.5" E). The leaves collected from those paulownia clones contained the most secondary metabolites and antioxidants [Dżugan et al. 2021]. The paulownia leaves were dried at room temperature, without exposure to sunlight and grounded in a laboratory mill (MMK-06M, MPM, Milanówek, Poland). Two grams of air-dried plant material were then dissolved in 30 mL of solvent, shaken at 160 RPM at room temperature for 24 hours and filtered. The extracts were stored at 4 °C for further analyses. Several solvent types in various proportions were used to extract bioactive compounds from paulownia leaves (Table 1).

Bacteriostatic properties determination

The bacteriostatic properties of paulownia leaf extracts were tested against Gram-positive bacteria *Clavibacter michiganensis* ssp. *michiganensis* and five Gram-negative bacteria: *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae* pv. *lachrymans*, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas hortorum* pv. *carotae*. The *A. tumefaciens* was obtained from the Research Institute of Horticulture in Skierniewice, Poland. Five other bacterial isolates were purchased from the Bank of Pathogens at the Institute of Plant Protection – National Research Institute in Poznań, Poland.

The assessment of the bacteriostatic properties of paulownia extracts was carried out using the agar well diffusion method. Fresh bacterial suspensions were adjusted to 0.5 McFarland standard and were

Table 1. Composition of solvents used to extract active compounds from paulownia leaves

Solvent designation	Solvent composition
W	water (100%)
MetW	methanol/water (50%/50%)
MetWA	methanol/water/acetic acid (50%/49.5%/0.5%)
MetA	methanol/acetic acid (99.5%/0.5%)
EtW	ethanol/water (50%/50%)
EtWA	ethanol/water/acetic acid (50%/49.5%/0.5%)
EtA	ethanol/acetic acid (99.5%/0.5%)
AcWA2	acetone/water/acetic acid (70%/28%/2%)
AcWA05	acetone/water/acetic acid (70%/29.5%/0.5%)
AcWA02	acetone/water/acetic acid (70%/29.8%/0.2%)

evenly spread on Petri dishes. The *A. tumefaciens* was inoculated on the YEB medium (Thermo Fisher Scientific, Waltham, MA, USA). The *C. michiganensis* ssp. *michiganensis* and *X. hortorum* pv. *carotae* were grown on the PDA medium (BioMaxima, Lublin, Poland). Czapek medium (BioMaxima, Lublin, Poland) was used for *P. syringae* pv. *lachrymans* and *P. syringae* pv. *tomato*. The *E. carotovora* was inoculated on the TSA medium (BTL, Łódź, Poland).

Three wells with a diameter of 5 mm were made in the culture medium using a sterile cork borer. Next, 100 µL of solvent or paulownia extract solution was introduced into the wells. The Petri dishes were sealed with laboratory film and incubated at 28 °C for 48 hours after bacteria inoculation. After this time, the real inhibition zone of bacterial growth was obtained by subtracting the diameter of the zone of inhibition caused by the extract and solvents from the zone of inhibition due to the solvent. In this way, the antibacterial activity of paulownia extracts alone was determined.

Total flavonoids and polyphenolic compound content determination

The total flavonoid content was determined using the spectrophotometric method with aluminum chloride described by Yadav et al. [2013] with slight modifications. The volumes of 0.1 mL of paulownia extracts, 1.6 mL of methyl alcohol, 0.1 mL of 10% $AlCl_3 \times 6\ H_2O$, 0.1 mL of 1M CH_3COONa and 2.8 mL of distilled water were added. After 40 minutes of incubation at room temperature, the absorption of the tested solutions was measured at 415 nm. Quercetin dihydrate was used as a standard to make the calibration curve. Once the readings were obtained, the total flavonoid content was calculated. The results were expressed in milligrams QE per gram of leaf dry mass. Eight independent replicates were completed for each solvent.

The total content of polyphenolic compounds following the Folin-Ciocâlteu method was carried out according to Żbik et al. [2023]. The volumes of 0.1 mL of paulownia extract, 6 mL of distilled water and 0.5 mL of Folin-Ciocâlteu reagent were mixed. After 3 minutes, 1.5 mL of saturated sodium carbonate and 1.9 mL of distilled water were added. The mixture was then incubated at 40 °C for 30 minutes. The absorbance was measured at 765 nm. A calibration curve was prepared based on the gallic acid concentration. The results were expressed in milligrams GAE per gram of leaf dry mass. Eight independent replicates were performed for each solvent.

Antioxidant activity determination by DPPH and FRAP tests

The DPPH was conducted according to Uğuz and Kara [2019]. A solution of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) diluted with methanol was prepared, with the absorbance of approximately 0.9 at 517 nm. Then, 0.2 mL of paulownia extract and 1.8 mL of DPPH solution were mixed. After five minutes of incubation, the absorbance of tested samples was measured again at 517 nm against methanol as a reference. The results were expressed in µmol Trolox per gram of leaf dry mass. For each solvent, six independent replications were completed.

Antioxidant properties of paulownia extract were additionally determined using the FRAP test according to Bertoncelj et al. [2007]. The FRAP reagent contained 25 mL of 0.3 M acetate buffer (pH 3.6), 2.5 mL

of a 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) and 2.5 mL of 20 mM $FeCl_3$. The volumes of 0.2 mL paulownia extract and 1.8 mL of FRAP solution were mixed. After incubation at room temperature for 10 minutes, the absorbance of tested samples was measured at 593 nm. A calibration curve was prepared based on the Trolox solution, and results were expressed in µmol Trolox per gram of leaf dry mass. For each solvent, six independent replications were performed.

Statistical analyses

The obtained results were statistically analyzed using the Statistica 13.3 program software. The collected data were subjected to ANOVA and LSD mean separation tests at $p < 0.05$ significance level, and a correlation analysis among the obtained parameters was performed. Cluster analysis according to Ward's method and Euclidean distance were also performed. The following traits were used for agglomeration: the diameter of the bacterial inhibition zone, total flavonoid and polyphenol contents, and antioxidant activity of the extracts.

RESULTS

Bacteriostatic properties of paulownia leaf extract

Paulownia leaf extracts have a bacteriostatic effect against five bacteria. Plant extracts were most effective against the *C. michiganensis* ssp. *michiganensis* and *X. hortorum* pv. *carotae* (Table 2). The average diameter of the growth inhibition zone was over 2 mm. The tested plant extracts were less effective for *P. syringae* pv. *lachrymans* and *A. tumefaciens* with a zone of inhibition of almost 2 mm. Small inhibition zones were also found for *P. syringae* pv. *tomato*. Nevertheless, *E. carotovora* was generally resistant to paulownia leaf extracts (Table 2).

The extraction solvent type influenced the antibacterial activity of paulownia leaf extracts (Fig. 1). Water-acetone-acetic extracts were more effective than alcohol and water extracts. The antimicrobial activity was significantly higher in 'AcWA05' and 'AcWA02' extracts against tested bacteria. For *X. hortorum* pv. *carotae*, the maximum inhibition zone was over 6 mm (Figure 2A). For the *C. michiganensis* ssp. *michiganensis*, the bacteriostatic activity was slightly lower, with a zone of inhibition almost 6 mm in diameter (Figures 2B and C). These two acetone extracts also exhibited high bacteriostatic activities against the other tested bacteria. However, the third 'AcWA2' acetone extract had a slightly weaker bacteriostatic effect (Table 2). For *E. carotovora*, the growth inhibition zone for acetone solvents was approximately 1 mm.

High antimicrobial activity was also found for ethanol paulownia leaf extracts ('EtWA' and 'EtA'). *P. syringae* pv. *lachrymans* was the most sensitive to these extracts. Maximum zones of inhibition reached 3.5 mm.

Table 2. Diameter of growth inhibition zone of tested bacterial isolates by paulownia leaf extracts

Solvent	Growth inhibition zone diameter (mm)*					
	Agrobacterium tumefaciens	*Clavibacter michiganensis* ssp. *michiganensis*	*Erwinia carotovora*	*Pseudomonas syringae* pv. *lachrymans*	*Pseudomonas syringae* pv. *tomato*	*Xanthomonas hortorum* pv. *carotae*
W	0.4 a	0.4 a	0.3 ab	0.1 a	0.2 a	0.3 a
MetW	0.3 a	0.7 a	0.2 a	0.2 a	0.4 ab	0.3 a
MetWA	1.2 bc	1.5 b	0.5 bc	0.8 b	0.8 bc	0.6 a
MetA	1.4 cd	2.2 cd	0.5 bc	1.4 c	0.9 c	2.3 c
EtW	0.5 ab	0.4 a	0.4 ab	0.3 ab	0.4 ab	0.4 a
EtWA	2.3 e	2.3 cd	0.7 c	3.4 e	1.7 d	2.1 bc
EtA	2.1 e	1.8 bc	0.5 bc	2.4 d	1.1 c	1.6 b
AcWA2	2.1 de	2.5 d	0.5 bc	3.7 ef	2.5 e	3.3 d
AcWA05	4.0 f	5.8 f	1.1 d	4.1 f	3.6 f	6.1 e
AcWA02	4.5 f	5.1 e	1.0 d	3.1 e	3.3 f	5.6 e
Mean	1.9	2.3	0.6	2.0	1.5	2.3

* real growth inhibition zone diameter, i.e., difference between the growth inhibition zone diameter of paulownia leaf extract and the growth inhibition zone diameter of solvent used for extraction (different letters indicate significant differences at $p = 0.05$)

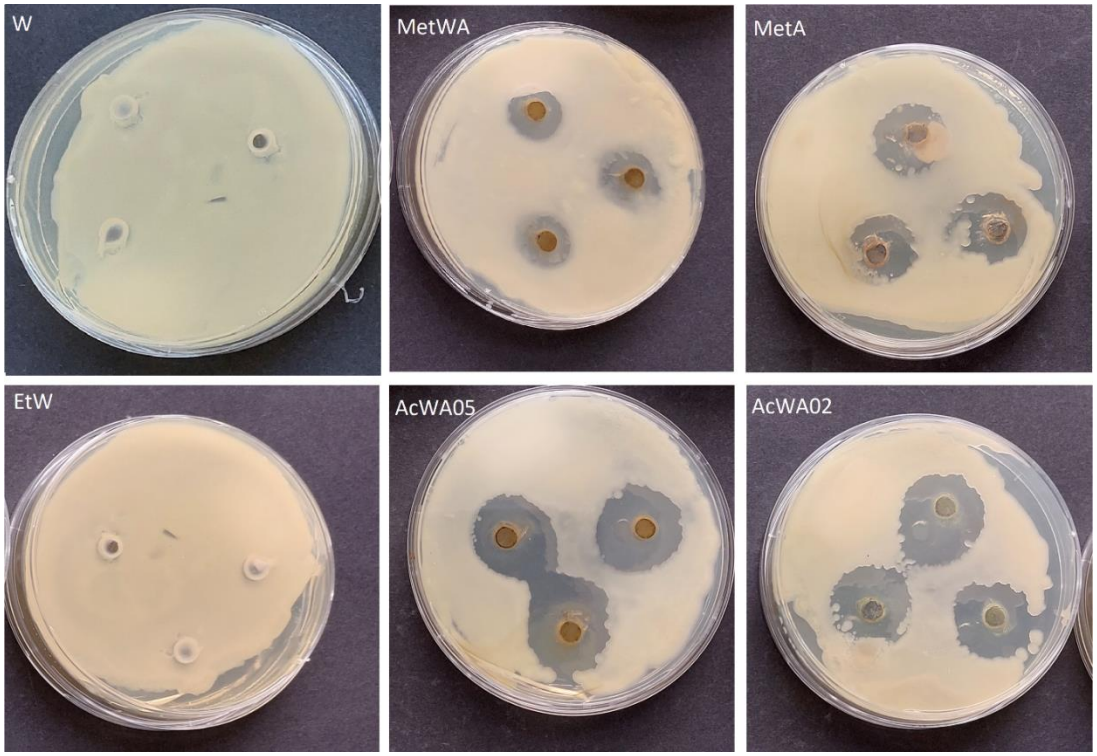

Fig. 1. Effect of paulownia leaf extracts on the growth of *Clavibacter michiganensis* ssp. *michiganensis* depending on extraction solvent type

The ethanol extracts also showed bacteriostatic properties against *A. tumefaciens* and *C. michiganensis* ssp. *michiganensis* with an inhibition zone of approximately 2 mm. The only water-ethanol extract ('EtW') did not inhibit the growth of any of the tested bacteria. The diameter of the growth inhibition zone did not exceed 0.5 mm.

However, the least bacteriostatic properties against tested bacteria were shown by methanol extracts (Figures 2D and E). Nevertheless, *X. hortorum* pv. *carotae* and *C. michiganensis* ssp. *michiganensis* were the most sensitive to 'MetA' extract. The highest inhibition zone diameter of methanol extract was almost 2.5 mm. Water-methanol extract ('MetW') was the least effective for tested bacteria. Nevertheless, the paulownia water extract had the weakest inhibitory effect against all tested bacteria (Table 2). The diameter of the growth inhibition zone did not exceed 0.5 mm.

Total flavonoid and polyphenol contents in paulownia extracts

The total flavonoid content in paulownia leaf extracts depended on the solvent types (Fig. 3). The content of these compounds ranged from 11 to 56 mg QE·g^{-1} DM. The highest total flavonoid content was found in the acetone extracts, followed by the ethanol, methanol and water extracts. The total flavonoid content of acetone extracts was over 50 mg QE·g^{-1} DM. The highest content of these compounds was determined in the 'AcWA02' extract. However, the total flavonoid content of ethanol and methanol extracts ranged from 29 to 46 mg QE·g^{-1} DM. Among the tested alcohol extracts, the contents of these compounds were higher in 'EtW' and 'MetA' extracts. Meanwhile, the total flavonoid content in water extract was approximately 5 and 3.5 times lower than in acetone and alcohol extracts, respectively.

The total polyphenol content in paulownia leaf extracts was determined using the Folin-Ciocâlteu method. The content of these compounds ranged from 30 to 62 mg GAE·g^{-1} DM. The total polyphenol content of the acetone extracts is higher than other extracts (Fig. 4). The highest total polyphenol content was found in the 'AcWA05' acetone extract. Two other acetone extracts ('AcWA2' and 'AcWA02') were also

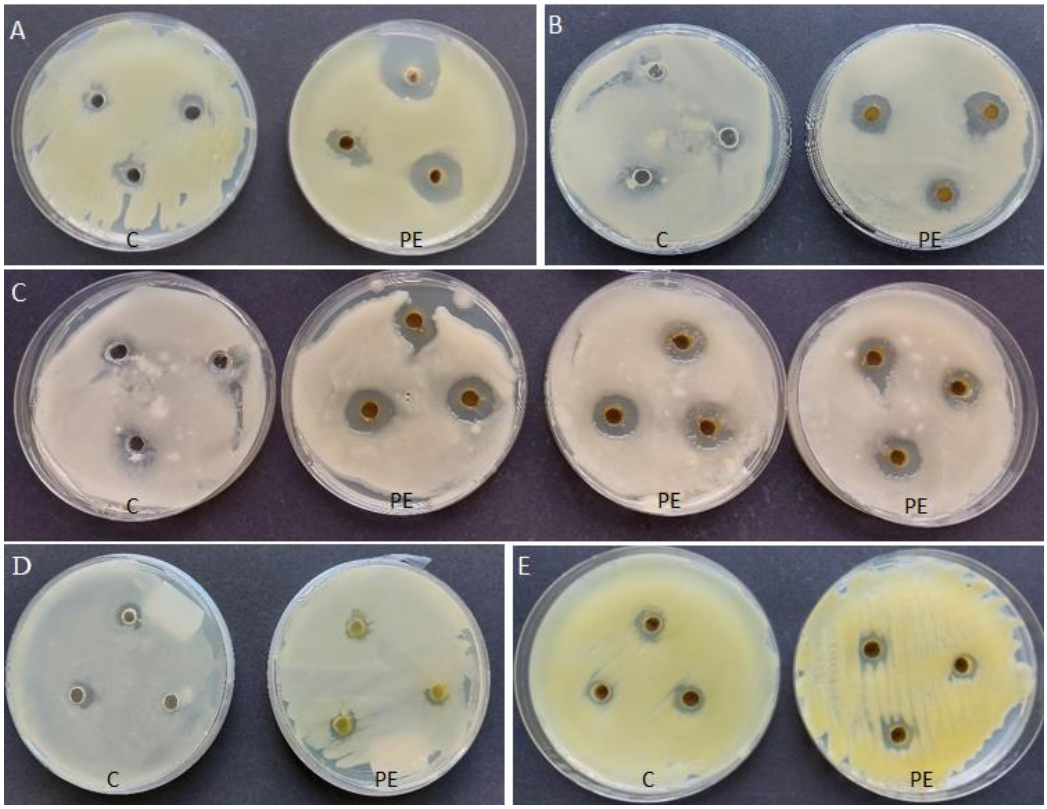

Fig. 2. Growth inhibition zone of bacterial isolates, where C – control and PE – paulownia leaf extracts; *Xanthomonas hortorum* pv. *carotae* 'AcWA05' (A), *Clavibacter michiganensis* ssp. *michiganensis* 'AcWA05' (B), *C. michiganensis* ssp. *michiganensis* 'AcWA02' (C), *Agrobacterium tumefaciens* 'MetA' (D) and *X. hortorum* pv. *carotae* 'MetA' extract (E)

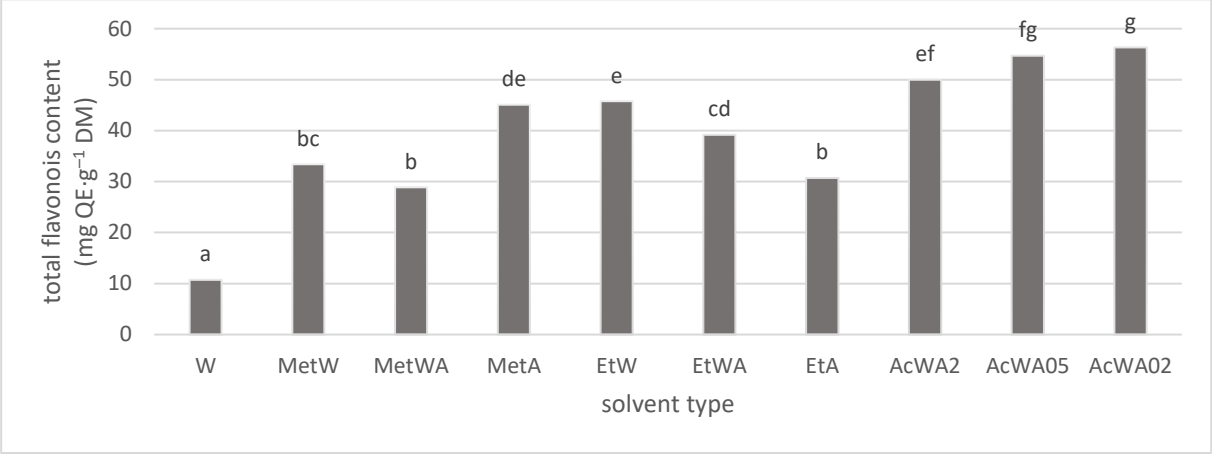

Fig. 3. The total flavonoid content in paulownia leaf extracts depending on the solvent type (different letters indicate significant differences among means at $p = 0.05$)

characterized by a high content of polyphenols. However, the total polyphenol content of the ethanol and methanol extracts was slightly lower (Figure 4), especially for the 'MetW' and 'EtA' extracts. Nevertheless, the lowest content of polyphenolic compounds was found in the water extract. The total polyphenol content in water extract was up to two times lower than in acetone extracts.

