

The combined treatments of brassinolide and zeaxanthin better alleviate oxidative damage and improve hypocotyl length, biomass, and the quality of radish sprouts stored at low temperature

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Chemical compounds studied in this article:

Phenylalanine ammonia-lyase (EC: 4.3.1.24)
Peroxidase (EC 1.11.1.7)
Superoxide dimutase (SOD, EC 1.15.1.1)
Glutathione (PubChem CID: 124886)
Zeaxanthin (PubChem CID: 5280899)
Proline (PubChem CID: 145742)
Anthocyanin (PubChem CID: 145858)
Vitamin C (PubChem CID: 54670067)
Malondialdehyde (PubChem CID: 10964)
Hydrogen peroxide (PubChem CID: 784)

ABSTRACT

The rot and deterioration of sprouts are closely related to their physiological state and postharvest storage quality. The study investigated the influences of brassinolide, zeaxanthin, and their combination on physiological metabolism, chlorophyll fluorescence, and nutritional quality of radish sprouts stored at 4 °C. The combined treatments enhanced hypocotyl length, fresh weight, contents of secondary metabolites, nutritional ingredients, glutathione, the photoprotective capacity of PSII, and FRAP level in radish sprouts compared with zeaxanthin alone. The combined treatments enhanced hypocotyl length, fresh weight, glutathione content, F_v/F_m value, and antioxidant capacity in sprouts compared to brassinolide alone. The combined treatment of zeaxanthin and brassinolide could make radish sprouts keep high biomass and antioxidant capacity by increasing the contents of stress-resistant metabolites and by weakening the photoinhibition of PSII in radish sprouts stored at 4 °C.

1. Introduction

The consumption of cruciferous sprouts, a new type of plant food, has spread all over the world in the past decades because they are rich in active ingredients and nutrients when compared with adult plants (O'Hare, Wong, Force, & Irving, 2007). However, the market value of vegetables (such as sprouts) is related to water loss, shrinking, and decay caused by vigorous metabolic activities after harvest (Zhu, Wang, Cao, & Jiang, 2008). Low-temperature storage is usually used to slow down the metabolic activity of fruits and vegetables after harvest, but with the extension of storage time, oxidative damage and photoinhibition still occur in plant tissues (Bourne, 2004; Tang et al., 2021). Therefore, how to improve the storage characteristics and prolong the storage time of sprouts by enhancing their growth and physiological quality is a crucial

problem.

Carotenoids are a class of natural lipid-soluble pigments, especially lutein and zeaxanthin, which are widely used in antioxidant and anti-cancer research (Gulcin, 2012; Gulcin, 2020). Antioxidant activity increased linearly with the increase of total carotenoid content in high-yield corn hybrids, and carotenoids played a synergistic role in membrane antioxidant activity (Kljak & Grbesa, 2015; Liang, Tian, Yang, Zhang, & Skibsted, 2009). Several studies have found that xanthophylls and zeaxanthin can act as molecular rivets on the cell membrane that protect the membrane structure and resist the damage of reactive oxygen species (Gabrielska & Gruszecki, 1996; Gruszecki & Strzalka, 2005). Zhang et al. (2020) found that photoinhibition in pepper was alleviated by the accumulation of carotenoid-related metabolic compounds under the combined stress of low temperature and low light. The increase in

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violaxanthin de-epoxidase gene expression eased the photoinhibition of tomato and tobacco under low temperatures (Gao, Han, Feng, Zhao, & Meng, 2010; Han et al., 2010). Zeaxanthin was used to mitigate photoinhibition of plants under low temperatures, which has become a research hotspot in recent years.

Brassinosteroids are widely used in plant growth regulation, such as stem and leaf growth, fruit ripening, and enhancing plant resistance to stress (Bajguz & Hayat, 2009). Fariduddin, Yusuf, Chalkoo, Hayat, and Ahmad (2011) found that 28-homobrassinolide could promote the growth and photosynthesis of cucumber by activating the antioxidant system under low-temperature stress. Aghdam and Mohammadkhani (2013) reported that postharvest brassinolide treatment ($6 \mu\text{mol L}^{-1}$) enhanced the quality of tomato fruit stored at low temperature by decreasing electrolyte leakage and malondialdehyde (MDA) content and by the increasing proline content. Gao et al. (2015) suggested that 24-epibrassinolide might relieve chilling injury and browning reaction in eggplant fruit stored at low temperature. 24-epibrassinolide treatment could improve the storage quality and shelf life of daylily flower buds by decreasing the metabolite of active oxygen and increasing the activities of antioxidant enzymes (Yao et al., 2017). However, there are few studies on the application of BR in sprouts.

The corruption of postharvest sprouts is related to their quality and physiological state. Therefore, it is of great significance to study how to improve the growth and postharvest storage quality of sprouts. At present, it has been found that exogenous carotenoids have the good antioxidant capacity and alleviate photoinhibition, but there are few studies on the effects of carotenoids on the growth and physiological characteristics of sprouts and other plants. At the same time, although exogenous brassinosteroids have been applied to improve plant quality and preserve fruits and vegetables, there are few studies on their application in sprout storage and other fields, and there are relatively few studies on the interaction between exogenous antioxidants and brassinosteroids. Therefore, the paper mainly explored the influences of zeaxanthin, brassinolide, and their combined treatments on nutritional quality, physiological metabolism, and chlorophyll fluorescence of postharvest radish sprouts stored for 7 d at low temperature ($4 \text{ }^\circ\text{C}$), in order to provide theoretical support for zeaxanthin and brassinolide in improving the quality and delaying aging of sprouts. We hypothesized that: (1) the combined treatments of zeaxanthin and brassinolide could better alleviate the membrane oxidation process of sprouts during low-temperature storage; (2) the combined treatment of zeaxanthin and brassinolide on the preservation quality of radish sprouts was better than that of zeaxanthin or brassinolide alone, which was beneficial to prolong the storage time of radish sprouts.

2. Materials and methods

2.1. Materials and treatments

The study was carried out at Hebei University, Baoding, China. Radish seeds (*Raphanus sativus*) were bought from Tianjin Gengyun seed Inc in Tianjin province, China. Brassinolide was bought from ANPEL Laboratory Technologies (Shanghai, China) Inc. (Number: 200030). Zeaxanthin was purchased from Aladdin Chemical Reagent (Shanghai, China) Inc. (Number: 688179).

Radish seeds were sterilized in 0.5 % sodium hypochlorite solution for 5 min and washed with distilled water for 4–5 times. The seeds were soaked in distilled water at room temperature for 12 h. Seeds (5 g) were evenly planted in the plastic basket ($31 * 22.5 * 7.5 \text{ cm}$) and cultured with distilled water (16 h light, 8 h dark, $20 \text{ }^\circ\text{C}$). Five-day-old sprouts were divided into four groups (4 replicates per group): (1) the first group was sprayed with 10 mL distilled water (CK); (2) the second group was sprayed with 10 mL brassinolide solution (1 mg L^{-1}) (BR); (3) the third group was sprayed with 10 mL zeaxanthin (20 mg L^{-1}) solution (Z); and (4) the fourth group was sprayed with 10 mL mixed solution of brassinolide and zeaxanthin. The distilled water, brassinolide, zeaxanthin, and

the mixed solution used in the four groups contained 0.02 % Tween-20 and 1 % ethanol. Brassinolide concentration was according to our previous research results (Xue et al., 2021), and zeaxanthin concentration was based on our preliminary works (data unpublished). There were ten days from sowing to harvesting. Each group samples were weighed to the same weight and kept in polystyrene foam boxes at $4 \text{ }^\circ\text{C}$ for 7 d without light. After that, the roots of the sprouts were discarded. Some of the sprouts were rapidly frozen at $-80 \text{ }^\circ\text{C}$. Some were dried and were ground in a laboratory grinder.

