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The effects of thiamine on *in vitro* organ development and anthocyanin accumulation in *Raphanus sativus*

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Abstract: Thiamine (Vitamin B1) has an important role as an enzymatic cofactor in metabolic processes such as central carbon pathways, which are necessary for biosynthesis and energy production in plants. The current study investigated concentration-dependent effects of *in vitro* thiamine treatments on seed germination, organ development, and anthocyanin accumulation in hypocotyls of *Raphanus sativus* (radish) 'Cherry Belle'. Radish seeds (30 per treatment across 6 replicates) were cultured for 15 days on Murashige and Skoog medium supplemented with six additional thiamine HCl concentrations: 0.4, 1.6, 6.4, 12.8, 25.6, and 51.2 mg L^{-1}. Thiamine supplementation did not result in statistically significant changes in seed germination, whereas concentrations above 6.4 mg L^{-1} reduced leaf production (to 2.40 leaves per seedling at 12.8 mg L^{-1}). All thiamine treatments enhanced hypocotyl elongation, while treatments above 6.4 mg L^{-1} induced root elongation, reaching a maximum length of 8.64 cm at 25.6 mg L^{-1}. The treatment at 25.6 mg L^{-1} gave the maximum seedling vigor index (3737.23 ± 510.44). The treatment at 12.8 mg L^{-1} induced the highest anthocyanin accumulation in the hypocotyls (3136.05 ± 684.52 µg CGE g^{-1} DW). The findings suggested that thiamine treatment at specific concentrations may be useful for optimizing early *in vitro* seedling development and anthocyanin accumulation in radish. Also, the need for further studies to explain the underlying physiological mechanisms responsible for these concentration-dependent effects of thiamine in radish plants was suggested.

Keywords: Leaf production; Radish; Rhizogenesis; Seed germination; Vitamin B1

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1 Introduction

Thiamine (vitamin B1) functions as thiamine pyrophosphate, one of the vital cofactors for several enzymes associated with central carbon pathways such as the tricarboxylic acid cycle and pentose phosphate pathway in plant metabolism (Li et al. 2022). These pathways are vital for energy production and biosynthetic processes during *in vitro* culture (Fitzpatrick and Chapman, 2020). For this reason, thiamine has been applied as a stimulant in horticultural and agricultural species to improve growth, rooting, and stress tolerance. In sweet potato, plant height and root elongation were promoted, while exogenous thiamine treatment improved stress tolerance in cotton (Li et al. 2022; Vollmer et al. 2023). In tissue culture, thiamine supplementation promotes organogenesis, rooting, and acclimation, often synergizing with auxin and cytokinin treatments (Abrahamian and Kantharajah, 2011). Additionally, thiamine modulates secondary metabolism, such as phenylpropanoid pathways, which contribute to phenolic accumulation and lignin deposition, and may be associated with pigment production (Boubakri et al. 2013). One of such pigments is anthocyanin. Anthocyanins are flavonoid pigments that mainly contribute to red, blue, and purple colorations, and they contribute to the regulation of reactive oxygen species homeostasis, enhance plant resilience against stress, and function as pollinator attractants or photoprotectants in vegetative tissues. Anthocyanins are biosynthesized through the phenylpropanoid pathway, and their biosynthesis is controlled by both the MBW protein complex (MYB-bHLH-WD40) and environmental factors (Mannino et al. 2021; Shi et al. 2023; Li et al. 2025).

The radish plant (*Raphanus sativus* L.) is a fast-growing root vegetable belonging to the Brassicaceae family. Both its tuberized hypocotyls and leaves are edible, with the hypocotyls being the primary part consumed (Sarhang et al. 2023). Radish cultivars display significant diversity in size, color, and soil requirements. Due to their high stability,

anthocyanins extracted from red radish are extensively utilized as natural food colorants (Park et al. 2011). Furthermore, candidate genes that play key roles in phenotypic and spatiotemporal anthocyanin accumulation in radish have been identified (Muleke et al. 2017). Also, increased anthocyanin accumulation in hypocotyls reflects metabolic changes during development and may be associated with enhanced stress tolerance and phytochemical potential (Landi et al. 2015; Gamba et al. 2021). These characteristics make radish a valuable model organism for studying root and hypocotyl development as well as anthocyanin accumulation.