Antioxidant activity of paulownia extracts

The antioxidant activity of paulownia leaf extracts determined by the DPPH method ranged from 128 to 169 µmol Trolox·g^{-1} DM. Meanwhile, the antioxidant activity of these extracts measured by the FRAP method ranged from 121 to 167 µmol Trolox·g^{-1} DM. The antioxidant activity of the extracts depended on the type of solvent (Figure 5). The alcohol extracts acidified with acetic acid ('EtA' and 'MetA') were found to have the highest antioxidant activity. High antioxidant activity was also found in the water-methanol-acetic ('MetWA') and water-acetone-acetic extracts ('AcWA2'). However, the lowest antioxidant activity was observed in the ethanol-water extract, which was approximately 25% lower compared with the 'EtA' extract.

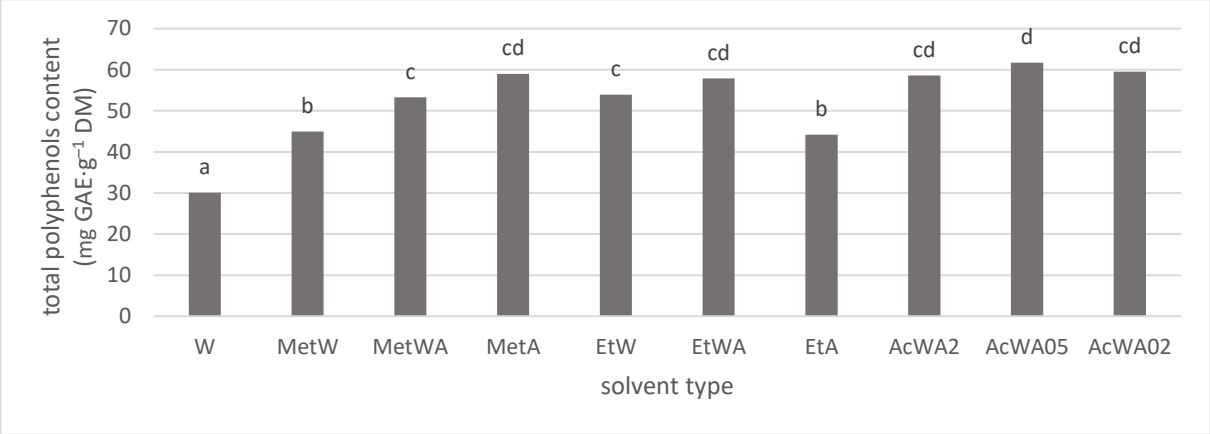

Fig. 4. The total polyphenol contents in paulownia leaf extracts depending on the solvent type (different letters indicate significant differences among means at $p = 0.05$)

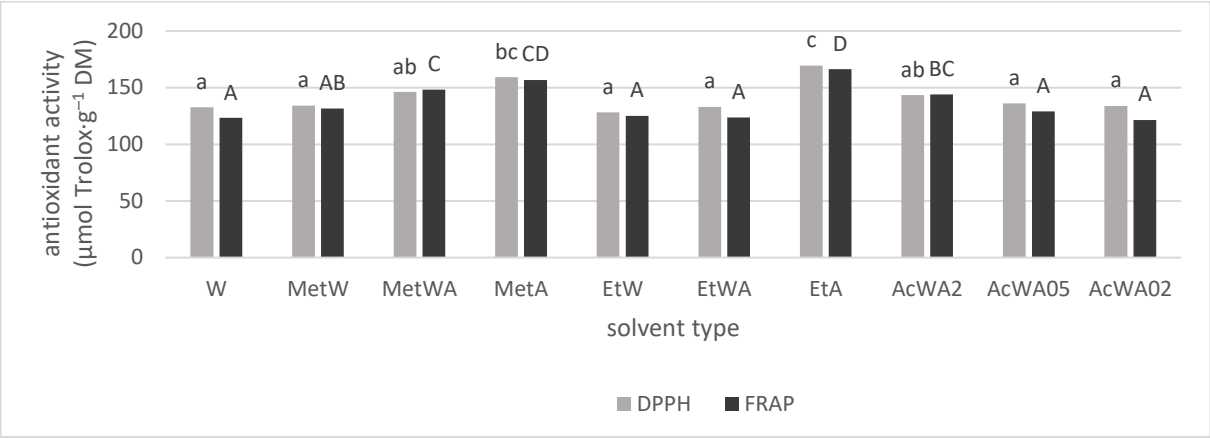

Fig. 5. Antioxidant activity of paulownia leaf extracts (different letters indicate significant differences among means at $p = 0.05$)

Relationships between the analyzed traits

The relationships between the bacterial growth inhibition zone, the total content of flavonoids and polyphenols and the total antioxidant activity of paulownia leaf extracts were determined. The *zone* of *growth inhibition* of the tested bacteria and the content of flavonoids and polyphenols were significantly positively correlated (r = 0.61*–0.77*). However, the bacteriostatic properties of the paulownia extracts and their antioxidant activity were not significantly correlated ($r = –0.22^{ns}–0.11^{ns}$). A significantly high positive correlation was only found between the DPPH and FRAP methods (r = 0.96*).

A cluster analysis divided the tested bacteria into three groups depending on their sensitivity to paulownia leaf extracts (Figure 6A). The first group consisted of *C. michiganensis* ssp. *michiganensis* (CMM), *X. hortorum* pv. *carotae* (XHC) and *P. syringae* pv. *tomato* (PST). The second group were *A. tumefaciens* (AT) and *E. carotovora* (EC). However, *P. syringae* pv. *lachrymans* (PSL) were separated into the third group.

Ten paulownia leaf extracts were divided into four groups depending on bacteriostatic properties by cluster analysis (Figure 6B). The first group consisted of 'AcWA02' and 'AcWA05' extracts and was separated from the other samples. These acetone solvents had the highest inhibition zone of bacterial growth. The second group comprised 'AcWA2' and 'EtWA' extracts. The 'MetWA', 'MetA' and 'EtA' extracts formed a separate group. The 'W', 'EtW' and 'MetW' extracts were separated into a fourth cluster with the least bacteriostatic activity.

DISCUSSION

Many secondary metabolites were isolated from the paulownia plants [Schneiderová and Šmetkal 2015, Sławińska et al. 2023]. Paulownia leaves, flowers, wood, fruit and bark were used in traditional Chinese medicine. The antibacterial activity of paulownia extracts on human pathogens has been found [Popova and Baykov 2013, Ferdosi et al. 2021, İnci et al. 2021, Škovranová et al. 2024]. These plant extracts were used, among others, against *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria. Paulownia leaf extracts demonstrated the bacteriostatic effect against pathogenic bacteria in animal fodder, i.e., *Bacillus cereus*, *Staphylococcus aureus* and *Yersinia enterocolitica* [Dżugan et al. 2021]. However, there is still no reliable information about their properties against pathogenic bacteria in plants. Due to the number of registered chemical plant protection products in Poland is limited, there is a growing interest in natural and ecological alternative for their bacteriostatic

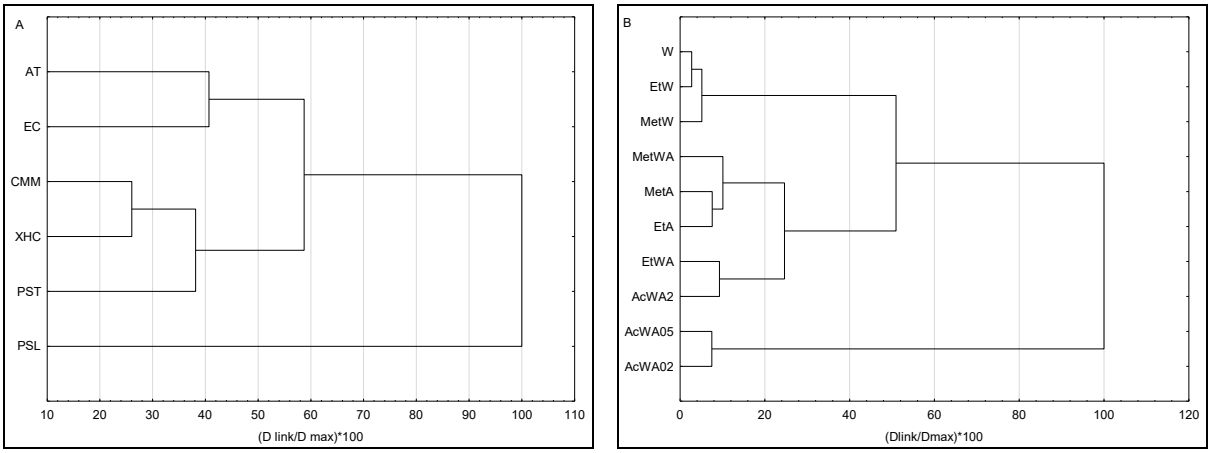

Fig. 6. Dendrogram showing the similarity of paulownia leaf extract effect; *Agrobacterium tumefaciens* (AT), *Clavibacter michiganensis* ssp. *michiganensis* (CMM), *Erwinia carotovora* (EC), *Pseudomonas syringae* pv. *lachrymans* (PSL), *P. syringae* pv. *tomato* (PST) and *Xanthomonas hortorum* pv. *carotae* (XHC)

effects. Moreover, large leaves of paulownia plants are not commonly used in the biomass industry [Jacek and Litwińczuk 2016].

Many studies have reported that plant extracts are more effective against Gram-positive bacteria than Gram-negative bacteria [e.g., Parekh et al. 2005, Limsuwan et al. 2009, Modarresi-Chahardehi et al. 2012, Koohsari et al. 2015]. Only a few studies have shown that plant extracts have a better inhibitory effect against Gram-negative bacteria [Parekh and Chanda 2007, Popova and Baykov 2013, El Mannoubi 2023]. The first results of the bacteriostatic activity of paulownia plants are promising. In the present study, paulownia leaf extracts were confirmed to have bacteriostatic properties against some pathogenic bacteria causing diseases of vegetable and fruit plants. The paulownia leaf extracts were effective against both Gram-positive (*C. michiganensis* ssp. *michiganensis*) and Gram-negative bacteria (*A. tumefaciens*, *P. syringae* pv. *lachrymans*, *P. syringae* pv. *tomato* and *X. hortorum* pv. *carotae*). Furthermore, paulownia extracts can be used in integrated and organic cultivation.

Paulownia leaf extracts were most effective against the *C. michiganensis* ssp. *michiganensis* and *X. hortorum* pv. *carotae*. These bacteria cause common diseases, such as bacterial canker of tomatoes and bacterial blight of carrots [Scott and Dung 2020, Peritore-Galve et al. 2021]. This is probably the first study of the antimicrobial activity of paulownia leaf extracts against these pathogens. Nevertheless, the Moroccan plant extracts [Talibi et al. 2011], common marigold (*Calendula officinalis* L.) and purple coneflower (*Echinacea purpurea* L.) extracts [Aksoy et al. 2021] were tested against the bacterial canker of tomato. The growth inhibition diameter ranged from 5 to 50 mm. However, the peppermint (*Peganum harmala* L.) and Syrian rue (*Peganum harmala* L.) extracts [Siddique et al. 2020] were less effective. The growth inhibition diameter slightly exceeded 10 mm. On the other hand, Jacobo-Salcedo et al. [2011] and Makhubu et al. [2023] reported that plant extracts were ineffective against *C. michiganensis* ssp. *michiganensis*. However, there is very little information on the effectiveness of plant extracts against *Xanthomonas hortorum* pv. *carotae*.

The paulownia leaf extracts were also effective for *A. tumefaciens*. It is worth underlining that the soil bacteria *Agrobacterium* is polyphagous with a wide host range [Mańka and Grzywacz 2023]. This is particularly important because of the lack of effective chemical plant protection products on crown gall. Therefore, plant extracts can be useful for plant protection. The mugwort (*Artemisia* L.) and ginger (*Zingiber* Rosc.) extracts [Njagi et al. 2021], as well as g*arlic (Allium sativum L.) extracts* [Nabatanzi 2018] were also tested against *A. tumefaciens*. The growth inhibition diameter was approximately 12 mm for these plant extracts. The bacteriostatic effect of the eucalyptus leaf (*Eucalyptus cinerea* L.) extracts against crown gall was also found. The growth inhibition diameter ranged from 0 to 15 mm, depending on the extraction solvent [Kahla et al. 2017]. Okla et al. [2019] also found a bacteriostatic effect of the orange leaf and branch (*Citrus aurantium* L.) extracts on *A. tumefaciens*. The highest diameter of the growth inhibition zone of this bacterium was up to 18 mm. However, branch bark and branch wood extracts were ineffective.

Bacterial soft rot is one of the most common diseases of potatoes [Viswanath et al. 2018]. However, paulownia leaf extracts did not significantly inhibit the growth of *E. carotovora*. Bdliya and Dahiru [2006] found that aqueous leaf and seed extracts of neem (*Azadirachta indica* L.) significantly reduced bacterial soft rot. However, the aqueous leaf extract of river redgum (*Eucalyptus camaldulensis*) was ineffective. In turn, Viswanath et al. [2018] evaluated 13 aqueous plant extracts against soft rot caused by *E. carotovora*. The highest diameter of the inhibition zone was found for jimsonweed (*Datura stramonium* L.) and common fig (*Ficus carica* L.) extracts and was approximately 10 mm. However, the other plant extracts were less effective. Bhardwaj and Laura [2008] tested aqueous extracts of twenty plants against *E. carotovora*. The highest diameter of growth inhibition zone was found for leaf tea (*Camellia sinensis* L.), arabic tree bark (*Acacia arabica* Willd.) and katha bark (*Acacia catechu* Willd.) extracts. However, 13 other plant extracts were ineffective.

In the present study, paulownia leaf extracts demonstrated a bacteriostatic effect against five tested bacteria. Nevertheless, the growth inhibition zones obtained are weaker than the results of the discussed literature. There are no unified methods for plant extract preparation. Various extraction techniques are used to extract bioactive compounds, including hot water ex-

traction, ultrasound or microwave. The bacteriostatic activity also depends on the extraction solvent type, extraction time, sample weight and different plant parts. Plant extracts are prepared from leaves, stems, branches, fruits, flowers and roots. The differences in the bacteriostatic activity of plant extracts may also depend on the plant cultivar, growth conditions, plant part, plant maturity, extraction and storage method [Rashmi and Negi 2022].

The extraction solvent type significantly influenced the antibacterial activity of paulownia extracts. The solvents were ranked by the inhibition zone in the following order: acetone, ethanol, methanol and water. Based on the cluster analysis, 'AcWA02' and 'AcWA05' solvents were separated into a separate group. Acetone extracts obtained from paulownia plants were significantly more effective than water extracts, possibly due to the higher content of flavonoids and polyphenols. Similarly, Basri and Nor [2014] reported that the acetone extracts of tropical fruit trees (*Canarium odontophyllum* Miq.) against *Staphylococcus aureus* had a higher inhibition zone than the methanol extracts. Nevertheless, it is noteworthy that the 'EtWA' and 'EtA' extracts of paulownia leaves also had significantly high inhibition zones, especially against the *P. syringae* pv. *lachrymans*. These two ethanol extracts also exhibited moderate bacteriostatic activity against the *A. tumefaciens* and *C. michiganensis* ssp. *michiganensis*. However, the lowest bacteriostatic activity was found for water ('W') and water-alcohol ('MetW' and 'EtW') extracts. Similarly, Krupiński and Sobiczewski [2001] reported higher bacteriostatic activity of ethanol extracts against *E. amylovora* than water extracts. Likewise, Gniewosz et al. [2012] discovered that the ethanol extracts of common sage (*Salvia officinalis* L.) were more effective than water extracts. In another study, the plant methanol extracts were also more active than the water extracts [Parekh et al. 2005].

Moreover, the flavonoid and polyphenol contents in paulownia leaf extracts and their antioxidant activity depend on extraction solvents. These compounds are the most important secondary metabolites that may be responsible for antibacterial properties [e.g., Safari and Ahmady-Asbchin 2019, Krzepiłko et al. 2020]. The antimicrobial activity of flavonoids involves disrupting cell membrane integrity, inhibiting nucleic acid synthesis and paralyzing the energy metabolism of bacteria [Tagousop et al. 2018, Liga et al. 2023]. A significantly positive correlation was found between the flavonoid content and the diameter of the *growth inhibition* zone of the tested bacteria (r = 0.65*–0.77*). Nevertheless, the flavonoid content in the plant extracts varies significantly depending on the solvents used [Gong et al. 2012, Dirar et al. 2019, El Mannoubi 2023], which agrees with the current results. The most effective solvent for flavonoid extraction was acetone. Paulownia acetone extracts contained five times higher total flavonoid content than a water extract. Likewise, Munhoz et al. [2014] reported that water-acetone and water-ethanol extracts contained approximately three times more flavonoids than water extracts. Sasadara and Wirawan [2021] also confirmed a higher total flavonoid content in alcohol and acetone than in water extracts. A high content of these compounds in paulownia water-ethanol extracts was found by Dżugan et al. [2021]. Total flavonoid content ranged from 111 to 234 mg QE·g^{-1} DM, depending on the paulownia clone. However, Na-Young and Ki-Tae found a slightly lower content of total flavonoids in paulownia ethanol extracts [2019]. The content of these compounds was 115 mg CE·g^{-1} DM. Similarly, Yadav et al. [2013] found that the total flavonoid content in the fresh leaf extracts of paulownia ranged from 103 to 158 µg·mL^{-1}.