2.2. Hypocotyl length, fresh and dry weight of radish sprouts

Hypocotyl length was measured by a ruler, and the average value was calculated with 20 seedlings per treatment. Weight loss, fresh weight (FW), and dry weight (DW) of sprouts were measured by an electronic balance (Sartorius, China).

2.3. Polyphenol, anthocyanin, flavonoids, alkaloid content, and phenylalanine ammonia-lyase enzyme (PAL) activity

Polyphenol was determined with the ferrous tartrate method at 540 nm and was shown as g kg^{-1} on a dry weight basis (Qadir, Muhammad, Bakri, & Gao, 2017). Anthocyanin content was determined using acidified methanol method (methanol: HCl = 99:1, v: v) at 530 and 657 nm according to the description by Xue et al. (2021) and was expressed as ($A_{530} - 0.25 * A_{657}$) kg^{-1} on a fresh weight basis. Flavonoids were measured by the AlCl_3 method at 420 nm and were shown as g kg^{-1} on a dry weight basis (Shi, Chu, Zhang, Liu, & Yao, 2017). Alkaloid content was determined using the bromothymol blue colorimetry at 416 nm described by Guo et al. (2021) and was shown as g kg^{-1} on a dry weight basis. PAL activity was determined with a spectrophotometer at 290 nm according to Shi et al. (2017) and was expressed as $\text{U kg}^{-1}\text{h}^{-1}$ on a fresh weight basis.

2.4. Soluble protein, vitamin C content, and soluble sugar content

Soluble protein content was determined by the Coomassie brilliant blue R250 staining method at 595 nm according to the description of Guo et al. (2021) and was shown as g kg^{-1} on a fresh weight basis. Vitamin C was measured with UV-spectrophotometer at 265 nm according to the description of Akbıyık, Sönmezöglü, Güçlü, Tor, and Apak (2012) with minor modification and was shown as g kg^{-1} on a fresh weight basis. Soluble sugar was determined by the anthrone- H_2SO_4 method at 625 nm and was shown as g kg^{-1} on a dry weight basis (Shi et al., 2017).

2.5. Antioxidant capacity measurement

The antioxidant capacities included the ferric reducing antioxidant power (FRAP, g kg^{-1} on a dry weight basis), diphenylpicrylhydrazyl (DPPH, %), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, g kg^{-1} on a dry weight basis), they were determined with a spectrophotometer at 593 nm, 734 nm, 517 nm, respectively, based on the description of Balaydin, Gulcin, Menzek, Goksu, and Sahin (2010), Durmaz et al. (2022), and Polat Kose and Gulcin (2021).

2.6. Antioxidant enzyme activity

Superoxide dismutase (SOD) was determined using the NBT method and was shown as $\text{U kg}^{-1} \text{ min}^{-1}$ on a fresh weight basis (Apak et al., 2022). Peroxidase (POD) was measured with the guaiacol method at 470 nm according to Wang, Li, Li, Li, and Luo (2021) and was expressed as $\text{U kg}^{-1} \text{ min}^{-1}$ on a fresh weight basis.

2.7. MDA, H₂O₂, and superoxide radical production rate

MDA was measured with the thiobarbituric acid method and was shown as mmol kg⁻¹ on a fresh weight basis (Shi et al., 2017). H₂O₂ content was determined with a spectrophotometer at 460 nm according to the description of Velikova, Yordanov, and Edreva (2000) and was expressed as g kg⁻¹ on a fresh weight basis. The rate of superoxide radical (O₂⁻) production was measured with a spectrophotometer at 530 nm according to Wang et al. (2021), and was expressed as mmol kg⁻¹ min⁻¹ on a fresh weight basis.

2.8. Proline and glutathione content

Proline was measured with the acid ninhydrin method at 520 nm based on Zhang, Shen, Li, Meng, and Sheng (2013) and was expressed as mg kg⁻¹ on a fresh weight basis. Glutathione was measured with the 5,5-dithio-bis-(2-nitrobenzoic acid) method at 412 nm according to Szarka, Lőrincz, Czobor, and Hajdinák (2018) and was expressed as mol kg⁻¹ on a fresh weight basis.

2.9. Measurement of weight loss

Weight loss was determined by weighing polystyrene foam boxes per treatment at intervals during the storage by an electronic balance (Sartorius, China). The data were expressed as a percentage of the initial weight.

2.10. Photosynthetic pigment content

Total chlorophyll (chlorophyll *a* + *b*), carotenoids, and zeaxanthin were measured by the 80 % acetone extraction method. Total chlorophyll and carotenoids contents were calculated according to Lichtenthaler and Wellburn (1983), zeaxanthin content was determined according to Hong and Xu (1999), and the results were shown as g kg⁻¹ on a fresh weight basis.

2.11. Measurement of chlorophyll fluorescence parameters

An Imaging-PAM Chlorophyll Fluorometer (Model IMG-K6, Heinz Walz GmbH, Effeltrich, Germany) was used to determine the chlorophyll fluorescence parameters of leaves after dark adaption for 25 min. The chlorophyll fluorescence parameters were measured after an 8000 μmol m⁻² s⁻¹ photosynthetically active radiation for 0.8 s. They included the light-adapted minimal fluorescence (F₀), the maximum photochemical efficiency of PSII (F_v/F_m), the quantum efficiency of PSII photochemistry (Y(II)), the non-photochemical quenching coefficient (NPQ), the quantum yields of regulated energy dissipation Y(NPQ), and the quantum yields of non-regulated dissipation Y(NO).

2.10. Statistical analysis

Statistical analyses were executed utilizing SPSS 23 (IBM, Chicago, USA). Homogeneity of variance was tested using the Levene test before analysis. The data were analyzed using one-way ANOVA, followed by Tukey's test to determine the significant difference (*p* ≤ 0.05). The multi-way ANOVA was calculated to investigate the interaction effects of zeaxanthin and brassinolide.

3. Results

3.1. Sprout growth and weight loss rate

Fig. 1A–D showed that hypocotyl length, FW, and DW of radish sprouts treated with zeaxanthin, brassinolide, and their combined treatments increased by 8 %, 9 % and 19 %, 3 %, 13 % and 29 %, 11 %, 9 % and 9 %, respectively, when compared with the control group (CK).

Hypocotyl length, FW, and DW were the highest in the combined-treated sprouts. There was shown a marked interaction in zeaxanthin × brassinolide on the DW of sprouts (*p* = 0.021) (Table S1).