Although efficient micropropagation protocols exist for *R. sativus* (Karthik et al. 2022; Kim et al. 2023), the effects of *in vitro* thiamine treatments on secondary metabolite production, such as anthocyanin accumulation, and on plant development in this species have not been fully explored. This study investigated how different thiamine concentrations affect *R. sativus* cultures *in vitro*, focusing on organ development and hypocotyl pigmentation. Thus, it was hypothesized that thiamine levels in the culture medium would significantly affect organ development and anthocyanin accumulation in hypocotyls. Also, thiamine may serve as a modulator of pigmentation during *in vitro* regeneration of radish plants.

2 Material and Methods

2.1 Plant material and disinfection method

The commercial *Raphanus sativus* 'Cherry Belle' seeds were obtained from a local vendor in Kocaeli, Türkiye, and they were kept in their original packaging until the disinfection treatment. The seeds were disinfected by rinsing them in a 1% (*v:v*) NaOCl solution for 5 min, then washed with sterile distilled water.

2.2 Culture medium preparation, thiamine treatments, and culture conditions

Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962), which already includes thiamine HCl at 0.1 mg L^{-1}, was supplemented with additional thiamine HCl (Sigma-Aldrich, T3902) at 0.4, 1.6, 6.4, 12.8, 25.6, and 51.2 mg L^{-1} concentrations. The culture medium was supplemented with 30 g L^{-1} sucrose and was solidified using plant agar at 7 g L^{-1}. The pH of the medium was adjusted to 5.7-5.8. After sterilization at 121 °C and 118 kPa for 20 minutes, 40 mL of the medium was dispensed into sterile MK-5 culture vessels (Caisson Labs). Cultures were maintained at 23 ± 1 °C under a photon flux density of 60 µmol m^{-2} s^{-1} from fluorescent lamps, with a 16/8 h light/dark photoperiod for 15 days.

2.3 Developmental parameters measured and anthocyanin content estimation

Developmental parameters, including germination rate, hypocotyl and root elongation, and leaf production, were measured at the end of the incubation period. The seedling vigor index (SVI) was calculated as SVI = (root length + shoot length) × germination rate, where shoot length is the aboveground height.

Anthocyanin in the hypocotyls was measured as total monomeric anthocyanin pigment content, following the same incubation period. Hypocotyls were harvested, dried for 24 hours at 50 °C in the dark, and ground into a fine powder. Subsequently, 200 mg of the powdered sample was extracted with 4 mL of 80% (*v:v*) aqueous methanol using an ultrasonic bath at 60 °C for 60 minutes (ISOLAB, 300 W, 50 kHz). The extract was centrifuged at 4400 × g for 10 minutes, and the supernatant was collected for further analysis. The analytical procedure described by Lee et al. (2005) was employed. Results were reported as micrograms of cyanidin-3-glucoside equivalents per gram of dry weight (µg CGE g^{-1} DW).

2.4 Statistical analyses and data visualization

A completely randomized design was employed in the study. Each treatment was repeated 6 times, with each repeat consisting of 5 seeds, for a total of 30 seeds per treatment. Biochemical assays were performed in triplicate. Results are presented as mean ± standard deviation. Duncan's Multiple Range Test (DMRT) was performed after variance analysis at $p < 0.05$ using IBM SPSS Statistics 22 software. Developmental data were normalized to the [0, 1] interval and processed with the Interactive Clustered Heat Map Builder (Ryan et al. 2020) for hierarchical cluster analysis (HCA) using Euclidean distance and the complete-linkage clustering method.

3 Results

3.1 Effects of thiamine on *in vitro* organ development

Thiamine treatments did not produce a statistically significant difference in seed germination rates. The control group exhibited a germination rate of 88 ± 4%, while supplementation with 0.4 mg L^{-1} additional thiamine increased the rate to 96 ± 4%. The lowest germination rate, 76 ± 9%, was observed in the medium containing an additional 12.8 mg L^{-1} thiamine (Fig. 1A).