Polyphenols damage the cytoplasmic membrane, cell wall and nucleic acid, as well as denature enzymes of microorganisms [Skroza et al. 2019, Krzepiłko et al. 2020]. In the present study, the total polyphenol contents and the diameter of the *growth inhibition* zone of the six tested bacteria were significantly positively correlated (r = 0.61* – 0.67*). The acetone solvent was the most effective in the extraction of polyphenols. The content of polyphenolic compounds was two times higher in acetone than in water extracts. This may partly explain their higher bacteriostatic properties. These results were similar to those obtained by Złotek et al. [2016]. The water-acetone extracts with acetic acid significantly increased the total polyphenol content of basil plants (*Ocimum basilicum* L.). However, the combination of methanol, water and acetic acid was less effective. In studies carried out by Michiels et al. [2012], the acetone/

water/acetic acid system (70/28/2, v/v/v) also proved to be more effective in the extraction of polyphenolic compounds from fruits and vegetables than methanol solvents. Likewise, Abozed et al. [2014] and Dirar et al. [2019] recommended acetone solvents for extracting polyphenols. However, Hęś et al. [2012], Tomsone et al. [2012], Salih et al. [2021], and Palaiogiannis et al. [2023] reported that alcohol extracts were more effective for the extraction of polyphenols. Nino et al. [2016] also found that methanol extracts of medicinal plants had higher phenolic and flavonoid contents than other solvents used for extraction.

Additionally, flavonoids and polyphenols are perfect sources of antioxidants, which deactivate free radicals and protect cells [Proestos et al. 2013, Krzepiłko et al. 2020]. The antioxidant activity of paulownia extracts was determined using the DPPH and FRAP methods. A significantly high positive correlation between the above tests was found (r = 0.96*). The other researchers [Turkmen et al. 2006, Nino et al. 2016, Złotek et al. 2016, Sasadara and Wirawan 2021] reported a positive correlation between the phenolic content and antioxidant activity. However, this relationship was not confirmed in the present study. The diameter of the growth inhibition zone of tested bacteria was also not significantly correlated with the antioxidant activity of paulownia extracts. Nevertheless, Sithisarn et al. [2015] also found no correlation between the total phenolic and flavonoid contents, antibacterial activity, and antibacterial activity of the extracts from *C. orientalis*. Similarly, Adamu et al. [2014] and Ispiryan et al. [2024] found no high correlation between antioxidant activity and antibacterial activity of plant extracts.

The present study found the highest antioxidant activity for ethanol-acetic and methanol-acetic paulownia extracts. It can be assumed that acetic acid improves the antioxidant properties of plant extracts. Żbik et al. [2023] obtained similar results, which also showed that the acidification of methanol solvent enhanced the stability of the extracted antioxidant compounds. Sowndhararajan and Kang [2013] and Nino et al. [2016] reported the highest antioxidant properties in methanol extracts. According to Karthikumar et al. [2007], Sasadara and Wirawan [2021] and Palaiogiannis et al. [2023], the highest antioxidant activity was demonstrated by ethanol extracts.

CONCLUSION

The bacteriostatic properties of paulownia leaf extracts against several Gram-positive and Gram-negative pathogens in the cultivation of vegetable and fruit plants were analyzed. These extracts were most effective against the *C. michiganensis* ssp. *michiganensis* and *X. hortorum* pv. *carotae*. They were slightly less effective against *A. tumefaciens*, *P. syringae* pv. *lachrymans* and *P. syringae* pv. *tomato*. However, only *E. carotovora* was resistant to these plant extracts. It is worth emphasizing that the extraction solvent type influenced the antibacterial activity of plant extracts. Acetone extracts were more effective than alcohol and water extracts. Generally, acetone extracts contained more flavonoids and polyphenolic compounds than other extracts, which probably resulted in their better bacteriostatic properties.

Paulownia leaf extracts could enrich the offer of preparations available on the market and replace chemical plant protection products. Importantly, these preparations can be used in integrated and organic cultivation systems. However, research will continue with paulownia extracts. It is worth assessing the potential bacteriostatic activity of extracts obtained from various plant parts. It is also necessary to expand the research to include pot and field experiments and assessment of phytotoxic effects to use the preparation on a wider scale. A detailed analysis of biologically active compounds that can be extracted from paulownia leaves is also necessary. Nevertheless, the first research results are promising.

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INFLUENCE OF NITROGEN-SULFUR BALANCE ON TOMATO PRODUCTIVITY AND QUALITY TRAITS IN SOILLESS CULTIVATION

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ABSTRACT

Nitrogen (N) and sulfur (S) are crucial macronutrient elements for physiological and biochemical processes in tomato plants. However, synergistic effects of lowering N and increasing S application on tomato yield and quality have not been documented. The effect of four N/S balances: 50.00, 9.20, 4.66 and 2.92, prepared by varying the concentration of N and S, were evaluated in tomatoes grown in soilless medium (peat + perlite). The experiment was conducted in a completely randomized design with three replicates. The results of the study showed that the optimal N/S balance in the nutrient solution differed depending on the properties investigated. The N/S balance of 9.20 resulted in the highest overall fruit yield, average fruit weight, fruit size and diameter. Moreover, the N/S balances required in the nutrient solution for the highest lycopene content (7.69 mg 100 g^{-1} fresh weight) and vitamin C content (20.63 mg 100 g^{-1} fresh weight) in tomato fruits were 50.00 and 9.20, respectively. It was found that the N/S balance above or below 9.20 had negative effects on yield and yield components as well as on some biophysical quality characteristics of the fruit. However, the N/S balance had no influence on the number of fruits, the firmness and shape index and the pH value of the fruits. Therefore, lowering N and increasing S application to the balance of 9.20 would have great potential to enhance the synergistic effect on the productivity and quality of tomato cultivation.

Keywords: *Lycopersicon esculentum*, soilless culture, nitrogen to sulfur balance, nutrient concentration, yield, fruit quality

INTRODUCTION

Over a long period of time, there has been a concerted effort to address the challenges associated with soil-based farming, particularly in greenhouse environments. Issues such as soil-borne diseases, poor soil fertility, and high salt content have prompted the exploration of alternative cultivation methods. Soilless culture, including both hydroponic and substrate-based systems, has gained prominence as a solution to these challenges [Fussy and Papenbrock 2022]. In the coming years, it will be essential to produce sufficient food for a growing population while minimizing the use of natural resources per capita and reducing environmental impact, a goal that can be supported through sustainable farming practices like soilless culture [Cámara-Zapata et al. 2019].

Soilless culture is increasingly popular as an innovative and effective form of technology [Lucke et al. 2019]. The method of soilless cultivation involves growing plants without using soil as a rooting medium. It is widely used to improve the regulation of environmental conditions for growth and to avoid soil ambiguity [Tzortzakis et al. 2020]. The plants are grown in a substrate with a continual supply of nutrient solution, allowing for optimal mineral nutrition

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management [Lu et al. 2022]. Commercially, substrate culture has been used successfully for fruiting vegetables [Tüzel et al. 2019].

The tomato (*Lycopersicon esculentum* L.) from the *Solanaceae* family has become one of the most significant horticultural crops in the past fifty years [Panno et al. 2021]. In 2020, global production of tomatoes reached roughly 187 million metric tons, positioning it as one of the most extensively cultivated crops worldwide [Dasgan et al. 2024]. China leads tomato production with about 65 million metric tons, while India and Türkiye rank second and third, producing 20.5 million and 13.2 million metric tons, respectively [FAOSTAT 2020].

Tomato fruit contains many phytochemical compounds that can improve human health [Khan et al. 2021]. This fruit is an important dietary source of lycopene, potassium, calcium, iron, folic acid and vitamin C [Feng et al. 2020, Gonçalves et al. 2020, Wu et al. 2022].

Managing substrate fertility for tomato crops is essential for maintaining fruit yield and quality, as tomatoes are among the vegetables with the highest nutrient requirements [Du et al. 2017]. Different sources of individual nutrient elements can be used as fertilizers to meet the plant's need for particular elements [Souri and Dehnavard 2018]. Tomato production requires optimal fertilization [Frías-Moreno et al. 2020]. Nitrogen (N) is one of the most important nutrients needed for plant growth and development and often the most restrictive nutrient in tomato production [Cheng et al. 2021]. Since nitrogen is an essential component of proteins, enzymes and nucleic acids, it plays a crucial role in various physiological and metabolic processes. Nitrogen is also essential for maintaining the structural integrity of tomato plants [Albornoz 2016]. Sulfur (S) is a vital constituent of all forms of life, including plants [Narayan et al. 2023]. Sulfur is a constituent of the proteinaceous amino acids (including cysteine and methionine), vitamins (biotin and thiamine), chlorophyll, phytochelatins, coenzyme A, and S-adenosyl methionine [Nakai and Maruyama-Nakashita 2020, Narayan et al. 2023]. Sulfur affects various aspects of plant life, including growth, development, nutritional quality and disease resistance [Kopriva et al. 2019, Abdalla et al. 2020, Meschede et al. 2020]. Attaining optimal nutrient usage efficiency is a primary target for sustainable agricultural systems, both from an economic and environmental perspective [Çakmakçı et al. 2023]. Various variables influence the total efficiency of nutrient use, including the availability of water [Shewangizaw et al. 2024], genetic acquisitions [Peng et al. 2022], and the availability of other nutrients [Duncan et al. 2018]. The presence of N and S in the growing medium can affect nutrient uptake in plant tissues and the concentration of both nutrients [Sutradhar et al. 2017, Carciochi et al. 2019]. Sulfur and nitrogen are crucial for protein synthesis in plants, and their availability is closely interrelated [Zhou et al. 2024]. Furthermore, the S status of the plant has a potent influence on N metabolism, and the S requirement is closely related to N nutrition [Zenda et al. 2021].

In general, plant performance can only be maximized by the application of N if sufficient S is present. Similarly, the maximum response to the application of S can only occur if sufficient N is present. Thus, S deficiency can reduce the efficiency of N utilization, and a similar reaction is expected in the opposite direction, i.e., N deficiency affects the efficiency of S utilization [Jobe et al. 2019]. In plants, N is present in the form of protein, and S is a component of two essential amino acids, methionine and cysteine. The absence of these elements results in a reduction in the synthesis of these amino acids, and proteins comprising them are incapable of being formed. For this reason, the plant's metabolism can be altered depending on the N form together with the S when fertilizing [Marschner 2012]. Moreover, both N and S can have synergistic effects and influence fruit production, ripening, and quality to some extent [Marschner 2012]. Siueia et al. [2020] reported that the interaction between N and S rates positively influenced fruit firmness, soluble solids (SS), titratable acidity (TA), and the SS/TA ratio while negatively affecting vitamin C, lycopene, and beta-carotene levels. However, this interaction did not change the quality characteristics of tomatoes compared to recommended values. Additionally, pH was solely influenced by increasing N rates, reaching a maximum of 4.2, which achieved the desired acidity level.

A literature review found a lack of knowledge concerning the effects of the interaction between N and S in terms of nutrient balance and nutrient concentration. It is hypothesized that the effects of N and S are not only due to the respective concentration in

the nutrient solution but also to the balance of the two macronutrients. In this context, the aim of the present study was to evaluate the influence of the N/S balance in the nutrient solution at different N and S concentrations on the yield and yield components, biophysical, organoleptic and nutraceutical quality characteristics of tomato grown in a soilless medium.

MATERIAL AND METHODS

Plant material and growth conditions. Tomato (*Lycopersicon esculentum* L. cv. Kardelen F1) was used as plant material. Seedlings were produced in a commercial nursery located in Antalya (Türkiye). The tomato seedlings, which were almost 10 cm tall and with their second set of leaves, were transplanted singly into pots on 7 July 2022. In the experiment, the growth medium was prepared by mixing peat and perlite at a ratio of 2:1 (v/v). Peat moss (Klasmann) is a moss that belongs to the genus of peat moss (Sphagnum) and has a high water-holding capacity and a pH value between 5.5 and 6.0. The expanded mineral perlite is an inert, salt-free substrate with a neutral pH and a high aeration capacity. A total of 1500 g of medium was placed in each 3 L pot having a diameter of 16.5 cm and a depth of 19.0 cm. Drainage holes were drilled into the bottom of the pots. The present experiment was conducted in a greenhouse at the Experimental Field of Ondokuz Mayıs University (41°21' N, 36°11' E), Samsun, Türkiye. The conditions in the greenhouse were as follows: temperature of 29 ±4 °C/22 ±2 °C (day/night), photoperiod of 12 h, and relative humidity of 55% ±5%. The greenhouse used in the experiment had a metal structure measuring 6.0 × 16.0 m (96 m² of total area), with a ceiling height of 4.5 m and an arched frame. It was covered with a transparent plastic film to prevent UV degradation and direct sunlight exposure to the plants.

Experimental design and N/S balance. The experiment was arranged in a completely randomized block design with a one-factor setup. Each treatment was replicated three times, resulting in a total of 12 pots. The concentrations of macro- and microelements in the nutrient solution for the tomato plants were applied according to the procedures described by Alpaslan et al. [1998]. The treatments in relation to the balance of nitrogen and sulfur and the concentration of macroelements in the nutrient solution applied to the substrate medium are given in Table 1. In all nutrient solutions with different N and S concentrations, the total cations ($NH_4^+ + K^+ + Ca^{+2} + Mg^{+2} = 13.5$ me L^{-1}) and the total anions ($NO_3^- + H_2PO_4^- + SO_4^{-2} = 13.5$ me L^{-1}) are in equilibrium. The N/S balance in the nutrient solution was calculated using the following equation:

$$\text{N/S balance} = \text{N concentration (meq L}^{-1}) / \text{S concentration (meq L}^{-1})$$

The concentrations of microelements in four different nutrient solutions containing varying concentrations of N and S used in the experiment are the same and are given below.

Manganese chloride dihydrate ($MnCl_2 \cdot 2H_2O$), iron (Fe)-EDDHA [ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid)], boric acid (H_3BO_3), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), and ammonium molybdate tetrahydrate [$(NH_4)_6Mo_7O_{27} \cdot 4H_2O$] were used to prepare a micro-nutrient solution at 5 µM manganese (Mn), 40 µM iron (Fe), 30 µM boron (B), 0.75 µM copper (Cu), 4 µM zinc (Zn) and 0.5 µM molybdenum (Mo). The pH of the plant nutrient solutions was modified to 5.5 with 1.0 M KOH or H_2SO_4 solution. Analytical-grade reagents were used throughout the experiment.

The growing media were moistened with tap water before transplanting the tomato seedlings. Irrigation with the nutrient solution was initiated 3 days after transplanting and was provided every day, allowing a 20% ±5% leaching fraction. The supply of 150 mL of nutrient solution per day to each pot started at transplanting and was maintained until 14 August 2022. Subsequent to that specific date, a quantity of 300 mL of nutrient solution was provided daily to each pot until the completion of the harvesting process. The volumes of nutrient solution applied to tomato plants were determined based on plant growth observed in previous experiments [Korkmaz et al. 2018]. In the soilless culture system, additional daily irrigation with tap water was carried out alongside the nutrient solution. The substrate moisture dynamics were monitored daily using a gravimetric method. To compensate for water loss due to evapotranspiration, the plants were watered daily before sunset, based on the weight loss of the pots. Plants were harvested on 7 October 2022.

Table 1. Nitrogen (N) and sulfur (S) balance and concentration of macroelements in the nutrient solution

N/S balance meq L^{-1} / meq L^{-1}	N:S stoichiometry mM : mM	Macro-nutrient solution compositions	NO_3^-	$H_2PO_4^-$	SO_4^{-2}	NH_4^+	K^+	Ca^{+2}	Mg^{+2}
			mM						
50.00	**$N_{12.5}$: $S_{0.125}$**	12.5 mM N	**12.00**	1.25	**0.125**	**0.5**	5.25	2.75	1.125
		1.25 mM KH_2PO_4	–	1.25	–	–	1.25	–	–
		2.75 mM $Ca(NO_3)_2{\cdot}4H_2O$	5.50	–	–	–	–	2.75	–
		0.50 mM NH_4NO_3	0.50	–	–	0.50	–	–	–
		4.00 mM KNO_3	4.00	–	–	–	4.00	–	–
		0.125 mM $MgSO_4{\cdot}7H_2O$	–	–	0.125	–	–	–	0.125
		1.00 mM $Mg(NO_3)_2$	2.00	–	–	–	–	–	1.00
9.20	**$N_{11.5}$: $S_{0.625}$**	11.5 mM N	**11.00**	1.25	**0.625**	**0.5**	5.25	2.75	1.125
		1.25 mM KH_2PO_4	–	1.25	–	–	1.25	–	–
		2.75 mM $Ca(NO_3)_2{\cdot}4H_2O$	5.50	–	–	–	–	2.75	–
		0.50 mM NH_4NO_3	0.50	–	–	0.50	–	–	–
		4.00 mM KNO_3	4.00	–	–	–	4.00	–	–
		0.625 mM $MgSO_4{\cdot}7H_2O$	–	–	0.625	–		–	0.625
		0.50 mM $Mg(NO_3)_2$	1.00	–	–	–	–	–	0.50
4.66	**$N_{10.5}$: $S_{1.125}$**	10.5 mM N	**10.00**	1.25	**1.125**	**0.5**	5.25	2.75	1.125
		1.25 mM KH_2PO_4	–	1.25	–	–	1.25	–	–
		2.75 mM $Ca(NO_3)_2{\cdot}4H_2O$	5.50	–	–	–	–	2.75	–
		0.50 mM NH_4NO_3	0.50	–	–	0.50	–	–	–
		4.00 mM KNO_3	4.00	–	–	–	4.00	–	–
		1.125 mM $MgSO_4{\cdot}7H_2O$	–	–	1.125	–	–	–	1.125
2.92	**$N_{9.5}$: $S_{1.625}$**	9.5 mM N	**9.00**	1.25	**1.625**	**0.5**	5.25	2.75	1.125
		1.25 mM KH_2PO_4	–	1.25	–	–	1.25	–	–
		2.75 mM $Ca(NO_3)_2{\cdot}4H_2O$	5.50	–	–	–	–	2.75	–
		0.50 mM NH_4NO_3	0.50	–	–	0.50	–	–	–
		3.00 mM KNO_3	3.00	–	–		3.00	–	–
		0.5 mM K_2SO_4	–	–	0.50	–	1.00	–	–
		1.125 mM $MgSO_4{\cdot}7H_2O$	–	–	1.125	–	–	–	1.125

Measurement of fruit yield and physical characteristics. The fruit yield was measured in the lab using a sensitive scale (Precisa, XB-620M, Switzerland). The fruit yield was calculated for each plant based on the cumulative fruit weight and the number of fruits during the six pickings. The average fruit weight was then calculated.