Weight loss was considered to be a vital indicator of sprout freshness. The weight loss of radish sprouts was enhanced with the extension of storage time (Fig. 1E). On the 7th day, zeaxanthin, brassinolide, and their combined treatments reduced the weight loss of sprouts by 5.48 %, 5.77 %, and 3.59 %, respectively, compared with that of the CK group. An evident interaction in brassinolide × T was found on the weight loss of sprouts (*p* = 0.003) (Table S4).

3.2. Malondialdehyde and H₂O₂ contents and the rate of O₂⁻ production

MDA content in sprouts stored for 7 d (CK7) was more than that in the other groups, and zeaxanthin, brassinolide, and their combined treatments significantly reduced MDA content in radish sprouts over the control (CK0, day 0) or CK7 (Fig. 2A). There was a significant interaction in zeaxanthin × brassinolide on the MDA content of sprouts (*p* = 0.000) (Supplementary Table 1). H₂O₂ content and the rate of O₂⁻ production in the CK7 group was enhanced by 43 % and 31 %, respectively, when compared with the CK0 group (Fig. 2B and C). Zeaxanthin, brassinolide, and their combined treatments reduced the H₂O₂ content, and the rate of O₂⁻ production in sprouts stored for 7 d, and the levels were 12 %, 13 %, 16 %, 20 %, 20 %, and 26 %, respectively, when compared with the CK7 group. And the values of the rate of O₂⁻ production in zeaxanthin-treated, brassinolide-treated, and their combined-treated groups decreased to the level of the CK0 group. There was shown a marked interaction in zeaxanthin × brassinolide on the H₂O₂ content of sprouts (*p* = 0.000) (Table S1).

3.3. Proline and glutathione contents

Fig. 3A showed that proline content in radish sprouts was reduced by CK7 and zeaxanthin treatments while was enhanced by brassinolide and the combined treatments of zeaxanthin + brassinolide when compared with the CK0 group. There was no marked interaction in zeaxanthin × brassinolide on the proline content of sprouts (*p* = 0.777) (Table S3).

Glutathione content of radish sprouts in the CK7 group was reduced compared with CK0 (Fig. 3B). In contrast, brassinolide and the combined treatments of zeaxanthin + brassinolide increased glutathione content in sprouts by 3 % and 6 %, respectively, compared with CK0. There was no marked interaction in zeaxanthin × brassinolide on the glutathione content of sprouts (*p* = 0.286) (Table S3).

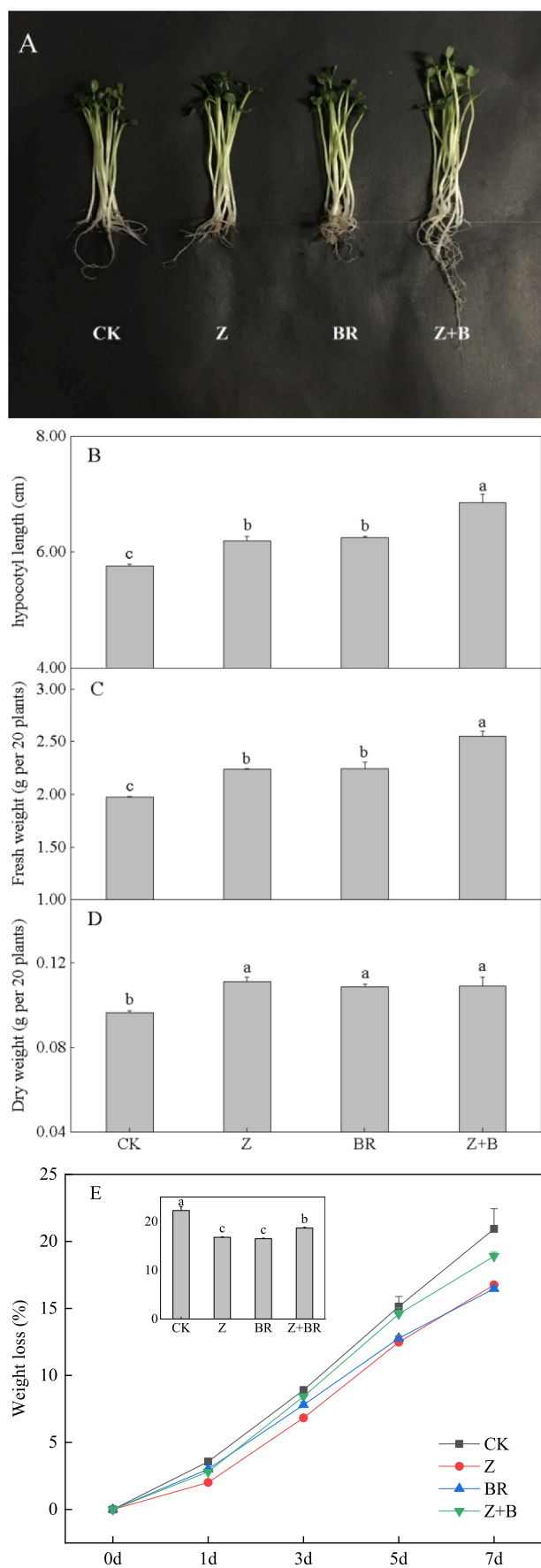
3.4. Anthocyanin and polyphenol contents

Anthocyanin content was increased by zeaxanthin, brassinolide, and their combined treatments, and the levels rose 34 %, 14 %, and 16 %, respectively, when compared with the CK0 or CK7 group (Fig. 4A). Anthocyanin content was the highest in zeaxanthin-treated sprouts. There was a marked interaction in zeaxanthin × brassinolide on the anthocyanin content of sprouts (*p* = 0.000) (Table S2).

CK7, zeaxanthin, brassinolide, and the combined treatments of zeaxanthin + brassinolide increased polyphenol content in radish sprouts by 0.39-, 0.51-, 0.76-, and 0.75- fold, respectively, over the CK0 group, and polyphenol content was the highest in sprouts treated with brassinolide and the combined treatments of zeaxanthin + brassinolide (Fig. 4B). There was shown a marked interaction in zeaxanthin × brassinolide on the polyphenol content of sprouts (*p* = 0.001) (Table S2).

3.5. Alkaloid and flavonoids content and PAL activity

Alkaloid content in the CK7 group was decreased by 48 % compared with the CK0 (Fig. 5A). While zeaxanthin, brassinolide, and their combined treatments increased alkaloid content in radish sprouts by 84 %, 84 %, and 84 %, respectively, when compared with the CK0 group.



(caption on next column)

Fig. 1. Effects of brassinolide and zeaxanthin on hypocotyl length (A, B), fresh weight (C), dry weight (D) of fresh radish sprouts, and weight loss (E) of radish sprouts during storage for 7 d at 4 °C. The CK, Z, BR and Z + BR represent control, Z-treated, BR-treated and the combined-treated samples, respectively. The insets in Fig. 1 E represent the values of 7 days of storage. Values are means of four replicates \pm standard error. The bars with different letters are significantly different from each other ($p < 0.05$).