Leaf production remained statistically stable with thiamine supplementation up to 6.4 mg L^{-1}. Increasing thiamine concentration beyond 6.4 mg L^{-1} resulted in a statistically significant reduction in leaf production. Seedlings in the control group produced a mean of 4.44 ± 0.18 leaves per seedling. The highest mean leaf number, 4.88 ± 0.20, was observed in seedlings grown with 6.4 mg L^{-1} additional thiamine. In contrast, supplementation with 12.8 mg L^{-1} thiamine reduced mean leaf production to 2.40 ± 0.25 leaves per seedling (Fig. 1B).

All thiamine treatments resulted in increased hypocotyl length compared with the control (3.79 ± 0.22 cm). The maximum hypocotyl length reached 5.09 ± 0.10 cm per seedling grown in the medium supplemented with 25.6 mg L^{-1} additional thiamine (Fig. 1C).

Thiamine concentrations above 6.4 mg L^{-1} led to a significant increase in mean root length. The maximum mean root length, 8.64 ± 0.40 cm per seedling, was recorded from the medium with 25.6 mg L^{-1} additional thiamine. In comparison, control group seedlings had a mean root length of 4.51 ± 0.22 cm. The shortest roots, measuring 4.18 ± 0.35 cm per seedling,

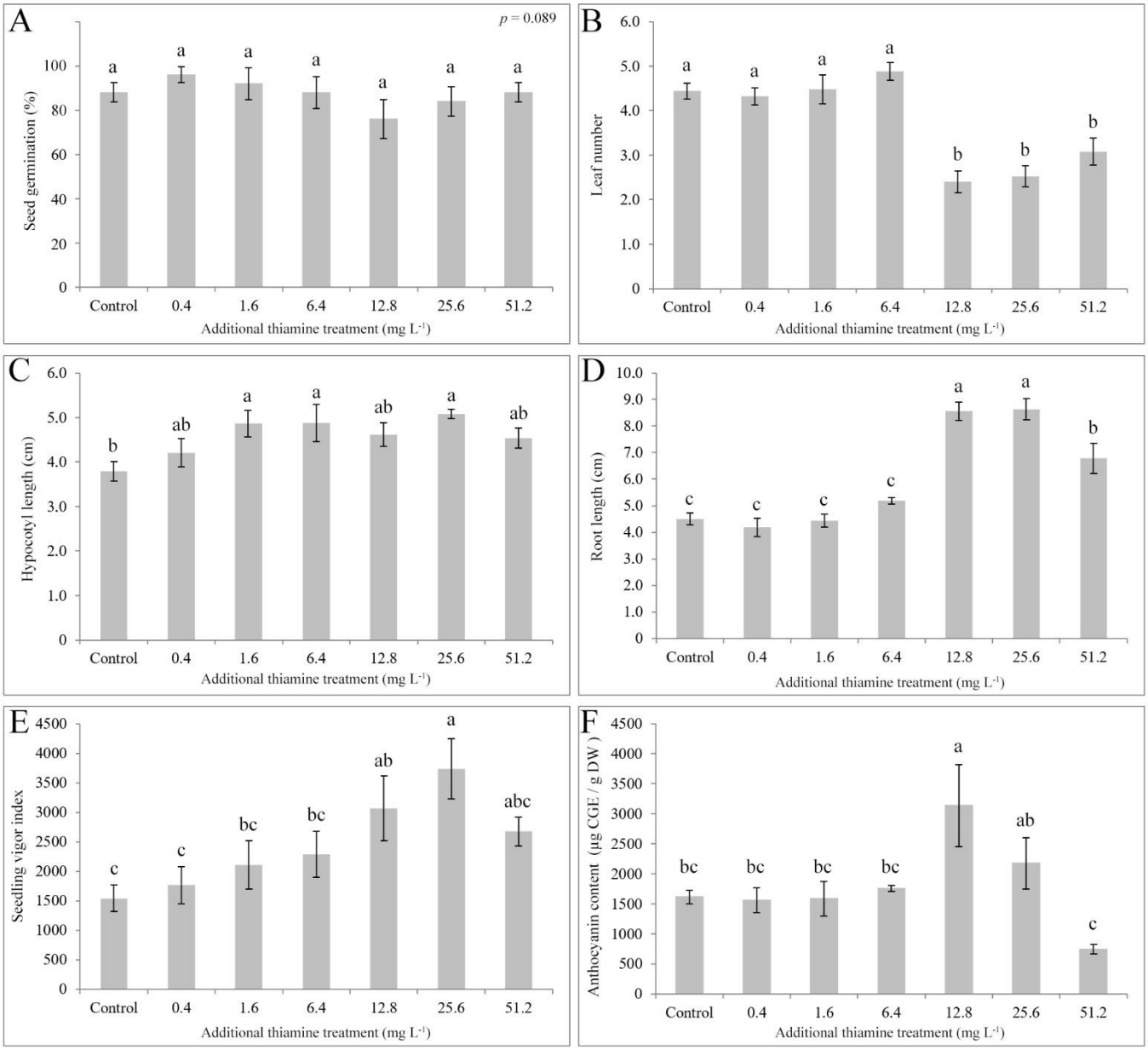


Fig. 1 Effects of thiamine treatments on *in vitro* seed germination (A), leaf production (B), hypocotyl elongation (C), root elongation (D), seedling vigor index (E), and anthocyanin content (F) in *Raphanus sativus* 'Cherry Belle'. Data represent mean ± standard deviation. The means having the same letters are not significantly different by Duncan's MRT ($p < 0.05$).