The height and diameter of the intact fruits were measured using a digital caliper (ASIMETO, Series 307). A measurement of fruit weight was made from the blossom end to the top of the fruit, and the diameter was taken as the maximal diameter of the equatorial section. The fruit shape index was determined as the vertical diameter divided by the horizontal diameter. Every hour, the caliper was washed with water to remove deposited plant parts. A digital penetrometer (PCE Instruments, PCE-FM 200) with a cone-shaped probe of Φ8 mm was used for measuring firmness in the equatorial zone. The resistance at the penetration of the probe was measured and expressed in kg cm^{-2}.

Fruit sampling and quality analysis. Immediately after collection, fully ripened tomato fruits of each replicate were washed in tap water, dried with a paper towel and then cut in half. The seeds were discarded, and the pericarp and mesocarp were crushed to a homogeneous puree in a blender (Tefal, Type: MB450, Türkiye) for about 2 minutes. Part of the sample was instantly used for some analyses (fruit dry matter, titratable acidity, total soluble solids, lycopene, ascorbic acid, and nitrate). In addition, the squeezed juice

was further filtered with a 120 mm Whatman paper filter. The clearly filtered juice was used for the pH analysis. pH was measured using a pH meter (Mettler Toledo Instruments, SevenCompact pH meter S220) [AOAC 1990].

The amount of dry matter (%) was determined gravimetrically by drying 5 g of tomato homogenate in a laboratory oven (Nüve, ES-500, Türkiye) set at 70 °C until a stable weight was attained. To determine the titratable acidity (TA), filtered tomato juice (10 mL) was titrated with 0.1 N standardized sodium hydroxide (NaOH) solution till equilibrium (pH of 8.1) and the measured TA was expressed as the concentration (%) of citric acid, a major organic acid in tomatoes. For the determination of total soluble solids (TSS), a single drop of the clear juice was measured using a digital refractometer Atago PAL-1 (3810), 0.0–53.0 Brix (Atago, Tokyo, Japan) and the measurement was represented in °Brix [AOAC 1990]. The method described by Fish et al. [2002] was used to determine the lycopene content in tomatoes. The vitamin C (ascorbic acid) content was determined by the titration method based on fresh weight (FW) described by Padayatt et al. [2001]. Nitrate was determined in fresh samples using the salicylic acid method [Cataldo et al. 1975].

Statistical analysis. Statistical analysis was performed using JMP version 5.1. Data were presented as means ±standard errors. A one-way analysis of variance (ANOVA) was conducted to assess overall treatment significance ($p < 0.05$). Pairwise comparisons among treatment means were performed using Fisher's Least Significant Difference (LSD).

RESULTS

Effect of the N/S balance on fruit productivity. The results of the effects of decreasing N or increasing S in the nutrient solution applied to substrate culture on the yield and its components of tomato are given in Fig. 1(A–C).

A reduction in the N concentration or an increase in the S concentration significantly affected ($p < 0.05$) total fruit yield. The highest total yield (1841.73 g plant^{-1}) was obtained at stoichiometry $N_{11.5}$: $S_{0.625}$ in the nutrient solution. The yield values obtained in other stoichiometric nutrient ratios were close to each other, and the difference between them was not statistically significant (Fig. 1A). The ideal N/S balance in the nutrient solution for the highest fruit yield was determined to be 9.20. When the N/S balance was

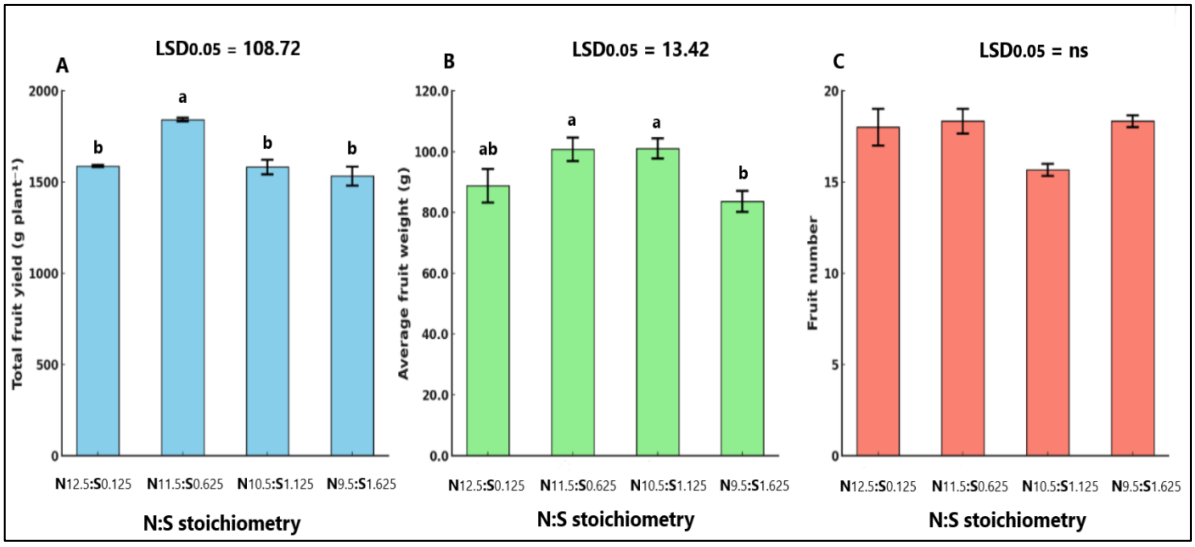

Fig. 1. Effect of nitrogen (N) and sulfur (S) concentration in the nutrient solution on the yield and yield components of tomato plants: (A) total fruit yield, (B) average fruit weight, (C) fruit number per plant. Values are presented as means ±SE. Distinct letters indicate statistically significant differences according to Fisher's least significant difference (LSD) test ($p < 0.05$); ns: non-significant.

Table 2. Effect of nitrogen (N) and sulfur (S) concentration in the nutrient solution on size, diameter, shape index, and firmness of tomato fruit cv. Kardelen F1

N:S stoichiometry	Fruit size (mm)	Fruit diameter (mm)	Fruit shape index	Fruit firmness (kg ·cm^{-2})
$N_{12.5}$:$S_{0.125}$	53.45 ±1.13 a	62.97 ±0.70 a	0.84 ±0.02	3.09 ±0.15
$N_{11.5}$:$S_{0.625}$	52.23 ±0.72 a	61.86 ±1.59 a	0.84 ±0.02	2.63 ±0.01
$N_{10.5}$:$S_{1.125}$	51.44 ±0.39 ab	59.23 ±1.41 ab	0.86 ±0.03	2.79 ±0.05
$N_{9.5}$:$S_{1.625}$	49.52 ±0.64 b	55.76 ±0.87 b	0.88 ±0.02	2.77 ±0.15
Level of significance	*	*	ns	ns
$LSD_{0.05}$	2.51	3.89	–	–

Each value represents mean ±SE (n = 3). There is no significant difference at 0.05 between the mean values given in the column with the same letters.
*significant at 5%; ns: non-significant

higher or lower than 9.2, a decrease in total fruit yield was observed. Increasing the N concentration from 11.5 to 12.5 mM and simultaneously reducing the S concentration in the nutrient solution from 0.625 to 0.125 mM reduced total fruit production by 32.63%. Compared to the treatment with an N/S balance of 9.20, the treatments with an N/S balance of 4.66 and 2.29 reduced the total fruit yield by 14.16% and 16.80%, respectively. A reduction in the N concentration or an increase in the S concentration significantly affected ($p < 0.05$) average fruit weight. The highest average fruit weight (101.00 g) was obtained at stoichiometry $N_{10.5}$:$S_{1.125}$ in nutrient solution. The average fruit weights were similar in the $N_{11.5}$:$S_{0.625}$ and $N_{10.5}$:$S_{1.125}$ treatments. An increase or decrease in the N/S balance in the nutrient solution led to a decrease in the average fruit weight. Moreover, the lowest fruit weight (83.64 g) was obtained when treated with $N_{9.5}$:$S_{1.625}$ (Fig. 1B) However, this change in the N:S ratio did not significantly affect the number of fruits per plant (Fig. 1C).

Effect of the N/S balance on biophysical quality characteristics of fruits. The N/S balance in the nutrient solution had a significant effect ($p < 0.05$) on fruit size and diameter. The largest fruit (53.45 mm) was obtained at stoichiometry $N_{12.5}$:$S_{0.125}$ in the nutrient solution. There was no difference between treatments $N_{12.5}$:$S_{0.125}$ and $N_{11.5}$:$S_{0.625}$ in terms of their effects on fruit size (Table 2). The fruit size of tomato plants grown in treatments with an N/S balance of 50.00 and 9.20 in the nutrient solution was found to be higher. Tomato grown in a treatment with a lower N/S ratio (2.92) than these N/S ratios showed a significant decrease in fruit size. The highest fruit diameter (62.97 mm) was obtained in the $N_{12.5}$:$S_{0.125}$ stoichiometry, but there was no difference between the $N_{12.5}$:$S_{0.125}$, $N_{11.5}$:$S_{0.625}$ and $N_{10.5}$:$S_{1.125}$ treatments in terms of fruit diameter (Table 2). Fruit diameter decreased significantly in the treatment with an N/S balance of 2.92 in the nutrient solution. However, this change in N:S stoichiometry did not significantly affect fruit shape index or fruit firmness (Table 2).

Effect of the N/S balance on physico-chemical quality characteristics of fruits. The results of the effects of decreasing N or increasing S in the nutrient solution applied to substrate culture on tomato physico-chemical quality traits are given in Fig. 2(A–D).

The reduction of N or the increase of S in the nutrient solution had no effect ($p > 0.05$) on the pH value of the tomato fruits (Fig. 2A). However, the N/S balance in the nutrient solution had a significant impact ($p < 0.05$) on the total soluble solids of the tomato fruit. The tomatoes cultivated in the nutrient solution with a N/S balance of 50.00 had the highest value (4.70 °Brix) in the fruit, while those grown in the solution with a N/S balance of 4.66 had the lowest value (4.16 °Brix) in the fruit. Increasing the S concentration from 0.125 to 1.125 mM and simultaneously reducing the N concentration in the solution of nutrients from 12.5 to 10.5 mM decreased the TSS of the tomato fruits (Fig. 2B).

The N/S balance in the nutrient solution had a significant effect ($p < 0.05$) on the titratable acidity of

the tomato fruit. The increase of the N concentration from 11.5 to 12.5 mM and the simultaneous reduction of the S concentration in the nutrient solution from 0.625 to 0.125 mM reduced the TA of the tomato fruits (Fig. 2C).

The N/S balance in the nutrient solution had a significant effect ($p < 0.05$) on the dry matter content of the fruit. The maximum dry matter content (7.37%) of the tomato fruit was achieved with the stoichiometry $N_{12.5}$:$S_{0.125}$, while the stoichiometry $N_{11.5}$:$S_{0.625}$ resulted in the lowest dry matter content (6.77%). Nevertheless, the dry matter content of tomato fruit remained unaffected by the stoichiometry $N_{10.5}$:$S_{1.125}$ and $N_{9.5}$:$S_{1.625}$, as per the stoichiometry $N_{12.5}$:$S_{0.125}$ (Fig. 2D).

Effect of the N/S balance on nutraceutical quality characteristics of fruits. The results of the effects of

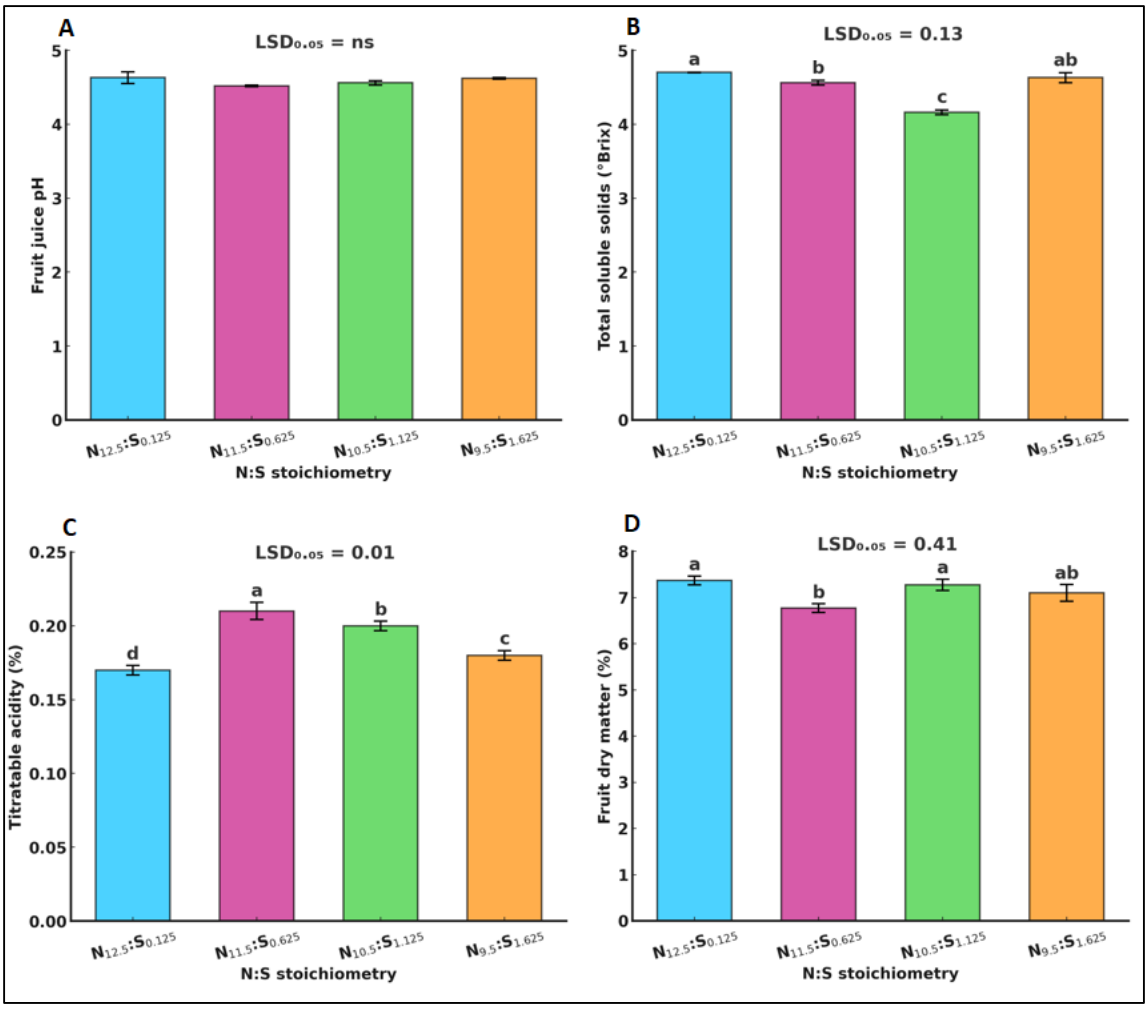

Fig. 2. Effects of nitrogen (N) and sulfur (S) concentration in the nutrient solution on the physico-chemical quality characteristics of tomato fruit: (A) pH, (B) total soluble solid, (C) titratable acid, (D) fruit dry matter (%). Values are presented as means ±SE. Distinct letters indicate statistically significant differences according to Fisher's least significant difference (LSD) test ($p < 0.05$)

decreasing N or increasing S in the nutrient solution applied to substrate culture on nutraceutical quality traits of tomato are given in Fig. 3(A–C).

The change in the N to S ratio had a substantial statistical influence ($p < 0.05$) on the lycopene content in tomato fruit. This carotenoid content in the fruit was the highest (7.69 mg 100 g^{-1} FW) in tomato plants grown in nutrient solution with N/S balance of 50.00, whereas it was the lowest (5.18 mg 100 g^{-1} FW) in tomato plants grown in the solution with a N/S balance of 2.92 (Fig. 3A). Increasing the S concentration from 0.125 to 1.625 mM and simultaneously reducing the N concentration in the nutrient solution from 12.5 to 9.5 mM reduced the lycopene content in tomato fruits by 32.63%.

The variation in N:S stoichiometry had a significant effect ($p < 0.05$) on the vitamin C content in tomato fruit. The tomatoes cultivated in the nutrient solution with a N/S balance of 9.20 had the highest vitamin C content (20.63 mg 100 g^{-1} FW), while those grown in the solution with a N/S balance of 50.00 had the lowest vitamin C content (13.16 mg 100 g^{-1} FW) (Fig. 3B). Increasing the N content from 11.5 to 12.5 mM and reducing the S content from 0.625 to 0.125 mM reduced the vitamin C content in tomato fruit by 36.20%. Similarly, elevating the S concentration in the nutrient solution from 0.625 to 1.125 mM and 1.625 mM and lowering the nitrogen concentration from 11.5 to 10.5 mM and 9.5 mM resulted in a decrease in the vitamin C content in the fruit by 32.81% and 21.95%, respectively.

The change in the N to S ratio had a substantial statistical influence ($p < 0.05$) on the nitrate (NO_3^-) content in tomato fruit. The tomatoes cultivated in the nutrient solution with a N/S balance of 50.00 had the highest NO_3^- content (13.75 mg·kg^{-1} FW), while those grown in the solution with a N/S balance of 2.92 had the lowest NO_3^- content (9.18 mg·kg^{-1} FW) (Fig. 3C). In other words, decreasing the N content from 12.5 to 9.5 mM and increasing the S content from 0.125 to 0.625 mM reduced the NO_3^- content in tomato fruit by 33.23%.