66 %, and 59 %, respectively, compared with the CK7 group, the alkaloid content in zeaxanthin-treated sprouts reached the levels in the CK0 group. There was shown a marked interaction in zeaxanthin \times brassinolide on the alkaloid content of sprouts ($p = 0.000$) (Table S2).

CK7 and zeaxanthin treatment had no significant influence on flavonoids content in radish sprouts compared with CK0 (Fig. 5B). However, brassinolide and the combined treatments of zeaxanthin + brassinolide increased flavonoids content in radish sprouts when compared with the CK7 or CK0 group, and there was no significant difference in flavonoids content between the brassinolide and the combined treatments. There was no marked interaction in zeaxanthin \times brassinolide on the flavonoids content of sprouts ($p = 0.144$) (Table S2).

No noticeable change in PAL activity was observed between CK7 and CK0, but zeaxanthin, brassinolide, and their combined treatments enhanced PAL activity in radish sprouts compared with CK0 (Fig. 5C). There was no evident difference in PAL activity between zeaxanthin, brassinolide, and their combined treatments. No marked interaction of zeaxanthin \times brassinolide was found on the PAL activity in sprouts ($p = 0.079$) (Table S2).

3.6. Vitamin C, soluble protein, and soluble sugar contents

Vitamin C content in the CK7 group decreased by 11.84 % compared with the CK0 (Fig. 6A). While zeaxanthin, brassinolide, and their combined treatments increased vitamin C content in radish sprouts by 12 %, 14 %, and 17 %, respectively, compared with CK7, and the vitamin C content in BR-treated and the combined-treated sprouts reached the levels in CK0 group. There was shown a marked interaction in zeaxanthin \times brassinolide on the vitamin C content in sprouts ($p = 0.002$) (Table S2).

CK7, zeaxanthin, brassinolide, and the combined treatments of zeaxanthin + brassinolide increased soluble protein content in radish sprouts by 0.06-, 0.04-, 0.11-, and 0.11-fold, respectively, compared with CK0, and soluble protein content was the highest in sprouts treated with brassinolide and the combined treatments of zeaxanthin + brassinolide (Fig. 6B). There was no marked interaction in zeaxanthin \times brassinolide on the soluble protein content in sprouts ($p = 0.569$) (Table S2).

CK7 treatment reduced soluble sugar content in radish sprouts compared with CK0 (Fig. 6C). Soluble sugar content was increased by zeaxanthin, brassinolide, and their combined treatments, and the levels rose 7 %, 8 %, and 8 %, respectively, when compared with the CK7 group. There was shown a marked interaction in zeaxanthin \times brassinolide on the soluble sugar content of sprouts ($p = 0.006$) (Table S2).

3.7. Antioxidant enzyme activity

CK7, zeaxanthin, brassinolide, and the combined treatments of zeaxanthin + brassinolide enhanced POD activity in radish sprouts by 0.38-, 0.69-, 0.52-, and 0.71-fold, respectively, compared with CK0, and POD activity got the maximum in sprouts treated with zeaxanthin and the combined treatments of zeaxanthin + brassinolide (Fig. S1A). There was shown a marked interaction in zeaxanthin \times brassinolide on the POD activity of sprouts ($p = 0.001$) (Table S1).

CK7 treatment reduced SOD activity in radish sprouts compared with CK0 (Fig. S1B). SOD activity was enhanced by zeaxanthin, brassinolide, and the combined treatments of zeaxanthin + brassinolide, and the levels rose 8 %, 9 %, and 4 %, respectively, compared with the CK7 group. A marked interaction in zeaxanthin \times brassinolide was found on

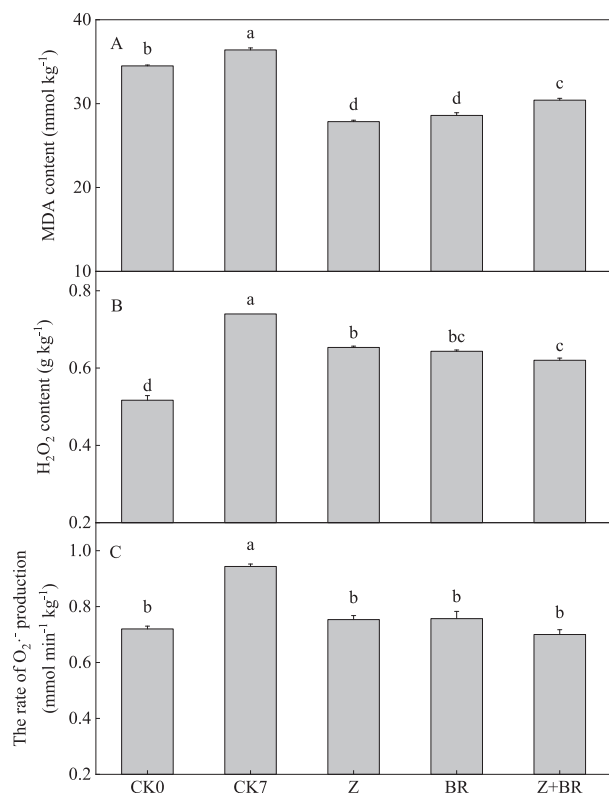


Fig. 2. Effects of brassinolide and zeaxanthin on contents of malondialdehyde (A) and hydrogen peroxide (H₂O₂) (B), the rate of O₂⁻ production (C) in radish sprouts stored for 7 d at 4 °C. CK0 represents control samples stored for 0 d. CK7, Z, BR and Z + BR represent control, Z-treated, BR-treated and the combined-treated samples stored for 7 d, respectively. Values are means of four replicates ± standard error. The bars with different letters are significantly different from each other (p < 0.05).

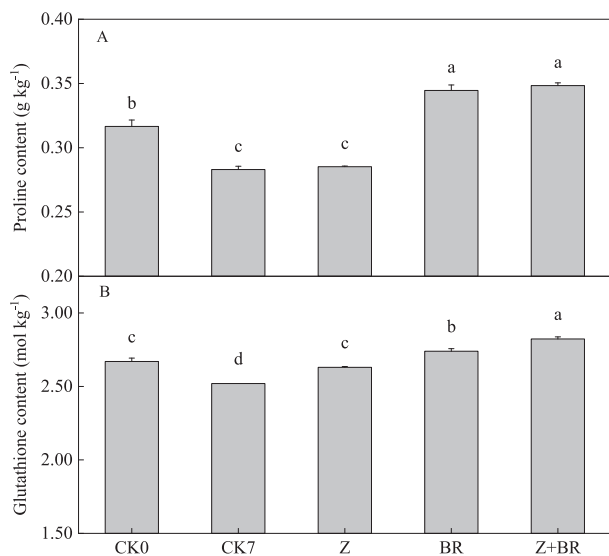


Fig. 3. Effects of brassinolide and zeaxanthin on contents of proline (A), and glutathione (B) in radish sprouts stored for 7 d at 4 °C. CK0 represents control samples stored for 0 d. CK7, Z, BR and Z + BR represent control, Z-treated, BR-treated and the combined-treated samples stored for 7 d, respectively. Values are means of four replicates ± standard error. The bars with different letters are significantly different from each other (p < 0.05).