were found in seedlings grown in the medium with 0.4 mg L^{-1} additional thiamine (Fig. 1D).

The SVI increased to 3737.23 ± 510.44 in seedlings grown in medium with an additional 25.6 mg L^{-1} thiamine. In contrast, the lowest SVI value (1541 ± 224.49) was observed in seedlings grown in the control medium. Media supplemented with 1.6 and 6.4 mg L^{-1} additional thiamine produced SVI results that were statistically similar (Fig. 1E).

3.2 Effects of thiamine on anthocyanin accumulation in hypocotyls

Total monomeric anthocyanin content was stable up to 6.4 mg L^{-1} additional thiamine treatment. However, above that concentration, the content rose to 3136.05 ± 684.52 µg CGE g^{-1} DW in seedlings grown in medium containing 12.8 mg L^{-1} additional thiamine. Above that concentration, the content gradually decreased to the minimum level (743.1 ± 75.99 µg CGE g^{-1} DW) in seedlings grown in the medium containing 51.2 mg L^{-1} additional thiamine. The control group had an anthocyanin content of 1616.45 ± 112.80 µg CGE g^{-1} DW (Fig. 1F). The images from *in vitro* cultures showing the visual differences in anthocyanin accumulation in the hypocotyls of radish plants are presented in Fig. 2, with all photographs captured under identical lighting and distance conditions.

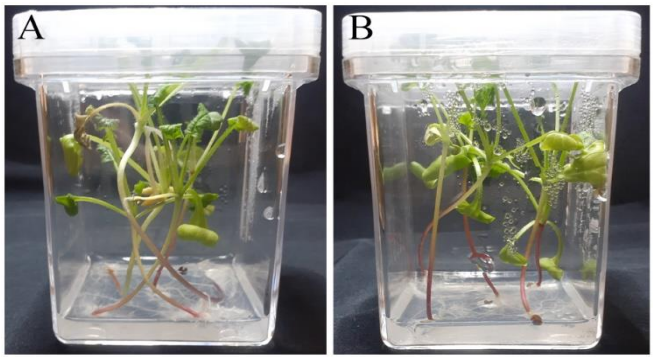


Fig. 2 The general view of *in vitro* cultures showing visual differences in anthocyanin accumulation in the hypocotyls of *Raphanus sativus* 'Cherry Belle' plants from the control (A) and thiamine-treated group at 12.8 mg L^{-1} (B).