DISCUSSION

Plants exhibit higher nutrient efficiency when nutrients are supplied in a balanced way, as the nutrient uptake rates are determined by the concentration in the root zone and by the interrelation between nutrients, which is as critical as the total concentration of ions [Alvarado-Camarillo et al. 2018]. As essential mineral nutrients for proteins, amino acids, enzymes,

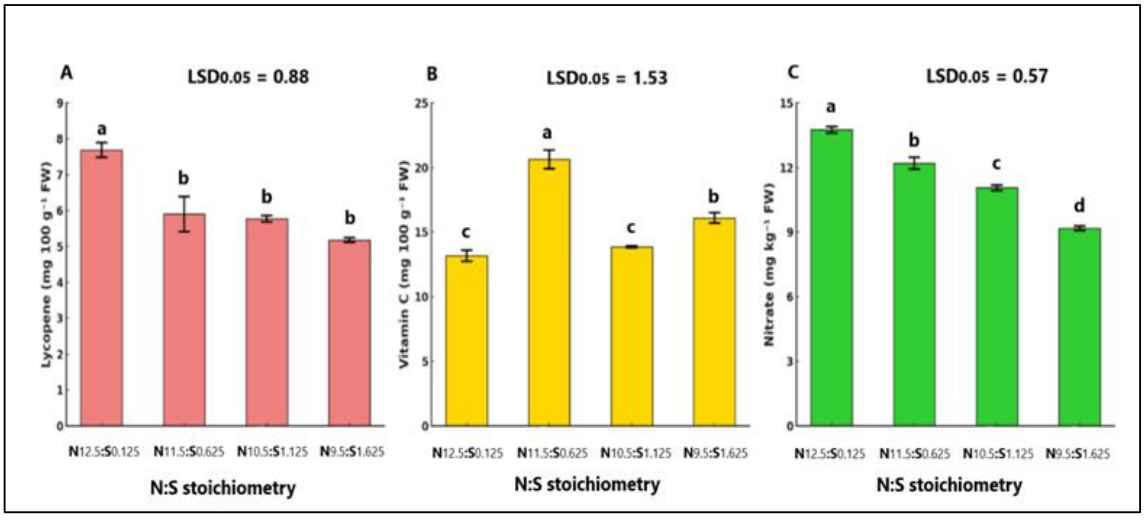

Fig. 3. Effects of nitrogen (N) and sulfur (S) concentration in the nutrient solution on the nutraceutical quality characteristics of tomato fruit: (A) lycopene (B), vitamin C, (C) nitrate. Values are presented as means ±SE. Distinct letters indicate statistically significant differences according to Fisher's least significant difference (LSD) test ($p < 0.05$)

coenzymes, prosthetic groups, vitamins, and secondary metabolites, N and S have substantial regulatory impacts on the growth, yield, and nutritional quality of crops [Gigolashvili and Kopriva 2014]. In agricultural productivity, N and S not only have individual roles but also interact with each other [Liu et al. 2020]. Khalili et al. [2024] emphasize that the synergistic effects between N and S play a crucial role in plant performance. Insufficient S supply impairs plant's efficient utilization of applied N [Kumar et al. 2017]. In the current study, the application of 11.5 mM N and 0.625 mM S, with an N/S balance of 9.20, resulted in the highest tomato yields and average fruit weight. Fruit yield decreased in applications where the N/S balance in the nutrient solution was above or below 9.20 (Fig. 1A). Furthermore, nutrient solutions with N/S balance above 9.20 and below 4.66 reduced the average fruit weight (Fig. 1B). However, the effect of N/S ratios in the nutrient solution on fruit number was insignificant (Fig. 1C). Janzen and Bettany [1984] found that optimum canola yields are achieved by maintaining a balanced availability of N and S. The authors confirmed that the optimal ratio was estimated to be 7:1 and reported that excessive S application in relation to N availability resulted in overwhelming S accumulation in the plant tissues and diminished seed production. Głowacka et al. [2023] highlight that an adequate supply of these nutrients is essential for plants to fully exploit their yield potential.

The shape of fleshy fruits considerably affects their utilization and consumer preference in distinct geographical locations [Li et al. 2023]. The fruit shape is usually defined by the fruit diameter, the fruit length and the fruit shape index, which indicates the ratio of fruit diameter to fruit length [Li et al. 2023]. It was observed that fruit size and diameter were larger in tomatoes fed with nutrient solutions with the N/S balance of 50.00 and 9.20. Nevertheless, the size and diameter of the fruit exhibited a decline when the N/S ratios reached 4.66 and 2.92 in the nutrient solution (Table 2). This was due to a decrease in the N concentration or an increase in the S concentration in the nutrient solution. The biophysical quality characteristics (fruit length, fruit diameter and core diameter) of pineapple fruits increased with increasing N dosage, as reported by Omotoso and Akinrinde [2013]. Grasso et al. [2022] noted that when the N supply to the melon plant was reduced too much, the formation of small fruits and fruit deformations was observed, resulting in a 58% increase in the proportion of fruit waste. In the current study, the fruit shape index showed no response to variations in N or S content, and no statistically significant differences were found between all treatments (Table 2). The current findings regarding this parameter were in agreement with those reported by Cai et al. [2023]. Fruit firmness is an important characteristic that ensures the storage and transport of tomatoes, as firmer fruit can better resist damage [Li et al. 2020]. Conversely, excessive softening during the ripening of fleshy fruit causes physical damage and increases susceptibility to infection, ultimately reducing quality and leading to significant losses in the supply chain [Shi et al. 2023]. In this study, there was no effect of increasing the level of N fertilization from 9.5 to 12 mM on the firmness of the fruit (Table 2), which is in agreement with Kaniszewski et al. [2019]. In contrast, Frías-Moreno et al. [2020] reported that the tomato fruit firmness increased with escalating doses (30 mM) of N till one point, but then firmness declined. Though a sufficient amount of N is required to have adequate firmness in tomatoes, higher amounts of N can lead to a weaker translocation of Ca into the fruit [Frías-Moreno et al. 2020]. A reduction in Ca accumulation in the fruit leads to loss of cell wall integrity and firmness [Zhang et al. 2020].

The pH value is an important quality characteristic in the processing of tomatoes. In the present study, depending on the treatment, the pH values in the tomato juices were between 4.52 and 4.63 (Fig. 2A). Tomatoes usually have adequate acidity to sustain a pH value below 4.6 and are, therefore, not categorized as low-acid foods. For this reason, tomatoes do not require the more drastic thermal treatments needed to kill spoilage microorganisms in foods classified as low in acids to ensure food safety [Siueia et al. 2020]. Desirable values of total soluble solids in tomato fruits are between 4 and 9% [Duma et al. 2015] for fresh consumption or for the processing industry. In the preset study, total soluble solid values were within this range (Fig. 2B). The soluble solids give the fruit a particular flavor depending on the sugar content. Nevertheless, the changes in this content probably depend on the genotype and various factors that influence the fruit's capability to import assimilated photosynthe-

sis. Therefore, using varieties in which this trait can be guaranteed is essential to allow greater consumer acceptance [Siueia et al. 2020]. The acidity of the fruit is a significant factor in determining the taste of the tomato products [Zhang et al. 2023a]. Citric acid is the primary carboxylic acid found in tomatoes and accounts for most of the total titratable acidity [Parra-Torrejón et al. 2023]. In the present study, significant differences in titratable acidity were observed in tomato fruits at different N/S ratios. Titratable acidity in tomato fruit was the lowest in the application ($N_{12.5}$:$S_{0.125}$) with the highest N/S ratio (Fig. 2C). This could be due to the degradation of organic acids by nitrogen, which is transported into the fruit as a result of an increased N concentration in the growth medium. One of the most notable indicators of the quality of tomato fruits and their technological traits is the dry matter content [Kurina et al. 2021]. In the current study, the dry matter content of the cultivated tomato fruits varied between 6.77 and 7.37% (Fig. 2D). The results of the current study on dry matter content are in agreement with the findings of another study [Alenazi et al. 2020], in which the dry matter content of tomato fruits was between 5.95 and 7.85%. Fruits with a high concentration of dry components taste good, produce a greater yield during processing, and have superior transportability and quality retention during storage [Kurina et al. 2021].

Crop productivity and sensory quality are among the aspects that receive the most attention. However, consumers have become more interested in the nutritional value of fruits and vegetables because they want to buy food that is good for their health [Scarano et al. 2020]. In this regard, tomato fruit is an important source of carotenoids such as lycopene, which has been linked to a lower risk of cancer and cardiovascular disease [Shah et al. 2021]. Lycopene is the main carotenoid that gives fruits their red pigmentation [Gupta et al. 2024]. The study showed that the treatment with the highest N concentration (12.5 mM) had the highest lycopene content (7.69 mg 100 g^{-1} FW) (Fig. 3A). In contrast, San-Martín-Hernández et al. [2022] observed a decrease in the lycopene content of the tomato when the N concentration in the nutrient solution increased from 10 to 16 mM. Lycopene concentration in tomato fruit can be affected by environmental factors, agronomic practices, and postharvest conditions [Lima et al. 2022]. Vitamin C is of great importance in human nutrition, not only for its role as an antioxidant but also for its positive effect on the availability of dietary iron [See et al. 2024]. In this study, vitamin C in tomato fruit was the lowest in the application ($N_{12.5}$:$S_{0.125}$) with the highest N/S ratio (Fig. 3B). These results are in line with Bénard et al. [2009] who found a decrease in the vitamin C content owing to an increase in the supply of N. The activities of crucial enzymes and metabolites involved in the degradation and recycling processes of vitamin C increased with an increasing N supply [Zhang et al. 2023b]. Nitrate is a chemical compound naturally present in fruits and vegetables [Uddin et al. 2021]. Nitrate acts as a signaling molecule that triggers the production of NO_3^--related genes responsible for the processes of absorption, transport, assimilation as well as vegetative and reproductive development [Aluko et al. 2023]. Plants take up NO_3^- from the root, assimilate NO_3^- and then translocate it to the shoot, where it can be re-mobilized in sink organs [Iqbal et al. 2020]. Excessive nitrate accumulation in vegetables is a prevalent concern that may present a risk to human health [Bian et al. 2020]. In this study, NO_3^- concentrations in tomatoes were considerably lower than World Health Organization (WHO) standards (Fig. 3C). Nitrate concentration in crops depends on the type and variety of the crop, environmental factors, and agricultural practices [Ferysiuk and Wójciak 2020].

CONCLUSIONS

The optimum N/S balance in the nutrient solution varied depending on the characteristics analyzed. The N/S balance of 9.20 in the nutrient solution resulted in high total fruit yield, average fruit weight, fruit size, and width. It was found that the N/S balance in the nutrient solution above or below 9.20 had negative effects on yield and yield parameters as well as on some biophysical quality characteristics of the fruit. However, the N/S balance in the nutrient solution had no influence on the number of fruits, the firmness and shape index and the pH value of the fruits. Based on these results, ensuring balanced plant nutrition is crucial to achieving a high-yielding, high-quality crop that meets the expectations of the end consumer.

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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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RECIPROCAL CROSS-COMPATIBILITY IN CUT ROSE BREEDING

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ABSTRACT

Rose breeding companies have developed new rose varieties in response to increasing demands for color, fragrance and shapes. Hybridization is one of the most important methods of creating new rose variations. Breeders focus on fertility, reproduction, and a high number of seeds per fruit. In the present study, four *Rosa* genotypes (‘Jumilia’, ‘Black Magic’, ‘Tineke’, ‘Black Baccara’) were crossed to assess genetic compatibility, seed formation potential and germination rate. The results showed that all genotypes were tetraploid, and pollen germination varied from 11.36% to 23.41%. The highest crossability rate (94.44%) was found in the ‘Black Baccara’ × ‘Jumilia’ combination, followed by ‘Black Magic’ × ‘Jumilia’ (60%). The highest seed yields were obtained in ‘Jumilia’ × ‘Tineke’ (60.50). ‘Tineke’ × ‘Jumilia’ (43.74) showed the second highest number of seeds per fruit, whereas limited success was determined in cross ‘Jumilia’ × ‘Black Magic (2.25). The maximum germination percentage was found in ‘Jumilia’ when crossed with ‘Tineke’. Significant variations were recorded for the weight of hips and weight of fruit. The PCA-biplot results indicated a positive correlation between crossability rate and seed production efficiency. Overall, the choice of parents was crucial for the crossability indices, which are the average crossability rate and seed production efficiency.

Key words: ACR (average crossability rate), compatibility, cut rose, hybridization, rose breeding

INTRODUCTION

Roses date back about 5,000 years in civilization, as shown in the history of cultivation. A wide variety of roses are grown for cut flowers, pot flowers, garden or landscape plants, perfumery, food, and medical purposes [Lawrence 1997, Liu et al. 2015, Dogan et al. 2020]. Rose breeding primarily involves cut flowers, as approximately 10 billion cut rose stems are sold annually worldwide [AIPH and Union Fleurs 2018]. However, sales of potted roses and garden or landscape roses are approximately 300 million per year. Cut roses are still the leader in rose production. Roses are known as the first non-edible species used in plant breeding with crossbreeding [Gudin 2001]. The hybrid tea rose genotype ‘1985’ was selected as the seed parent, and genotype ‘1848’ was selected as the pollen donor [Pipino et al. 2011]. Therefore, hybridization is the most widely used breeding method in cut flowers to obtain a new rose variety these days. The ability to produce new rose varieties is mandatory for growers to maintain and develop their market share.

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The taxonomic variation in the Rosa species offers an opportunity for success in the transition [De Vries and Dubois 1996]. As with all ornamental plants, aesthetic value is at the center of attention in cut rose cultivation. The breeders desire several further characteristics, such as yield, flower colors, stem quality, tough petals, double flowers, flower opening shape, the size of the flowers, vase life, fragrance and durability. The most fertile progenitors, such as seed or/and pollen donors, are preferred in gene pools because roses are known for their difficult sexual reproduction, from pollination to seed set. Problems with germination and genetic barriers are obstacles to frequently gaining interspecific hybrids [Perez and Moore 1985, Gudin 1992, Abdolmohammadi et al. 2014, Dogan et al. 2020]. Rose breeders demand to increase the efficiency of breeding programs by producing more offspring, where hybrid genotypes producing high seed yield can be evaluated based on different characteristics. F1 hybrids are selected for commercialization by breeders, and the decision regarding which parent will serve as the male or female line influences the distribution of progeny phenotypes in plants [Oh et al. 2005, Nadeem et al. 2014]. Estimating how male or female phenotypes will manifest in the progeny is difficult. For this reason, reciprocal hybridization provides information about the heritability of phenotypes.

The present study was designed to select hybrids that show relatively good performance, offer a good seed set and gain more insight into the inheritance of traits. Therefore, the purpose of this study was to determine the crossability indices in cut rose breeding between popular hybrid rose varieties.

MATERIAL AND METHODS

Plant material

The plant materials used for this study consisted of four hybrid tea roses 'Jumilia', 'Black Magic', 'Tineke', and 'Black Baccara' (Table 1). The study was conducted in the research area of the rose breeding greenhouse, Department of Horticulture, Faculty of Agriculture, Ankara University, Ankara, Türkiye (39°57'40.2" N 32°51'51.7" E), from 1 May 2021 to March 2022.

Ploidy levels of plant materials

The ploidy levels of all cultivars under study were determined using flow cytometry and confirmed through chromosome counting. Initially, the core DNA contents of the plants were analyzed, followed by chromosome counting in one of the plants exhibiting a distinct DNA content. The ploidy level was then correlated with the DNA content and chromosome number [Tuna 2016].

Pollen viability and germination

Anthers of pollen donors were collected after the removal of petals in one-third to one-half open stages and stored in glass Petri dishes in an incubator at temperatures above 24 °C and with humidity equal to 60%. The pollen viability was measured using the IKI (Iodine + Potassium Iodide) test described by Eti [1991]. The IKI solution was prepared by dissolving 1 g potassium iodide and 0.5 g iodine in 100 mL water for dyeing. A drop of the solution was placed on microscope slides, and pollen grains were then sprinkled on the stain with a brush [Abejide et al. 2013]. The viability of the pollen was examined under a light microscope (×100) after 5 minutes of incubation. Pollen grains were classified as viable or non-viable based on pollen color. Brown or black grains were viable, light red or orange ones were semi-viable and yellow ones were recorded as non-viable. A medium containing 20% sucrose and 10 ppm boric acid with 1% agar was prepared for the germination of fresh pollen in Petri dishes, and the pollen was splashed uniformly on the medium with a brush. After an eight-hour incubation period (24 °C and 60–65% humidity), pollen germination was counted when a pollen tube reached a length of at least 1.5 times the pollen diameter under the light microscope (×100) [Leus 2005, Nadeem et al. 2013, Kazaz et al. 2020]. The counting was conducted in four replicates in five randomized fields, and approximately 200 pollens were counted in each area.

Hybridization

Pollination was carried out by applying pollen onto the pistil of the female parents in the early morning by brush. Pollens were collected the day before and left for 24 h (24 °C and 60–65% humidity) for dusting in a dry place, and female parents were prepared by emasculation to prevent self-pollination [Roberts 2003]. The following day, pollinations were properly performed and labelled because it is believed that a 24-hour period is needed to induce the production of exudates in the stigmas necessary for pollen germination [Jacob and Ferrero 2003]. Reciprocal hybridizations

Table 1. Quantitative characteristics of plant materials

Varieties	Fragrance	Color	Blooming	Number of petals	Number of pistil per flower	Number of anther per flower
'Jumilia'	no	bicolor	repeat	30–40	120–150	90–120
'Black Magic'	no	black	repeat	30–40	100–135	130–150
'Tineke'	no	white	repeat	80–100	230–250	230–250
'Black Baccara'	no	black	repeat	35–50	130–220	70–90

were performed from 1 May to 15 June 2021. At least 30 hybridizations were obtained for each of the six combinations. The fruit set started after eight weeks of pollination, and mature fruits were harvested from the end of October until the beginning of December. An electric balance was used to measure the fresh weight of the fruit. Afterwards, seeds were extracted from the fruits, and the number of seeds per fruit, the total number of seeds and the weight of seeds were counted. Seeds were kept in bags and stored at a moderate moisture level at 4 °C for about 12 weeks in the peat. Following this, seeds were sown in mixed ingredients (cocopeat and peat 1:1), and germinated seeds were counted and recorded.