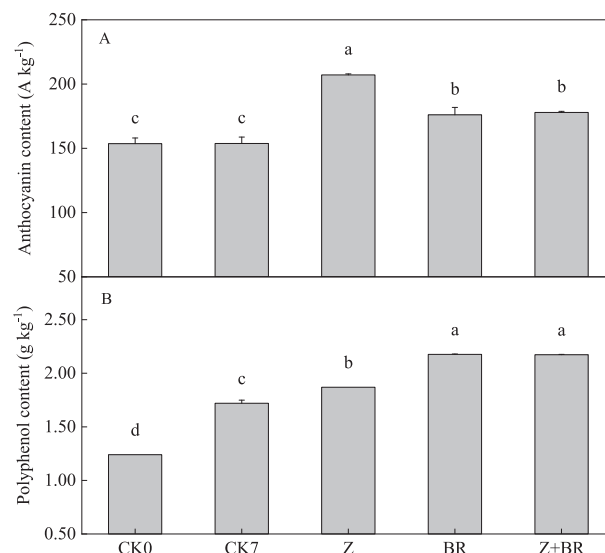


Fig. 4. Effects of brassinolide and zeaxanthin on contents of anthocyanin (A), and polyphenol (B) in radish sprouts stored for 7 d at 4 °C. CK0 represents control samples stored for 0 d. CK7, Z, BR and Z + BR represent control, Z-treated, BR-treated and the combined-treated samples stored for 7 d, respectively. Values are means of four replicates ± standard error. The bars with different letters are significantly different from each other (p < 0.05).

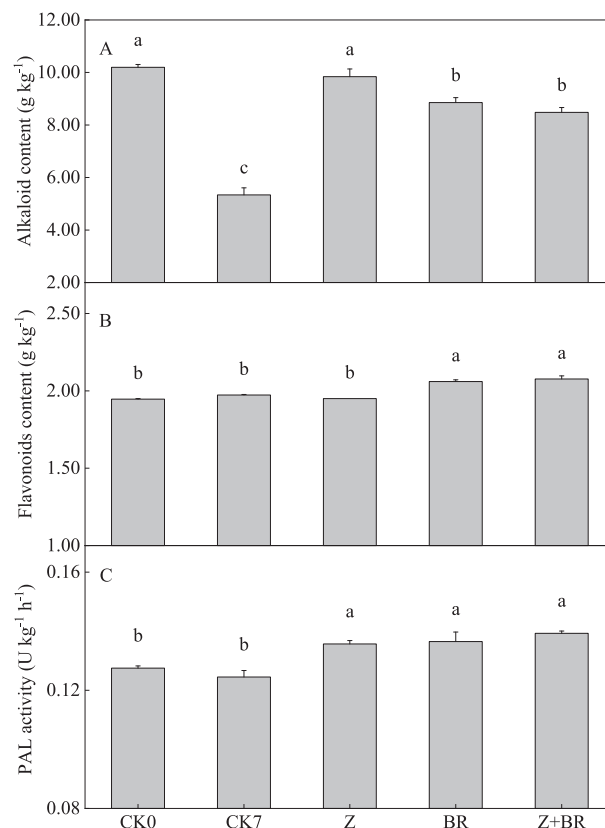


Fig. 5. Effects of brassinolide and zeaxanthin on contents of alkaloid (A), and flavonoids (B), the activity of phenylalanine ammonia lyase enzyme (PAL) (C) in radish sprouts stored for 7 d at 4 °C. CK0 represents control samples stored for 0 d. CK7, Z, BR and Z + BR represent control, Z-treated, BR-treated and the combined-treated samples stored for 7 d, respectively. Values are means of four replicates ± standard error. The bars with different letters are significantly different from each other (p < 0.05).

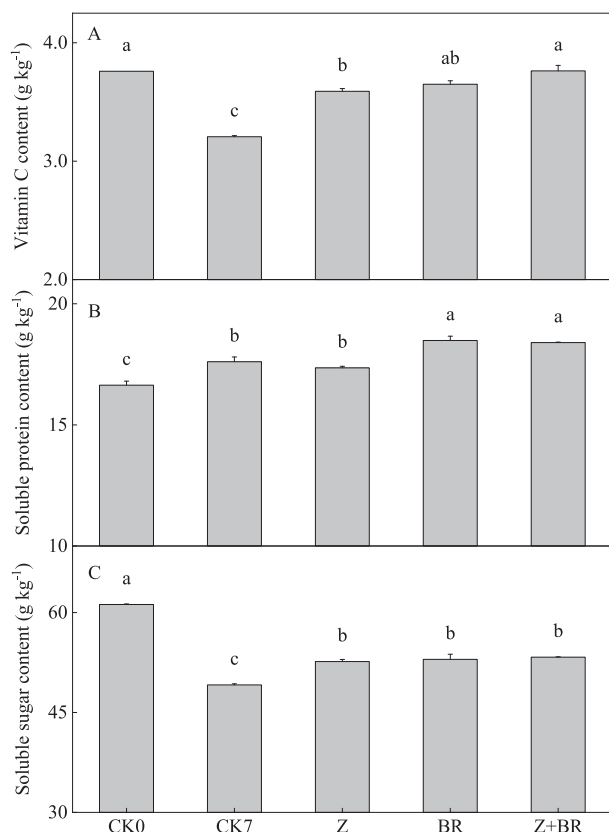


Fig. 6. Effects of brassinolide and zeaxanthin on contents of vitamin C (A), soluble protein (B) and soluble sugar (C) in radish sprouts stored for 7 d at 4 °C. CK0 represents control samples stored for 0d. CK7, Z, BR and Z + BR represent control, Z-treated, BR-treated and the combined-treated samples stored for 7 d, respectively. Values are means of four replicates \pm standard error. The bars with different letters are significantly different from each other ($p < 0.05$).

the SOD activity in sprouts ($p = 0.000$) (Table S1).

3.8. Antioxidant capacity

Fig. S2A showed that DPPH radical-scavenging activity in radish sprouts was increased by CK7, zeaxanthin, brassinolide, and their combined treatment over the CK group, and the levels rose 37 %, 70 %, 21 %, and 48 %, respectively. DPPH radical-scavenging activity was the highest in zeaxanthin-treated sprouts alone. There was no marked interaction in zeaxanthin \times brassinolide on the DPPH radical-scavenging activity of sprouts ($p = 0.253$) (Table S3).

CK7, zeaxanthin, brassinolide, and their combined treatments of zeaxanthin + brassinolide increased FRAP levels in radish sprouts by 139 %, 150 %, 142 %, and 168 %, respectively, compared with CK0, and FRAP levels got the maximum in sprouts of the combined treatments of zeaxanthin + brassinolide. (Fig. S2B). There was shown a marked interaction in zeaxanthin \times brassinolide on the FRAP levels of sprouts ($p = 0.003$) (Table S3).