3.3 Cluster-based overall comparison of the treatments' effects

The analysis of treatment effects revealed that thiamine treatments at 25.6 and 12.8 mg L^{-1} were grouped within the same main cluster, whereas the 51.2 mg L^{-1} treatment formed a distinct cluster. The control and 0.4 mg L^{-1} treatments were assigned to the same subcluster, while the 1.6 and 6.4 mg L^{-1} treatments were grouped in a separate subcluster within the same main cluster. Germination rate and leaf number were classified within the same main cluster, in contrast to hypocotyl length, which was isolated in its own cluster. The remaining parameters were grouped together in a single subcluster (Fig. 3).

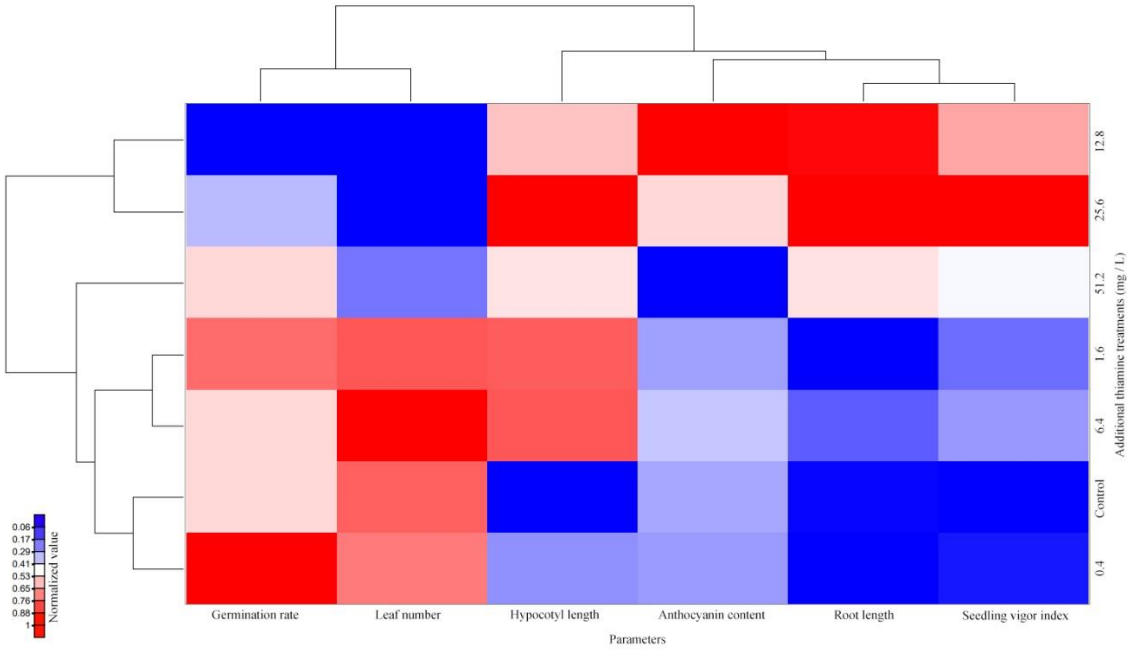


Fig. 3 The heat map representing the similarities among the effects of thiamine treatments based on their impact on the parameters measured in *in vitro* cultures of *Raphanus sativus* 'Cherry Belle'.

4 Discussion

In the current study, the germination rate parameters of *R. sativus* seeds were consistent when treated with different concentrations of thiamine. Seed germination had no statistically significant change from 0.4 to 51.2 mg L^{-1} of thiamine. Although a slight non-important increase in seed germination was recorded at 0.4 mg L^{-1}, the general conclusion is that thiamine has no effect on the pattern of this process and acts on growth after germination. Our result is consistent with previous findings that thiamine's positive effect on germination is more evident under stress conditions (Atif et al. 2022). Ezzat (2021) also observed statistically similar germination rates for *Triticum aestivum* treated with 1, 5, and 10 mg L^{-1} thiamine under both laboratory and field conditions.