Moreover, the data on the number of flowers crossed, fruit set and seed set were used to calculate the ACR (Average Crossability Rate) and SPE (Seed Production Efficiency), which are cross-compatibility indices. The cross-compatibility rate of a cross was calculated using the following formula:

$$\text{Crossability rate (\%)} = \frac{\text{Number of fruits set}}{\text{Number of flowers pollinated}} \times 100$$

ACR for a parent was calculated as the sum of cross-compatibility rates in specific crosses divided by the number of cross-combinations involving that particular parent:

$$\text{ACR} = \sum \frac{\text{Crossability rates}}{\text{Number of cross combinations}}$$

The SPE for a cross was calculated as the number of viable seeds divided by the number of stigmas of the seed parents in that cross-combination (the expected number of seeds in a rose fruit is equal to the number of stigmas), and the number of pollinated flowers multiplied by 100:

$$\text{SPE} = \frac{\text{Number of viable seeds set}}{\text{Number of flowers pollinated } x \text{ number of stigma}} \times 100$$

The sorted F_1 hybrid seeds were subjected to cold moist stratification at 4 ±1 °C for 100 days to eliminate the germination barrier [Gudin et al. 1990, Debener and Mattiesch 1995]. Perlite was used for the stratification medium, and seeds were treated with a fungicide with 25% tebuconazole as an active ingredient against fungal diseases. After cold moist stratification, the seeds were sown in vials containing peat and germinated in a plastic-covered greenhouse maintained at a temperature range of 18–21 °C to ensure optimal conditions for germination. The seeds were irrigated by the fogging method during the germination process. The operation of the fogging system, which maintains the greenhouse humidity level between 60–80% [Jones 2004, Kazaz et al. 2010] to accelerate seed germination, was adjusted based on internal humidity levels monitored by sensors using a stop-and-start mechanism. The germinated seeds (with the distinct formation of cotyledon leaves and the emergence of the shoot above the soil surface) were counted, and the seed germination rate (%) was determined by the following formula:

$$\text{Seed germination rate (\%)} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds sown}} \times 100$$

RESULTS

Ploidy levels

Ploidy levels were determined by analyzing the core DNA contents of genotypes and were found to

Table 2. Ploidy level of rose varieties

Varieties	DNA (pg/2C)	Ploidy level
'Jumilia'	2.33	tetraploid
'Black Magic'	2.42	tetraploid
'Tineke'	2.36	tetraploid
'Black Baccara'	2.34	tetraploid

Table 3. Pollen viability and germination of rose genotypes

Varieties	Pollen viability (%)	Pollen germination (%)
'Jumilia'	43.28 b	21.60 a
'Black Magic'	47.25 a	11.36 b
'Tineke'	33.29 c	20.50 a
'Black Baccara'	48.76 a	23.41 a

Statistically significant differences at $P \leq 0.05$

vary between 2.33 pg/2C and 2.42 pg/2C. All genotypes were identified as tetraploid (2n = 4x) with 2n = 28 chromosomes (Table 2).

Pollen viability and germination

The viability rate varied between 33.29% and 48.76%. Significant statistical differences ($P \leq 0.05$) were observed between varieties in pollen viability and pollen germination rates. The viability rate of 'Black Baccara' was the highest and reached 48.76%, followed by 47.25% in 'Black Magic' and 39.28% in 'Jumilia', respectively (Table 3). The lowest percentage of pollen viability was detected in 'Tineke' with a value of 33.29%. The percentage of pollen germination ranged between 11.36% and 40.88%. The maximum value of pollen germination rate was measured in 'Tineke' (25.79%), while the minimum value in 'Black Magic' (11.36%) (Table 3).

Crossability rate, seed set, and seed germination

Significant statistical differences ($P \leq 0.05$) were observed between crossability rate, number of seeds per fruit, germination of seed and seed production efficiency. As a male parent, 'Jumilia' excelled in the percentage of crossability rate (94.44%) when crossed with 'Black Baccara' followed by 'Black Magic' (seed parent) (60.0%) However, as a seed parent, 'Jumilia' showed limited success, achieving a crossability rate of only 14.44% when crossed with 'Tineke' and 20% with 'Black Magic'. As a female, 'Jumilia' showed a good number of seeds per fruit (60.50 seeds) when crossed with 'Tineke', while 'Tineke' × 'Jumilia' (43.74 seeds) showed the second highest value for a number of seeds per fruit, whereas a limited success was found in the cross 'Jumilia' × 'Black Magic' (2.25 seeds). The maximum average fruit weight was found in 'Jumilia' when crossed with 'Tineke' (13.66 g), although the minimum value was observed in 'Jumilia' when crossed with 'Black Magic' (4.08 g). 'Jumilia' also excelled in the average weight of seed when crossed with 'Black Magic' (14.85 mg), although the lowest value was recorded for 'Black Baccara' when crossed with 'Jumilia' (0.31 mg) (Table 4). As a female parent, 'Black Baccara' showed the highest germination percentage. The germination percentage of 'Black Baccara' as a male parent of 'Jumilia' reached 41.27%, followed by 'Jumilia' × 'Black Baccara' combination at 37.86%. However, the lowest value was recorded for 'Jumilia' when crossed with 'Tineke' (11.27%). As a female parent, 'Black Baccara' excelled in seed production efficiency (5.05%) when crossed with 'Jumilia' followed by 'Tineke' × 'Jumilia' (1.56%), although as a seed parent 'Jumilia' when crossed with 'Black Magic' showed limited success in the percentage of seed production efficiency (0.073%) (Table 4).

Table 4. Crossability rate, number of seeds per fruit, germination and seed production efficiency from reciprocal crosses in some cut roses

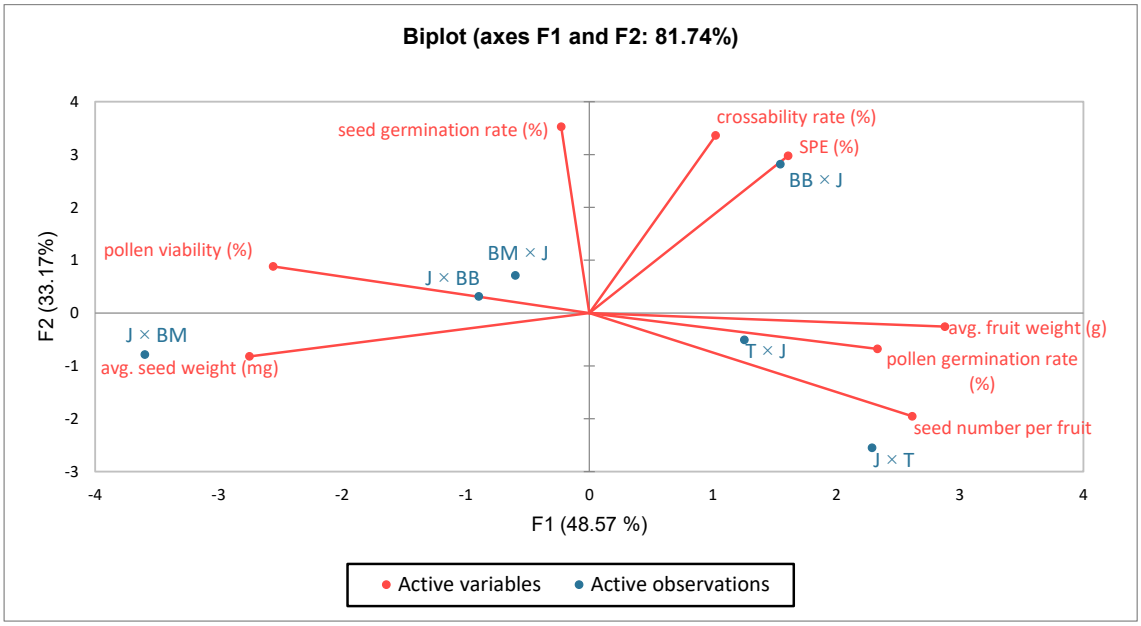
Cross combination	Crossability rate (%)	Seed number per fruit	Average fruit weight (g)	Average seed weight (mg)	Seed germination rate (%)	SPE (%)
'Jumilia' × 'Black Magic'	20.00 cd	2.25 d	4.08 c	14.85 a	20.63 ab	0.073 c
'Black Magic' × 'Jumilia'	60.00 b	6.88 d	5.07 c	1.57 b	31.53 ab	1.02 bc
'Jumilia' × 'Tineke'	14.44 d	60.50 a	13.66 a	0.71 b	11.27 b	0.74 bc
'Tineke' × 'Jumilia'	45.60 bc	43.74 b	13.46 a	0.34 b	22.66 ab	1.56 b
'Jumilia' × 'Black Baccara'	23.68 cd	12.37 cd	7.70 b	2.20 b	37.86 a	0.79 bc
'Black Baccara' × 'Jumilia'	94.44 a	22.94 c	12.85 a	0.31 b	41.27 a	5.05 a

$P \leq 0.05$, SPE: seed production efficiency

Table 5. Interspecific cross-compatibility indices among *Rosa hybrida* cultivar

Varieties	ACR (%)
'Tineke'	30.00 c
'Jumilia'	42.77 b
'Black Magic'	50.00 ab
'Black Baccara'	58.33 a

$P \leq 0.05$, ACR: average cross-compatibility/crossability rate



BM – 'Black Magic', J – 'Jumilia', BB – 'Black Baccara', T – 'Tineke'

Fig. 1. Principal component analysis

Cross-compatibility indices

Significant statistical differences ($P \leq 0.05$) were observed between average cross-compatibility rates. The average crossability rate (ACR) is presented in Table 5. The ACR of these genotypes ranged from 30% for 'Tineke' to 58.33% for 'Black Baccara'.

Principal component analysis

Principal component analysis (PCA) was performed, and the biplot was established for a greater approximation compared with the coefficient of correlation to describe the crossability success between rose varieties. Given the eigenvalue higher than 1, the first (F1) and second (F2) principal components explained 48.57% and 33.17% of the total variation, cumulatively accounting for 81.74% (Fig 3). The PCA-biplot results indicated a positive correlation between crossability rate and seed production efficiency because they were placed on the same side and had similar vector lengths. Average fruit weight, pollen germination rate and seed number per fruit also positively correlated (Fig. 3). Among all the cross-combinations, 'Black Baccara' × 'Jumilia' was found to have a higher crossability rate and seed production efficiency. 'Tineke' × 'Jumilia' and 'Jumilia' × 'Tineke' combinations were classified as the best based on average fruit weight, pollen germination rate, and seed number per fruit (Table 4).

DISCUSSION

Controlled hybridization has been used in cut rose breeding. The most important step in cross-breeding is to include genotypes in breeding programs to ensure high pollen viability/germination and a high seed set. Breeders are interested in enhancing the number of seeds per fruit. Therefore, fertile progenitors also yield high seeds, and due to the heterozygosity in roses, new rose varieties can be obtained more easily with the characteristics desired by breeders. Problems in rose hybridization arise from ploidy differences [Leus et al. 2018]. The base chromosome numbers of rose were reported as 2n = 2x, 3x, 4x, 6x, 8x, and 10x [Jian et al. 2010]. The ploidy levels of all genotypes used in the current study were found to be 2n = 4x (tetraploid). These results were compatible with research showing that the 2C/DNA levels of tetraploid roses varied from 1.85 pg/2C to 2.71 pg/2C [Yokoya et al. 2000]. According to reports, commercial-cut roses are usually tetraploid, while garden roses are either diploid or tetraploid [Datta 2018]. Tetraploid (2n = 4x) roses are more frequently utilized in breeding programs because of their beneficial production performance [Zlesak 2007]. However, it was found that different hybrid combinations produced different types of fruit and seeds. This variation results from the fact that plants with differing DNA contents but the same ploidy levels (e.g., tetraploid) can become incompatible with one another. This could be because variations in DNA content impact both physiological and genetic processes [Morey 1959, Rajapakse et al. 2001, Kazaz et al. 2020, Dogan et al. 2022]. Nadeem et al. [2013] reported that most *Rosa hybrida* cultivars are tetraploid and self-fertilized. For this reason, the low fruit set could be explained as cross incompatibility and ploidy level. In the present study, the findings are consistent with the findings of other researchers [Täckholm 1923, Erlanson 1938, Krussmann 1981, de Vries and Dubois 1996, Crespel et al. 2002, Zlesak 2007]. Although crossing efficiency typically remains consistent, with the number of fruits or seeds per fruit being similar regardless of whether the tetraploid parent is used as seed or pollen donor, the current study observed variations in fruit or seed formation. These differences can be attributed to the physiological and genetic factors of the parent plant.

The pollen germination rate in the current study ranged from 11.36% to 23.41%, whereas the pollen viability rate ranged from 33.29% to 47.25%. The lower and upper limit values varied among the investigations despite the fact that the results of the current study were largely consistent with the previous studies. The genotype, ploidy levels [Ueckert 2014], methods [Sulusoglu and Cavusoglu 2014], climate, plant nutritional status, pollen collection time (season, flowering period, and flower development period) [Martins et al. 2017], storage conditions, and storage duration are all thought to affect pollen quality [Miler and Wozny 2021]. Furthermore, it has been reported that wild and ancient garden roses produce higher-quality pollen than hybrid roses, which is consistent with the findings of the current study [Ueda and Hirata 1989, Gudin and Arene 1991, Meral 2023, Kılıç 2023]. According to Nadeem et al. [2013], interspe-

cific hybridizations, meiotic anomalies, heterozygous polyploidy parents, and the buildup of lethal recessive alleles may all contribute to the reduced pollen fertility of hybrid roses. High fertility in pollen enhances the efficiency of breeding programs and decreases the risk of infertile pollen related to using of new pollen donors. In the current study, differences in fruit set, seed formation per fruit, weight of fruit and weight of seed were observed. 'Jumilia' showed a higher crossability rate as a male parent than when it used as a maternal parent because 'Jumilia' featured high pollen viability and pollen germination. 'Black Baccara' achieved a good ACR of 58.33%, but the combination of 'Black Magic' × 'Jumilia' obtained 6.88 seeds per fruit. These reciprocal crossings showed that not all combinations were successful and some preferential crossing directions were observed (Table 3). However, the efficiency depended on the specific cross combination and varied between 2.25 and 60.50 seeds per fruit. The current study indicated the maximum crossability rate in 'Black Baccara' × 'Jumilia' (94.44%), while the ineffective result in crossability rate (23.68%) was obtained in 'Jumilia' × 'Black Baccara'. 'Black Baccara' showed a better SPE ratio than 'Jumilia', and this situation was paralleled by the success of crossability. The maximum number of seeds per fruit was obtained from the 'Jumilia' × 'Tineke' (60.50 seeds) combination, followed by 'Tineke' × 'Jumilia' (43.74 seeds). Regardless of the combination, Khan et al. [2021] found that the fruit set rate was 63.33% in their crossbreeding investigation. According to Abdolmohammadi et al. [2014], the combinations' fruit set rates ranged from 0% to 80.00%. It varied between 0% and 100.0% in the Atram et al. [2015] study and between 30.00% and 83.00% in the Nadeem et al. [2015] trials. According to research that determined the average number of seeds per fruit, roses typically had between 0 and 50.0 seeds per fruit [Zlesak 2007]. Abdolmohammadi et al. [2014] found that crosses between wild and old garden roses and modern roses resulted in an average of 0–35.30 seeds per fruit. Using contemporary roses as parents, Nadeem et al. [2015] found that the average number of seeds per fruit varied from 15.0 to 33.0. According to Farooq et al. [2016], crosses between five distinct rose species showed average numbers of seeds per fruit ranging from 0 to 17.0. According to Khan et al. [2020], hybrid roses had seed numbers ranging from 0.0 to 15.0 per fruit.

The complex genetic structures of genotypes [Ueckert 2014], parental fertility [Nadeem et al. 2015], incompatibility [MacPhail and Kevan 2009], meiotic abnormalities, and the accumulation of lethal alleles [Ogilvie et al. 1991, Nadeem et al. 2015] may all contribute to the variation in crossability rate among combinations. Furthermore, a poor crossability rate may result from hormonal regulation that affects the embryo and hip formation [Cruden and Lyon 1989, Stone et al. 1995]. According to Gudin [2001], fruit set in roses appears to be regulated by embryo development. The crossability rate and seed production efficiency were shown to be positively correlated in the current study. Zlesak [2007] asserts that hybrid roses are primarily self-pollinating and that the ability of the female gametes to accept foreign pollen is what determines whether the crossover is successful. The differences in seed set and crossability rate could also be attributed to petal counts [Nadeem 2012]. In the wild, there are more fruits and seeds, while old garden roses have fewer petals because of increased infertility. It has also been claimed that roses with fewer petals produce more fruits than those with more petals [Baydar et al. 2016]. Additionally, researchers found that sterility caused fewer fruits and seeds, which were correlated with fewer anthers as the number of petals increased. Other studies reported that the number of seeds per fruit may differ depending on pollination, fertilization, embryogenesis, ploidy levels and pollen viability and germination [Gudin and Arene 1992, Zlesak 2007, Farooq et al. 2016, Kazaz et al. 2020]. These differences were due to ACR, SPE, meiotic abnormality and accumulation of recessive alleles [Zlesak 2007]. The germination percentage differed for each combination. The low seed germination percentage could be attributed to a lack of a good seed set or viable embryos [Fagerlind 1954]. In addition, environmental factors such as daylight or daily temperature affect crossability rates and germination. A physiological dormancy, which is under hormonal control, may affect rose seeds [Semeniuk et al. 1963, Gudin 1995, Finch-Savage and Leubner-Metzger 2006]. Hence, the rose embryo may force the seed coat to open. Zlesak [2005] reported that hybrid tea roses demonstrate between 38.6% and 64.4% germination rates as these genotypes feature high germination rates. This confirms the findings of the current study that hybrid tea roses show different

germination characteristics of tetraploid genotypes as a result of different crosses. In the current study, the fertility status of genotypes varied from low to high.

CONCLUSIONS

Production and breeding in cut roses are continuously advancing, however, due to the limited scope of current rose breeding efforts, breeders are focused on increasing seed production to develop new rose varieties. The current study aimed to assess the compatibility among several Rosa varieties used in commercial cut rose breeding programs. It was found that 'Black Baccara' and 'Jumilia' exhibited excellent crossability rates and seed germination rates, with 'Jumilia' proving to be a superior male parent for all crosses. The findings emphasize the importance of compatible parent pairs in improving crossability indices (ACR, SPE), suggesting that further combinations of parents should be tested to fully explore their breeding potential. By improving crossability and seed sets, which are crucial for creating commercially viable rose varieties, this study provides valuable insights into current breeding strategies. The results offer practical applications that can guide future rose breeding programs. Moreover, further research is recommended into the effects of different ploidy levels across various cross combinations to optimize breeding outcomes and enhance the development of new rose varieties.

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Quantitative taxonomy of flower color in *Gladiolus × gandavensis* cultivars

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ABSTRACT

Gladiolus × *gandavensis* is one of the world's four famous cut flowers. Flower color is an important basis for the classification of *Gladiolus* × *gandavensis*. To define the flower color of *Gladiolus* × *gandavensis* more scientifically, twenty-three *Gladiolus* × *gandavensis* horticultural cultivars were studied for flower color phenotypes to provide a preliminary phenotypic quantitative basis for the determination of the affinity of unknown cultivars in the species and provide a scientific basis for the definition of flower color in the classification process. Based on the results of the RHSCC colorimetric card and colorimeter, the 23 *Gladiolus* × *gandavensis* cultivars were classified into six flower color categories, i.e. white, pink, purple, greenish-yellow, orange, and red. Clustering analysis further verified the above classification results according to the flower color, indicating that the two have good correspondence. At the same time, the characteristics of the distribution of the parameters of the CIELab color system of *Gladiolus* × *gandavensis* were analyzed and preliminarily determined the distribution range of the phenotypic parameters of each color group, and the results filled a gap in the study of the floral phenotype of *Gladiolus* × *gandavensis*.