As shown in Fig. S2C, ABTS levels were increased by CK7, zeaxanthin, brassinolide, and the combined treatments of zeaxanthin + brassinolide, and the levels rose 34 %, 57 %, 44 %, and 55 %, respectively, when compared with the CK0 group. ABTS levels got the maximum in sprouts treated with zeaxanthin alone and the combined treatments of zeaxanthin + brassinolide. There was shown a marked interaction in zeaxanthin \times brassinolide on the ABTS levels of sprouts ($p = 0.009$) (Table S3).

3.9. Total chlorophyll, carotenoids, and zeaxanthin contents

Total chlorophyll and carotenoids contents of radish sprouts in the CK7 group were reduced by 14 % and 16 %, respectively, when compared with CK0 (Fig. S3A and B). Total chlorophyll and carotenoids contents were increased by zeaxanthin, brassinolide, and their combined treatments, and the values reached the levels in the CK0 group. There was shown a marked interaction in zeaxanthin \times brassinolide on the total chlorophyll and carotenoids contents of sprouts ($p = 0.003$, $p = 0.020$) (Table S3).

Fig. S3C showed that zeaxanthin content in sprouts was reduced by CK7 treatments compared with CK0. However, zeaxanthin, brassinolide, and their combined treatments increased zeaxanthin content in radish sprouts by 22 %, 19 %, and 18 %, respectively, compared with CK7, and zeaxanthin content got the maximum in sprouts treated with the zeaxanthin alone. There was shown a marked interaction in zeaxanthin \times brassinolide on the zeaxanthin content of sprouts ($p = 0.000$) (Table S3).

3.10. Changes in chlorophyll fluorescence parameters

Figs. S4A–G showed that chlorophyll fluorescence parameters in sprouts changed with the increase of storage time. After seven days of storage, F_0 , F_v/F_m , Y (II), NPQ, and Y (NPQ) levels in radish sprouts were decreased, but Y (NO) value was increased when compared with the CK0. The effects of zeaxanthin treatment on these parameters were consistent with CK7. Brassinolide and the combined treatments of zeaxanthin + brassinolide increased F_0 (except for the combined treatment), F_v/F_m , NPQ, and Y (NPQ), and decreased Y(NO) levels in radish sprouts when compared with the CK7 group. F_v/F_m , NPQ, and Y(NPQ) levels were the highest in sprouts treated with the combined treatments of zeaxanthin + brassinolide. The marked interactions of zeaxanthin \times brassinolide were found on F_v/F_m ($p = 0.009$), Y (II) ($p = 0.045$), Y (NPQ) ($p = 0.000$), Y(NO) ($p = 0.000$), and NPQ ($p = 0.000$) values in sprouts (Table S4).

3.11. Correlations between storage quality and physiological metabolism and chlorophyll fluorescence

As shown in Fig. S5, Spearman's correlation coefficients were used to analyze the storage quality, physiological metabolism, and chlorophyll fluorescence in radish sprouts stored for 7 d. In the present study, a prominently positive correlation was found between proline and glutathione (0.810**), proline and glutathione contents had extremely negatively correlated with H_2O_2 content (-0.790**, -0.927**), and the rate of $O_2^{\cdot-}$ production (-0.735**, -0.847**), and Y(NO) (-0.713**, -0.725**), and exceedingly prominently positive correlations with vitamin C (0.799**, 0.979**) and F_v/F_m (0.833**, 0.931**).

H_2O_2 content had positive correlations with the rate of $O_2^{\cdot-}$ production (0.804**) and significant negative correlations with vitamin C (-0.860**), soluble protein (-0.679*), soluble sugar (-0.801**), and F_v/F_m (-0.851**).

FRAP showed a positive correlation with POD activity (0.898**). FRAP and POD activity had significant positive correlations with vitamin C (0.650*, 0.811**), anthocyanin (0.578*, 0.647*), and total chlorophyll (0.606*, 0.578*), and carotenoids (0.888**, 0.871**), and prominent negative correlations with the rate of $O_2^{\cdot-}$ production (-0.823**, -0.865**). Total chlorophyll showed a positive correlation with carotenoids (0.743**). Total chlorophyll and carotenoids had prominent positive correlations with anthocyanin (0.723**, 0.764**) and zeaxanthin (0.626*, 0.604*). Meanwhile, anthocyanin showed a positive correlation with zeaxanthin (0.912**).

F_v/F_m showed a positive correlation with NPQ (0.669*). F_v/F_m and NPQ had significant positive correlations with Y(NPQ) (0.795**, 0.848**), and significant negative correlations with Y(NO) (-0.781**, -0.860**), meanwhile, the Y(NPQ) showed a negative correlation with Y(NO) (-0.949**).

4. Discussion

In this study, we found that zeaxanthin, brassinolide, and their combined treatments enhanced hypocotyl length, fresh weight, dry weight of fresh radish sprouts compared with CK, and their levels in the combined-treated sprouts remained at the maximum (Fig. 1A–D). Similar to our results, zeaxanthin has been found to participate in photosensory transduction and protect the chloroplasts in the coleoptile (Zhu, Zeiger, & Zeiger, 1995). Tang et al. (2021) found that exogenous zeaxanthin pretreatment maintained a higher photosystem process and fresh weight of pepper seedlings than the control during low temperatures. Brassinolide could promote cell elongation in young rice seedlings (Fujii & Saka, 2001), and Sun et al. (2020) found that brassinolide treatment increased the net photosynthetic rate and plant biomass of maize under chilling stress. The increase in plant growth and dry matter accumulation might be related to maintaining chloroplast function by zeaxanthin treatment. Meanwhile, brassinolide treatment could promote cell elongation and enhance the biomass of plants by promoting photosynthesis.

The loss of water is the crucial factor leading to the devaluation of storage sprouts (Zhu et al., 2008). Our results showed that zeaxanthin, brassinolide, and their combined treatments reduced weight loss during storage and increased activities of enzymatic antioxidants compared with the CK group (Fig. 1E, Fig. S1). This research was similar to the results found by Wang, Ding, Gao, Pang, and Yang (2012), who found that brassinolide treatment could reduce the electrolyte leakage and induce the activity of antioxidant enzymes in pepper fruit with chilling stress. Kang et al. (2018) found that the increased resistance to stressful environments was related to the rise of carotenoids content in the transgenic sweet potato (cv. Shangshu 19). Other studies also suggested that carotenoids could react with free radicals to act as antioxidants in vivo and vitro (Kiokias & Gordon, 2004). These indicated that zeaxanthin, brassinolide, and their combined treatments could reduce the water loss of radish sprouts during storage by promoting the antioxidant capacity.

MDA is taken as an essential indicator to detect membrane damage, which is caused by the higher reactive oxygen species (ROS) levels (Ali et al., 2019). Sun et al. (2020) found that brassinolide treatment (0.1 mg L⁻¹) increased the antioxidant enzyme activities and reduced the MDA content in maize exposed to chilling stress. In chill-stressed plants, Tang et al. (2021) found that exogenous zeaxanthin pretreatment could reduce H₂O₂ and MDA content and the rate of O₂⁻ production by enhancing the antioxidant enzymes activities and transcriptional expression. This is also found in our study, zeaxanthin, brassinolide, and their combined treatments reduced MDA and H₂O₂ content and the rate of O₂⁻ production, and enhanced activities of antioxidant enzyme in sprouts stored at low temperature compared with CK7 (Fig. 2A–C), these indicated that zeaxanthin, brassinolide, and zeaxanthin + brassinolide application could inhibit membrane lipid peroxidation and reduced ROS levels in sprouts stored at low temperature by enhancing antioxidant enzyme activities.

Proline and glutathione are essential stress-resistant metabolites in the plant. He et al. (2019) found that under polyethylene-glycol stress, the navel orange mutant ‘Gannan No.1’ (*Citrus sinensis* Osbeck) accumulated higher proline and glutathione and decreased membrane electrolyte leakage and showed higher membrane stability compared with wild-type ‘Newhall’. Adding proline (75 mmol L⁻¹) could be against oxidative stress and improve sperm quality by enhancing the level of glutathione and activities of CAT and SOD (Feng et al., 2020). Aghdam, Moradi, Razavi, and Rabiei (2019) reported that exogenous application of phenylalanine (5 mmol L⁻¹) in tomato fruit could reduce H₂O₂ accumulation and enhance membrane integrity by improving the activities of enzymatic antioxidants and the contents of endogenous lycopene and proline. Our study showed that the contents of proline and glutathione in the combined-treated sprouts remained at the maximum, and the H₂O₂ content and the rate of O₂⁻ production remained at the

minimum (Fig. 3A and B). We also found a significant correlation between proline and glutathione ($r = 0.810^{**}$), and H₂O₂ content and the rate of O₂⁻ production were negatively correlated with proline and glutathione contents (Fig. S5). These indicated that zeaxanthin, brassinolide, and the combined treatments of zeaxanthin and brassinolide could reduce ROS levels of sprouts stored at low temperatures by enhancing proline and glutathione contents.

Anthocyanin, polyphenol, alkaloid, and flavonoids are the main secondary metabolites of plants, which are regulated by the phenylpropanoid pathway (Guo et al., 2021; Xue et al., 2021). Our results showed that zeaxanthin, brassinolide, and their combined treatments enhanced the contents of anthocyanin, polyphenol, flavonoids (except for zeaxanthin treatment) and alkaloid, and activity of phenylalanine ammonia-lyase (PAL) in radish sprouts compared with CK7 (Fig. 4A and B, and Fig. 5A–C). Aghdam, Asghari, Farmani, Mohayeji, and Moradbeygi (2012) found that postharvest brassinolide treatment (6 μmol L⁻¹ brassinolide solution, soaking for 5 min) could improve the response of tomato fruit to chilling stress (at 1 °C for 21 d) by enhancing total phenol content and PAL activity. However, Gao et al. (2015) found that total phenol content in eggplant fruit was reduced by 24-epibrassinolide treatment (10 μmol L⁻¹ solutions, soaking for 10 min) under chilling stress (at 1 °C for 15 days). The difference might be caused by experimental materials, brassinolide species, treatment concentration and time. Guo et al. (2021) found that brassinolide treatment (0.1 and 2 mg L⁻¹) could increase the contents of flavonoids and total alkaloids in the peach leaf shape of *Pinellia ternata*. In the low-temperature stress, zeaxanthin, as one of the carotenoids involved in the violaxanthin cycle, could improve the resistance of a rice cultivar (Kim, Hoang, Jeon, Wu, & Lee, 2017). Except for pro-vitamin A carotenoids, the metabolism of lycopene has also been found to have anticancer effects (Mein, Lian, & Wang, 2008). Xue et al. (2021) indicated that carotenoids might be involved in black bean sprouts as a new active substance. These showed that zeaxanthin, brassinolide, and the combined treatments of zeaxanthin + brassinolide could increase the accumulation of secondary metabolites in radish by promoting the phenylpropanoid pathway.

Soluble protein, soluble sugar, and vitamin C are essential nutritional ingredients in plant growth and development. In water stress, Li and Feng (2010) found that roots soaked in brassinolide solution (0.2 mg L⁻¹) could enhance soluble sugar, soluble protein, and ascorbic acid contents in *Xanthoceras sorbifolia* seedlings. Exogenous application of 5-aminolevulinic acid (ALA) could enhance soluble sugar, soluble protein, and ascorbic acid contents in tomato fruit by controlling carotenoids metabolism (Wang et al., 2021). Liu et al. (2014) found that ectopic expression of a *BZRI-1D* transcription factor in the brassinosteroids signaling pathway led to carotenoids accumulation and the increase of soluble sugar and vitamin C contents in tomatoes. In our study (Fig. 6A–C) zeaxanthin, brassinolide, and their combined treatments enhanced the contents of vitamin C, soluble protein (except for zeaxanthin treatment), and soluble sugar in radish sprouts compared with CK7. In the present study, we also found that H₂O₂ content was negatively correlated with the contents of vitamin C, soluble protein, and soluble sugar (Fig. S5). These demonstrated that zeaxanthin, brassinolide, and their combined treatments could alleviate the peroxidation damage of radish sprouts by enhancing the contents of vitamin C, soluble protein, and soluble sugar.

SOD and POD are important protective enzymes in the enzyme defense system. The content of ROS and their scavenger enzyme and scavenger can be used as physiological and biochemical indexes to measure the senescence degree of fruit and vegetables (Wang et al., 2021). Choudhary et al. (2010) found that radish seeds treated with 24-epibrassinolide (0.001 μmol L⁻¹) and putrescine (0.001 mol L⁻¹) could improve antioxidants levels, antioxidant enzymes activities, and free radical inactivation ability in sprouts under Cu ion stress. In our study, brassinolide treatment improved the antioxidant levels, POD and SOD activity, and ABTS levels in sprouts stored at low temperature (4 °C for 7 d) compared with CK7 (Fig. S1A and B and Fig. S2C). Interestingly, Gao

et al. (2015) found that chilling injury (at 1 °C for 15 d) could improve the POD activities of eggplant fruit. Our result also found that POD activity in stored sprouts (4 °C for 7 d) was increased (Fig. S1A), and was the highest under the combined treatments of zeaxanthin + brassinolide. Dall'Osto, Cazzaniga, North, Marion-Poll, and Bassi (2007) reported that neoxanthin could protect membrane lipids from ROS, which is related to the activity of antioxidant enzyme. Kang et al. (2017) found that the inhibition of β -carotene hydroxylase gene could improve β -carotene content, DPPH radical-scavenging ability, and stressful tolerance in transgenic sweet potato plants. The improvement of SOD activity and the toleration of oxidative stresses were affected by the increase of carotenoid content in the transgenic sweet potato plants (Kang et al., 2018). It was similar to our studies, zeaxanthin treatment and the combined treatments of zeaxanthin + brassinolide also improved the antioxidants levels (such as anthocyanin content), antioxidant enzymic activities, and free radical scavenging activities in sprouts compared with CK7 (Fig. S1A and B) (Fig. S2A–C), similar changes were also explained that the activities of antioxidant enzymic and free radical scavenging were positive correlation with anthocyanin content (Fig. S5). These indicated that zeaxanthin, brassinolide, and the combined treatments of zeaxanthin + brassinolide could improve the enzymic activities and free radical scavenging activities of radish sprouts by promoting the antioxidants levels.