Keywords: *Gladiolus* × *gandavensis,* RHSCC method of designating colors, CIELab color system, flower color phenotype, cluster analysist

INTRODUCTION

Gladiolus × *gandavensis* is a perennial monocotyledonous geophyte flower of the genus *Gladiolus* in the family Iridaceae [Zhao 1985]. Their corms are oblate, with yellowish-brown or brown membranous outer skins. Basal leaves are sword-shaped, alternate, and arranged in two rows. Flowering stems are erect and drawn from the leaf tufts [Zhao 1985]. They have terminal spikes, flowers symmetrical on both sides and colorful, beautiful flowers making them one of the world's four famous cut flowers [Yi et al. 2000]. *Gladiolus* × *gandavensis* is a hybrid species, probably formed by crossing *Gladiolus natalensis* and *Gladiolus oppositiflorus*. There are many cultivars of *Gladiolus* × *gandavensis*, and due to its complex genetic background, the classification of *Gladiolus* × *gandavensis* cultivars has been a difficult problem. According to the difference in blooming habit, it has been classified as a spring-flowering cultivar, which is planted in the fall

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and blooms in the following spring, as well as a summer-flowering cultivar, which is planted in the spring and blooms in the summer and fall [Chen 1990].

Flower color is a qualitative and quantitative trait controlled by multiple genes, involved in plant pollination, plays a role in plant defense against biotic and abiotic stresses and other physiological functions, and is an important basis for the classification of plant cultivars [Zeng et al. 2021]. Within the same color family, differences in color shade are exhibited due to environmental factors such as cultivation conditions (light intensity, temperature, nutrient availability, growth season), pigment system, types of pigments (anthocyanins, carotenoids, etc.) and cell sap pH. Between different flower color families, the trait is relatively stable unless mutations or transposons occur [Liu et al. 1998]. Currently, studies on flower color in ornamental plants mainly focus on flower color phenotype, flower color glycoside composition, and the flower color formation mechanism [Ding et al. 2019]. In flower color phenotype studies, flower color description methods are usually adopted from RHSCC (Royal Horticultural Society Color Chart) and ISCC-NBS (Inter-Society Color Council-National Bureau of Standard, collated by the Domestic Color Research Society and the National Bureau of Standards) color name representations are dominant, while RHSCC is the most widely used type of colorimetric color scale for describing floral colors [Sakata et al. 1995, Hashimoto et al. 2000, Torskangerpoll and Andersen 2005]. The CIELab tabular color system, on the other hand, has been widely used for the quantitative determination of flower color in ornamental plants, and its scientific validity has been confirmed by several studies, such as *Rhododendron mucronulatum* [Li et al. 2008], *Chrysanthemum* × *morifolium* [Sun et al. 2010, Hong et al. 2012], *Phalaenopsis* type [Li et al. 2013], *Paeonia* × *suffruticosapeony* [Wu et al. 2016, Han et al. 2017], *Rosa chinensis* [Wang et al. 2017], *Malus* spp. [Jiang et al. 2017], *Rosa damascena* [Rasouli et al. 2018], *Paeonia lactiflora* [Wang et al. 2018], *Bougainvillea spectabilis* [Zeng et al. 2018], *Freesia refracta* [Ding et al. 2019], *Nelumbo* sp. [Wu et al. 2020, Liu et al. 2020], *Yulania denudata* [Du et al. 2021], *Dianthus caryophyllus* [Teng et al. 2022], *Oxalis obtusa* [Li et al. 2022] and *Viola cornuta* [Jang et al. 2023].

As an important fresh-cut flower, the flower color of *Gladiolus* × *gandavensis* is one of the most important indicators of its ornamental properties. The flower color of the existing *Gladiolus* × *gandavensis* horticultural cultivars is mainly concentrated in white, pink, red, purple, yellow series, etc., and the solid color cultivars are the mainstream. However, there are fewer studies on the flower color of *Gladiolus* × *gandavensis*. Wang et al. [2007] conducted a principal compositional analysis of 76 traits in 25 cultivars of *Gladiolus* × *gandavensis* and found that the contribution rate of flower color reached 73.93%. Liu et al. [2016] qualitatively described the flower color of *Gladiolus* × *gandavensis.* Years of hybridization have also brought more difficulties in classifying the flower color of *Gladiolus* × *gandavensis*, which has limited its promotion and related research. The objective of the current study was to provide a quantitative classification of the flower color phenotypes of *Gladiolus* × *gandavensis* as a reference for future research on the identification and classification of new cultivars to aid in the genetic breeding of novel flower colors.

MATERIAL AND METHODS

Plant material and cultivation conditions

Samples of 23 Gladiolus × gandavensis horticultural cultivars were used as experimental material (Table 1), all of which were imported summer-flowering cultivars from the Netherlands, including corms of 'Adrenalin', 'Cartago', 'Flevo Shine', 'Flevo Snow', 'Indian Summer' and 'Natan' purchased from Sichuan Bella Horticulture Co. (Chengdu, Sichuan, China), and corms of the remaining 17 cultivars were purchased from Zhejiang Hongan Horticulture Co. (Jiaxing, Zhejiang, China). Gladiolus × gandavensis corms were planted in April 2024 in the Experimental Garden of the College of Life Science and Biotechnology, Mianyang Teachers' College. The base soil was yellow loam, loamy clay, with a planting density row spacing of 15 × 15 cm and a planting depth of 10–15 cm, with water and fertilizer applied per standard recommendations [Yi et al. 2000].

Flower color description and determination methods

During June–July 2024, when *Gladiolus* × *gandavensis* cultivars were in full bloom, six inflorescen-

Table 1. List of 23 *Gladiolus* × *gandavensis* cultivars used in the study at the College of Life Science and Biotechnology, Mianyang Teachers' College, China

No.	Cultivar
1	'Adrenalin'*
2	'Amsterdam'
3	'Bra Val'
4	'Cartago'*
5	'Dador De Pan'
6	'Essential'
7	'Fairytale Pink'
8	'Flevo Breezer'
9	'Flevo Fusion'
10	'Flevo Nautica'
11	'Flevo Quote'
12	'Flevo Shine'*
13	'Flevo Snow'*
14	'Green Star'
15	'Indian Summer'*
16	'Kio'
17	'Mojito'
18	'Natan'*
19	'Orange Sun'
20	'Prinses Margaret Rose'
21	'Priscilla'
22	'Sugar Babe'
23	'Zamora'

All cultivars were imported from the Netherlands: *purchased from Sichuan Bella Horticulture Co., China. Those without an asterisk were purchased from Zhejiang Hongan Horticulture Co., China

ces of each cultivar were selected, and three flowers that were in full bloom in each inflorescence were randomly selected. Since the middle and upper parts of the inner whorl petals of *Gladiolus* × *gandavensis* flowers are the main ornamental parts, and the main color expression of the petals is also determined by this part of the flower, the test was conducted only on the middle and upper parts of the inner whorl petals.

Colorimetry was performed using a visual inspection method and an RHSCC colorimetric card (6th edition, 920 colors) for the middle-upper center of the petal frontal surface in the inner whorl of each flower. At the same time, the lightness value (L^*), redness value (a^*), and yellowness value (b^*) of the middle -upper center of the adaxial surface of the petals of the inner whorl of each flower were measured sequentially, using a colorimeter (Linshang LS173) with a fixed light source of D65, according to the CIELab colorimetric system.

Data analysis

For each cultivar, the color number with the most repetitions was selected as the color number of that cultivar for the floral description. Using Excel software, the average of the L^*, a^*, and b^* values of multiple measurements for each cultivar were calculated as the flower color. The chroma value (C*) and hue angle value (h°) were calculated according to the formula: $C^* = (a^*2+b^*2)^{1/2}$, and $h^\circ = \arctan(b^*/a^*)$. L^*, a^*, and b* values were analyzed by squared Euclidean distance, intergroup linkage method of cluster analysis with IBM SPSS statistics 27.0 software. Correlation analy-

sis and graphing of L^*, a^*, b^*, and C^* values and different color groups were performed using Origin Pro 2020 software. The L* value is a measure of the lightness and darkness of the flower color, and changes in the C^* value (indicates the degree of color) affect the L^* value (indicates the degree of lightness).

RESULTS

Flower color classification based on flower color description

Based on the results of the RHSCC colorimetric card, the measured floral phenotypic values of 23 *Gladiolus* × *gandavensis* cultivars were named and systematically categorized. A total of 13 color names were obtained (Table 2), which can be classified into six color groups (Fig. 1): I – white group, including 'Amsterdam', 'Essential' and 'Flevo Snow'; II – pink group, including 'Adrenalin', 'Flevo Fusion', 'Flevo Quote', 'Priscilla' and 'Sugar Babe'; III – purple group, including 'Flevo Nautica', 'Mojito' and 'Zamora'; IV – greenish-yellow group, including 'Bra Val', 'Flevo Breezer', 'Green Star', 'Kio' and 'Prinses Margaret Rose'; V – orange group, including 'Cartago', 'Dador De Pan' and 'Orange Sun'; VI – red group, including 'Fairytale Pink', 'Flevo Shine', 'Indian Summer' and 'Natan'.

Flower color classification based on flower color determination

According to the CIELab spatial color system, the colors of *Gladiolus* × *gandavensis* cultivars were widely distributed in the CIELab spatial color system, and the distribution of L^*, a^*, b^*, C^* and $h°$ values ranged from 33.75 to 98.91, –4.65 to 61.95, –20.41 to 48.74, 3.35 to 67.68, and –1.39 to 1.52, respectively (Table 3). Among them, most of the a^*, b^*, and $h°$ values were distributed in the range of positive values.

A cluster analysis of L^*, a^*, and b^* values of the flower color of 23 *Gladiolus* × *gandavensis* cultivars were classified into six color groups at the classification distance of five (Fig. 2): Class I – white, comprising three cultivars, Class II – pink, comprising five cultivars, Class III – purple, comprising three cultivars, Class IV – greenish-yellow, comprising five cultivars, Class V – orange, comprising three cultivars, Class VI – red, comprising four cultivars. The grouping results of the cluster analysis basically coincided with the classification results based on the RHSCC colorimetric card (Table 4).

Evaluation of the CIELab color system based on the color group classified by the RHSCC color name representation method

The CIELab color system was used to evaluate the results of flower color classification of *Gladiolus* × *gandavensis* cultivars obtained by the RHSCC color name representation. By analyzing the CIELab color system parameters (i.e., L^*, a^*, and b^* values) of the six color groups of *Gladiolus* × *gandavensis*, it was found that the characteristics of each color group are obvious and can distinguish between the different color groups (Fig. 3), which are specifically expressed as follows. First, the L^* value of the white group was the largest, which was significantly higher than that of other color groups, and the L^* value of the red group was the smallest. There was an overlap between the L^* values of the purple and orange groups, but the a^* and b^* values of the orange group were much higher than those of the purple group, so the two could be clearly differentiated by the a^* and b^* values. The pink and greenish-yellow colors overlapped the L^* values, which could also be differentiated by the a^* and b^* values. Second, the a^* values of both the orange and red groups were larger and closer, but the b^* values were significantly different so that the two could be distinguished. The a^* value of the greenish-yellow group was the smallest and distributed over both the positive and negative range, while the a^* values of the remaining color groups were distributed in the positive range. Third, the b^* values of the greenish-yellow, white, pink, and orange groups were distributed in the positive range, and the greenish-yellow group was the largest. The b^* value of the purple group, which was distributed in the negative range, was the smallest, while the b^* value of the red group was distributed in both the positive and negative ranges. Although the b^* values of the white and pink groups overlapped, they could be differentiated by the a^* values. The results of the above analysis further showed that there is a good correspondence between the RHSCC color name representation and the CIELab color system, which can objectively differentiate different color groups. In addition, the classified color groups are reasonable and consistent with the phenotypic characteristics of *Gladiolus* × *gandavensis*.

Table 2. Flower color description (RHSCC numbers and their corresponding color description, and color groups) of petals of 23 tested *Gladiolus* × *gandavensis* cultivars

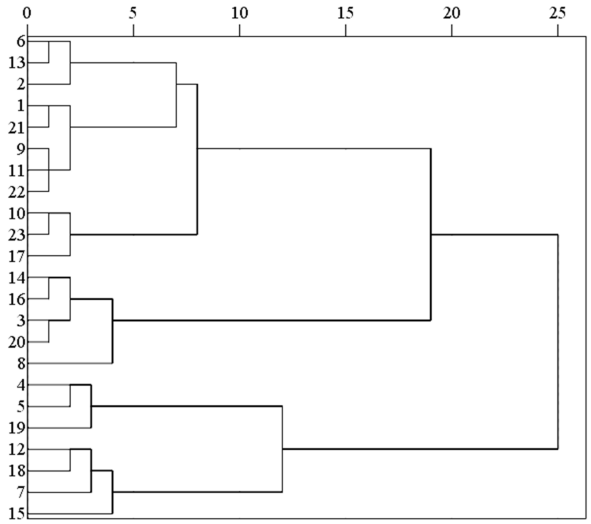
No.	Cultivars	RHSCC No.	Color description	Color group
1	'Adrenalin'	RHS 62C	light purplish-pink	pink
2	'Amsterdam'	RHS NN155C	white	white
3	'Bra Val'	RHS 8B	light greenish-yellow	greenish-yellow
4	'Cartago'	RHS N30B	vivid reddish-orange	orange
5	'Dador De Pan'	RHS 44B	vivid reddish-orange	orange
6	'Essential	RHS NN155C	white	white
7	'Fairytale Pink	RHS 58B	strong purplish-red	red
8	'Flevo Breezer'	RHS 5D	light greenish-yellow	greenish-yellow
9	'Flevo Fusion'	RHS 52D	strong pink	pink
10	'Flevo Nautica'	RHS N82C	light purple	purple
11	'Flevo Quote'	RHS 54D	moderate purplish-pink	pink
12	'Flevo Shine'	RHS 61B	strong purplish-red	red
13	'Flevo Snow'	RHS NN155D	white	white
14	'Green Star'	RHS N144D	strong yellow-green	greenish-yellow
15	'Indian Summer'	RHS 184D	moderate purplish-red	red
16	'Kio'	RHS 154C	brilliant yellow-green	greenish-yellow
17	'Mojito'	RHS N81C	brilliant purple	purple
18	'Natan	RHS 64A	moderate purplish-red	red
19	'Orange Sun'	RHS 33B	vivid reddish-orange	orange
20	'Prinses Margaret Rose'	RHS 7D	light greenish-yellow	greenish-yellow
21	'Priscilla'	RHS N66C	deep purplish-pink	pink
22	'Sugar Babe'	RHS 49A	strong pink	pink
23	'Zamora'	RHS 75A	light purple	purple

Fig. 1. Photographs showing the classification of 23 tested *Gladiolus* × *gandavensis* cultivars based on flower color description. I – 'Amsterdam', 'Essential', 'Flevo Snow'; II – 'Adrenalin', 'Flevo Fusion', 'Flevo Quote', 'Priscilla', 'Sugar Babe'; III – 'Flevo Nautica', 'Mojito', 'Zamora'; IV – 'Bra Val', 'Flevo Breezer', 'Green Star', 'Kio', 'Prinses Margaret Rose'; V – 'Cartago', 'Dador De Pan', 'Orange Sun'; VI – 'Fairytale Pink', 'Flevo Shine', 'Indian Summer', 'Natan'

Table 3. Determination of flower color on the middle and upper parts of the inner whorl petals of 23 tested *Gladiolus* × *gandavensis* cultivars

No.	Cultivars	CIELab				
		L^*	a^*	b^*	C^*	$h°$
1	'Adrenalin'	78.41	16.81	2.8	17.04	1.41
2	'Amsterdam'	98.91	5.04	8.94	10.26	0.51
3	'Bra Val'	86.62	1.93	41.17	38.69	0.05
4	'Cartago'	54.58	55.72	38.42	67.68	0.97
5	'Dador De Pan'	55.97	61.95	27.17	67.64	1.16
6	'Essential'	84.07	3.06	4.75	5.65	0.57
7	'Fairytale Pink'	50.90	58.03	3.93	58.16	1.5
8	'Flevo Breezer'	97.75	3.44	46.8	46.92	0.07
9	'Flevo Fusion'	75.72	31.71	7.64	32.62	1.33
10	'Flevo Nautica'	66.98	14.84	–14.14	20.5	–0.81
11	'Flevo Quote'	75.69	32.97	10.57	34.62	1.26
12	'Flevo Shine'	33.75	48.92	2.62	48.99	1.52
13	'Flevo Snow'	85.55	1.27	3.11	3.35	0.39
14	'Green Star'	70.28	–4.65	41.09	41.35	–0.11
15	'Indian Summer'	48.52	32.62	1.78	32.67	1.52
16	'Kio'	76.52	–1.8	37.37	37.41	–0.05
17	'Mojito'	58.41	14.42	–20.41	24.99	–0.62
18	'Natan'	43.71	52.05	–9.28	52.87	–1.39
19	'Orange Sun'	67.66	46.28	35.11	58.09	0.92
20	'Prinses Margaret Rose'	80.56	0.25	48.74	48.74	0.01
21	'Priscilla'	77.04	24.37	3.03	24.56	1.45
22	'Sugar Babe'	86.56	33.79	13.97	36.57	1.18
23	'Zamora'	68.75	15.83	–9.95	18.7	–1.01

L^* – lightness value; a^* – redness value; b^* – yellowness value; C^* – chroma value; $h°$ – hue angle value; $C^* = (a^{*2} + b^{*2})^{1/2}$, $h° = \arctan(b^*/a^*)$.



*L** – lightness value; *a** – redness value; *b** – yellowness value

Fig. 2. Cluster analysis, based on the *L**, *a**, *b** values, of 23 tested *Gladiolus* × *gandavensis* cultivars. The numbering of the cultivars in Figure 2 is the same as in Table 1

Characterization of phenotypic parameters distribution

Distribution of the L^*, a^*, b^* values. The flower color phenotypic parameters of *Gladiolus* × *gandavensis* were widely distributed. On a two-dimensional quadrant map of a^* and b^* values (Fig. 4A), the flower colors of *Gladiolus* × *gandavensis* cultivars in this experiment were mainly distributed in quadrants I, II, and IV. The number of germplasm resources in quadrant I was the most distributed, with the distribution of the pink group, orange group, and white group. In addition to the distribution of the greenish-yellow and red groups in quadrant I, they were also distributed in quadrants II and IV. Quadrant IV was the distribution of the purple group. There were no cultivars distributed in quadrant III, i.e., there were no flower colors with negative a^* and b^* values, indicating that there was no blue-green color group. The three-dimensional distribution of L^*, a^*, and b^* (Fig. 4B) showed that the color group was roughly distributed in three-dimensional bands.