During senescence or under stress conditions, chloroplast and chlorophyll degradation was accelerated (Jiang, Zhu, Wang, & Hou, 2020), and carotenoids synthesis was increased (Tattini et al., 2015). Meanwhile, carotenoids have the function of protecting the photosynthetic organs from damage and maintaining the chloroplast integrity (Polívka & Frank, 2010). In our study, zeaxanthin, brassinolide, and the combined treatments of zeaxanthin + brassinolide restored the levels of chlorophyll and carotenoids in stored sprouts compared with CK7. Zeaxanthin content in sprouts was higher in zeaxanthin-treated, brassinolide-treated, and the combined-treated groups than that in CK7 under storage (Fig. S3A–C). Some studies have also shown that exogenous 24-epibrassinolide enhanced chlorophyll content and photosynthetic capacity during low-temperature stress (Anwar et al., 2018; Cai et al., 2019). Zeaxanthin and tocopherol could act as antioxidants by working on the membrane lipid or lipoprotein (Havaux & García-Plazaola, 2014). Brunetti et al. (2019) also found that the increase of zeaxanthin (and neoxanthin) served an essential function as a chloroplast antioxidant. In our study, the contents of total chlorophyll, carotenoids, and zeaxanthin were significant positive correlations (Fig. S5). These indicated that the alleviating of total chlorophyll degradation and maintaining carotenoids content might be that the endogenous zeaxanthin increased the antioxidant capacity of membrane lipid under chilling storage.

PSII in plants always suffers from photoinhibition during low-temperature conditions (Zhang et al., 2020). F_0 value is the minimal fluorescence, indicating the openness of the PSII reaction center. (Li, Yang, Gan, Yu, & Xie, 2015). After 7 days of storage, F_0 values in radish sprouts were decreased, the F_0 value in stored sprouts treated with BR was the highest, the effects of zeaxanthin treatment and the combined treatments of zeaxanthin and brassinolide on F_0 value were consistent with CK7 (Fig. S4A), indicating that during low-temperature storage, the openness of the PSII reaction center in the cotyledon of radish sprouts to the light was diminished, zeaxanthin treatment and the combined treatments of zeaxanthin and brassinolide could not alleviate this change. Meanwhile, brassinolide might relieve this reaction to a certain extent.

The decrease in the F_v/F_m value means that the plants are affected by stress, while the greater NPQ value means that plants have a stronger photoprotective capacity (Maxwell & Johnson, 2000). Under low-temperature conditions, Li et al. (2015) found that 24-epibrassinolide treatment (foliar spray) relieved the photoinhibition by improving F_v/F_m , Y(II) of pepper seedlings. Tang et al. (2021) found that exogenous zeaxanthin pretreated peppers increased the transcription level of

CaPsbP, and Yi, Hargett, Liu, Frankel, and Bricker (2007) reported that F_v/F_m levels reduced with the reduction in PsbP protein expression of transgenic Arabidopsis plants. In the high light and low-temperature conditions, Gao et al. (2010) found that the expression of the violaxanthin de-epoxidase gene in transgenic tobacco relieved the decrease in F_v/F_m and the net photosynthetic rate. The F_v/F_m and NPQ value in radish sprouts were enhanced by brassinolide treatment and the combined treatments of zeaxanthin and brassinolide compared with CK7. While, the effects of zeaxanthin treatment on F_v/F_m and NPQ value were consistent with CK7 (Fig. S4B and F). Under storage, zeaxanthin might be played an essential role in maintaining chloroplast integrity instead of alleviating photoinhibition (Brunetti et al., 2019). F_v/F_m value was positively correlated with NPQ ($r = 0.669^*$) (Fig. S5). These indicated that brassinolide and the combined treatments of zeaxanthin and brassinolide might alleviate the stress of low-temperature storage on the photosystem of stored radish sprouts.

Y(II) values represent the actual quantum efficiency of the PSII reaction center (Meravi & Prajapati, 2018). After 7 days of storage, Y(II) values in radish sprouts were decreased, the effects of zeaxanthin, brassinolide, and the combined treatments of zeaxanthin and brassinolide on Y(II) values were consistent with CK7 (Fig. S4C). The results were different from Tang et al. (2021) and Yi et al. (2007), that may be because they used fresh plant leaves to measure fluorescence parameters, while we used stored postharvest leaves. These indicated that the actual quantum efficiency of PSII reaction center was reduced during storage of radish sprouts, and zeaxanthin, brassinolide, and the combined treatments of zeaxanthin and brassinolide could not alleviate the problem. High Y(NPQ) value means stronger light protection in plants, while a higher Y(NO) value indicates to have a strong inhibitory effect on the photosynthesis of plants (Tang et al., 2021). Our study showed that brassinolide and the combined treatments of zeaxanthin and brassinolide increased the value of Y(NPQ) and reduced the value of Y(NO) in radish sprouts during storage (Fig. S4D and E), indicating that brassinolide treatment might relieve photoinhibition by reallocating the energy of PSII in radish sprouts to a certain degree.

A comprehensive analysis of the above results showed that after 7 days of storage, PSII of the radish sprouts suffered from photoinhibition. Zeaxanthin treatment did not weaken this effect in sprouts. This may be because zeaxanthin mainly participates in low-temperature storage as a chloroplast antioxidant in stored radish sprouts. BR treatment and the combined treatments of zeaxanthin and brassinolide alleviated the photoinhibition of PSII in radish sprouts by increasing photosynthetic pigment content to a certain degree.

5. Conclusions

The membrane lipid peroxidation, ROS, photosynthetic pigment, F_v/F_m , and some antioxidant and quality parameters in sprouts stored for 7 days at low temperature were significantly lower than those in seedlings stored for 0 days. Zeaxanthin, brassinolide, and the combined treatments of zeaxanthin and brassinolide increased the storage quality of radish sprouts and didn't cause photoinhibition of PSII in sprouts during low-temperature storage compared with CK7. The hypocotyl length, fresh and dry weight, contents of glutathione and vitamin C, FRAP and ABTS levels, and F_v/F_m value in radish sprouts treated with the combined treatments of zeaxanthin and brassinolide were the highest, meanwhile ROS content was the lowest compared with brassinolide or zeaxanthin treatment alone. The combined treatments relieved the photoinhibition in radish sprouts during low-temperature storage. The comprehensive analysis indicated that the combined treatments of zeaxanthin and brassinolide could help radish sprouts maintain high biomass and antioxidant capacity by increasing the contents of stress-resistant metabolites and weakening photoinhibition of PSII in radish sprouts during low-temperature storage.

CRediT authorship contribution statement

Minghui Li: Investigation, Data curation, Writing – original draft. **Yanfen Zhang:** Investigation, Software. **Xihang Xu:** Data curation, Methodology, Writing – review & editing. **Ying Chen:** Data curation, Methodology, Writing – review & editing. **Jianzhou Chu:** Supervision, Writing – review & editing, Resources, Funding acquisition. **Xiaoqin Yao:** Supervision, Writing – review & editing, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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