Relationship between the L^* and C^* values for the different color groups. The relationship between the L^* and C^* values of *Gladiolus* × *gandavensis* cultivars was different for different color groups. The 23 *Gladiolus* × *gandavensis* cultivars could be classified into three groups based on the distribution of the L^* and C^* values in two-dimensional coordinates (Fig. 5). White was the first group, pink, purple, and greenish -yellow were the second group, and orange and red were the third group. A linear regression fit test of the L^* and C^* values of the three groups showed that the L^* value increased with the C^* value, i.e., the lightness and chroma of the floral phenotype were positively correlated, and the correlation between the L^* and C^* values of the three groups was not significant. The correlation coefficient between the L^* and C^* values for the first taxon is 0.6621 (Fig. 6A), the correlation coefficient between the L^* and C^* values for the second taxon is 0.3040 (Fig. 6B), and the correlation coefficient between the L^* and C^* values for the third taxon is 0.0271 (Fig. 6C).

DISCUSSION

Cluster classification of flower color phenotype in *Gladiolus* × *gandavensis* cultivars

In the current study, it was found that the L^*, a^*, and b^* values measured by the colorimeter could more accurately describe the petal color of *Gladiolus* × *gandavensis* cultivars. The classification based on

Table 4. Distribution range of flower color parameters L^*, a^*, b^*, C^*, $h°$ of each color group of 23 tested *Gladiolus* × *gandavensis* cultivars based o the cluster analysis

No.	Color group	Sample number	Percentage (%)	CIELab				
				L^*	a^*	b^*	C^*	$h°$
1	white	3	13.04	85.55～98.91	1.27～5.04	3.11～8.94	3.35～10.26	0.39～0.57
2	pink	5	21.74	75.69～85.56	16.81～33.79	2.80～13.97	17.04～36.57	1.18～1.45
3	purple	3	13.04	58.41～68.75	14.42～15.83	–20.41～–9.95	18.70～24.99	–1.01～–0.62
4	greenish-yellow	5	21.74	70.28～97.75	–4.65～3.44	37.37～48.74	37.41～48.74	–0.11～0.07
5	orange	3	13.04	54.58～67.66	46.28～61.95	27.17～38.42	58.09～67.68	0.92～1.16
6	red	4	17.39	33.75～50.90	32.62～58.03	–9.28～3.93	32.67～58.16	–1.39～1.52

L^* – lightness value; a^* – redness value; b^* – yellowness value; C^* – chroma value; $h°$ – hue angle value; $C^* = (a^{*2} + b^{*2})^{1/2}$, $h° = \arctan(b^*/a^*)$.

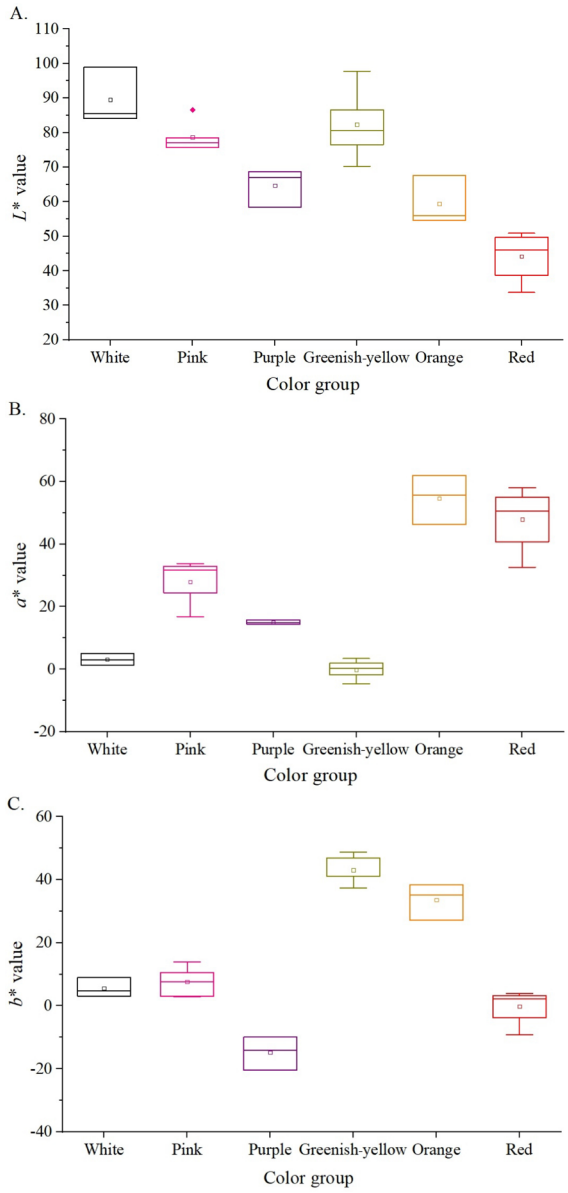


Fig. 3. Boxplot, based on the *L**, *a**, and *b** values, for each color group of 23 tested *Gladiolus* × *gandavensis* cultivars. A. Boxplot based on the *L** (lightness) values for each color group; B. Boxplot based on the *a** (redness) values for each color group; C. Boxplot based on the *b** (yellowness) values for each color group.

the description of the flower color of *Gladiolus* × *gandavensis* cultivars and the clustering results of the flower color measurements matched, and there was no phenomenon of individual clustering inaccuracy in the flower color classification studies such as those reported for *Freesia* × *hybrida* [Ding et al. 2019] and *Oxalis obtusa* [Li et al. 2022]. Previous researchers mostly used cluster analysis to classify flower color phenotype and found that the problem of not being able to differentiate the light color lineage, such as Hong et al. [2012] on a cluster analysis of *Chrysanthemum* × *morifolium*, found that the light pink cultivars were dispersed into the white group. Cluster analysis by Liu et al. [2020] on the flower color of *Nelumbo* sp. found

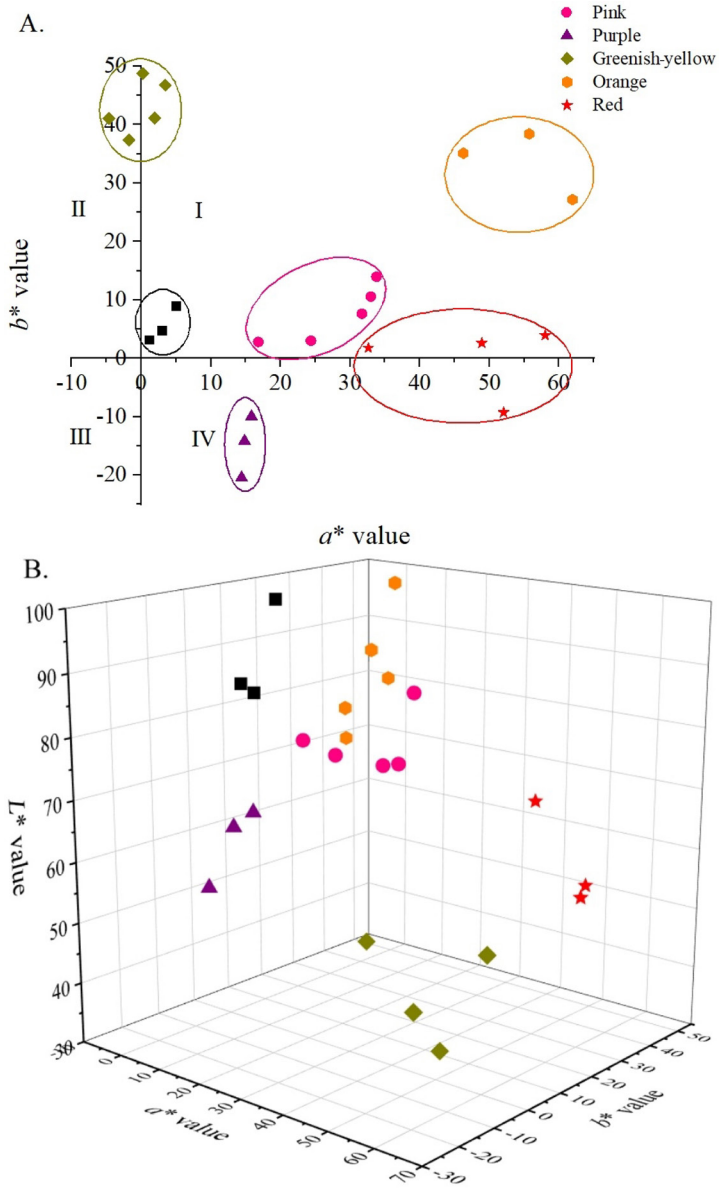
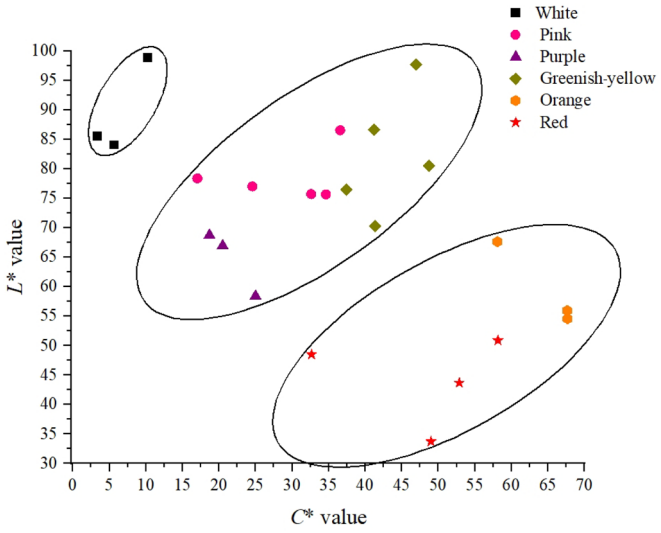


Fig. 4. Two-dimensional distribution map of the *a** and *b** values (A) and three-dimensional distribution map of the *L**, *a**, and *b** values (B) of each color group of 23 tested *Gladiolus* × *gandavensis cultivars*. *L** – lightness value, *a** – redness value, *b** – yellowness value

that it was not possible to differentiate the white group from the light color lineage, such as pale yellow and pink. Du et al. [2021] also found that it was not possible to distinguish the light color group on *Yulania denudata*, with white and light yellow crossing each other, but it was possible to cluster yellow-green into a separate category. The white, pink, and greenish-yellow groups of *Gladiolus* × *gandavensis* cultivars in the current experiment could be clustered into a separate category, probably because cultivars have more typical white, pink, and greenish-yellow petals.



*L** – lightness value; *C** – chroma value

Fig. 5. Two-dimensional scatterplot of the *L** and *C** values of 23 tested *Gladiolus* × *gandavensis* cultivars

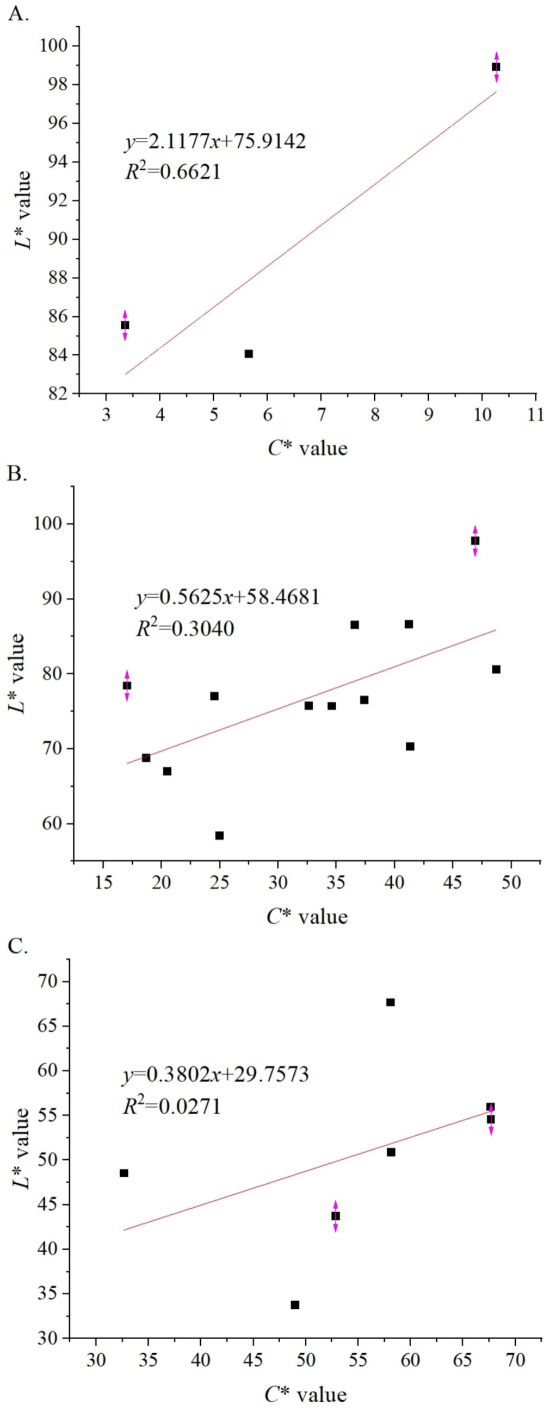
Distributional characteristics of flower color phenotype in *Gladiolus* × *gandavensis* cultivars

Gladiolus × *gandavensis* cultivars are rich in flower color and cover most color groups. On the two-dimensional quadrant map of a^* and b^*, they were mainly distributed in quadrants I, II, and IV, with no distribution in quadrant III, which is similar to some other ornamental plants, such as *Paeonia cathayana* [Han et al. 2010], *Chrysanthemum* × *morifolium* [Hong et al. 2012], *Rosa chinensis* [Wang et al. 2017], *Freesia refracta* [Ding et al. 2019], *Nelumbo* sp. [Liu et al. 2020], *Yulania denudate* [Du et al. 2021], and *Oxalis obtusa* [Li et al. 2022], in which blue-colored flowers are seldom seen. The main reason for the formation of blue flowers is the accumulation of blue-violet delphinidin glycosides in the petals. Many important ornamental plants in nature do not have blue flowers because they do not have the expression signal of the key enzyme gene for synthesizing delphinidin and do not have the core pigment that can show blue color [Li et al. 2021]. Therefore, in the breeding of *Gladiolus* × *gandavensis* for flower color improvement, it would be very meaningful to further study in depth the mechanism of flower color presentation, enrich the genetic resources of the synthesis pathway of anthocyanin glycosides in *Gladiolus* × *gandavensis*, and rebuild the pathway of fritillary in glycoside biosynthesis, to cultivate the blue *Gladiolus* × *gandavensis.*

Correlation of flower color phenotypic parameters in *Gladiolus* × *gandavensis* cultivars

In the quantitative classification of *Freesia refracta* [Ding et al. 2019] and *Nelumbo* sp. [Liu et al. 2020], the relationship between the L^* and C^* values was that the white and yellow groups were classified into one category, and the rest of the color groups were classified into another category. *Oxalis obtusa* [Li et al. 2022]. On the other hand, the yellow group classified into one category and the rest classified into another category. In the present experiment, in the two-dimensional scatter plot of L^* and C^* values, the white group was farther away from the greenish-yellow group, while the greenish-yellow group was closer to the pink and purple groups, and the orange group was closer to the red group. Therefore, the white group of *Gladiolus* × *gandavensis* cultivars was classified into one category alone, the greenish-yellow group was classified into one category with the pink and purple group, and the orange group was classified into one category with the red group.

The L^* and C^* values of flower color phenotype of *Gladiolus* × *gandavensis* cultivars in this experiment show a positive correlation, i.e., as the chroma increases, the lightness also increases. This is contrary to the findings that L^* and C^* values of flower color phenotype of *Consolida ajacis* [Hashimoto et al. 2000], *Freesia refracta* [Ding et al. 2019], and *Oxalis obtusa*



*L** – lightness value; *C** – chroma value

Fig. 6. . Linear regression analysis for the relationship between the *L** and *C** values of 23 tested *Gladiolus* × *gandavensis* cultivars. A. Relationship between the *L** and *C** values for the white group; B. Relationship between the *L** and *C** values for the greenish-yellow, pink, and purple groups. C. Relationship between the *L** and *C** values for the orange and red groups

[Li et al. 2022] were negatively correlated. Previous studies also found that total anthocyanin glycoside content, total carotenoid content, and total flavonoid content were negatively correlated with flower color lightness, while the magnitude of the colorimetric value was determined by the content of key pigments. Currently, there are fewer studies on the flower color of *Gladiolus* × *gandavensis*, and the reasons for the positive correlation between lightness and chroma in *Gladiolus* × *gandavensis* may need to be further determined through accurate pigment content testing.

Genetic diversity and taxonomic complexity of *Gladiolus* × *gandavensis* cultivars

Widely cultivated *Gladiolus* × *gandavensis* cultivars are made by interspecific, interspecies, intercultivar, species-hybrid, and intercultivar crosses of different species [Zhang 1982]. At present, there is no uniform method for their classification internationally, and most of them are classified according to their biological characteristics, reproductive period, flower shape, flower diameter, flower color, etc. Liu et al. [2016] used SRAP technology to construct fingerprints of *Gladiolus* × *gandavensis* and found that the classification of cultivars had no significant correlation with phenotypic traits and had certain genetic diversity at both the morphological and DNA molecular levels, which indicated that cultivars had a complex genetic background. The results of the cluster analysis of the flower color of *Gladiolus* × *gandavensis* cultivars in the current experiment provide a reference for the kinship relationship of *Gladiolus* × *gandavensis*. Due to the limited research resources, there are still many *Gladiolus* × *gandavensis* cultivars that have not been determined for flower color phenotype. In the future, the number of research cultivars can be increased to further optimize the quantitative classification of the flower color phenotype of *Gladiolus* × *gandavensis* and provide a basis for the study of the mechanism of flower color presentation.

CONCLUSIONS

This experiment used an RHSCC colorimetric card and colorimeter to describe and measure the flower colors of 23 *Gladiolus* × *gandavensis* cultivars, which can be roughly classified into six color groups, i.e. white, pink, purple, greenish-yellow, orange, and red, through cluster analysis. Overall, there was a positive correlation between the lightness and the chroma of *Gladiolus* × *gandavensis* flower colors. The flower colors could be classified into three groups on the two-dimensional coordinate system of lightness and chroma values. The results of this study provide a theoretical basis for the selection and breeding of new cultivars and flower colors of *Gladiolus* × *gandavensis* and provide a scientific basis for the molecular breeding study of flower colors of other ornamental plants, as well as the determination and classification of flower colors.

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