The promotive effect of thiamine on hypocotyl elongation in *R. sativus* was found when used at moderate concentrations in our study. Treatments between 1.6 and 25.6 mg L^{-1} produced significantly greater hypocotyl lengths compared to the control, with the most substantial increases occurring at concentrations from 6.4 to 25.6 mg L^{-1}. The promotive effect of thiamine in our study may be attributed to the fact that it is a cofactor in carbohydrate metabolism and ATP production, which provides energy for cell division and elongation, resulting in the early development and elongation of the shoot (Nagae et al. 2016; Al-Sultani and AlFahham, 2021).

Root development was positively affected by thiamine in *R. sativus*. Treatment at the 12.8 mg L^{-1} and 25.6 mg L^{-1} levels resulted in the most important enhancement to root length, which was nearly twice that of the untreated control plants. This result is consistent with observations of other species where thiamine positively affects rooting and root development. For example, in sweet potato, supplementation with thiamine at 0.1 mg L^{-1} increased the root length by 51% *in vitro* (Vollmer et al. 2023). Also, Sepahvand et al. (2012) found that thiamine treatments in combination with indole-3-butyric acid enhanced root production and elongation in a peach × almond hybrid (GF677). Furthermore, thiamine is synthesized in leaves and subsequently translocated to the

roots, where it plays a significant role in development (Nagae et al. 2016; Al-Sultani and AlFahham, 2021). However, at the highest tested concentration (51.2 mg L^{-1}), root length decreased from its highest value, suggesting an optimal threshold. Nevertheless, root length at this concentration remained significantly greater than in the control. The root length-increasing effect of thiamine treatment can be attributed to its role in carbon metabolism and cellular energy production (Luo et al. 2026). This may provide increased precursors and energy for secondary metabolite synthesis. Collectively, these findings highlight the concentration-dependent effects of thiamine on plant morphology (Amorim et al. 2019).

Unlike the effects on root and hypocotyl growth in *R. sativus*, higher thiamine concentrations reduced the number of leaves per plant. Lower to moderate concentrations (0.4-6.4 mg L^{-1}) kept leaf numbers steady or slightly increased them, but concentrations of 12.8 mg L^{-1} and above led to a clear drop in leaf number. This suggests a potential negative effect of excessive thiamine on leaf development. On the other hand, thiamine deficiency can lead to chlorotic leaves (Nagae et al. 2016), which suggests a possible vitamin balance required for healthy foliage. Thiamine is synthesized in young leaves and transported to roots, where it acts as a growth factor. If thiamine synthesis in leaves or its transport to roots decreases during specific developmental stages or environmental conditions, plants may benefit from absorbing thiamine present in the soil (Mozafar and Oertli, 1992). Thus, a decline in leaf production due to exogenous thiamine treatment at supra-optimal levels could limit endogenous thiamine synthesis and its translocation to the roots. However, these should be proven in further laboratory and field-based studies.

Thiamine treatment significantly improved the seedling vigor index, which is a measure of overall seedling strength. This finding is consistent with improvements in hypocotyl and root lengths. Thiamine's role in providing energy for active cell division (Nagae et al. 2016) and its general promotion of growth attributes (Jabeen et al. 2020) contribute to the observed increase in overall seedling vigor. The decline in vigor at 51.2 mg L^{-1} further emphasizes the importance of concentration specificity for maximizing plant benefits. Enhanced seedling vigor following thiamine treatment, which supports plant growth and uniform seedling establishment, also suggests improved adaptation to environmental conditions (Basu and Groot, 2023).

Anthocyanin content in *R. sativus* exhibited a pronounced, concentration-dependent response. At 12.8 mg L^{-1}, a statistically significant increase was observed, nearly doubling the levels found in the control group. Anthocyanins, as secondary metabolites, are primarily responsible for plant coloration and can provide protective effects, including defense against inhibitory light conditions (Mano et al. 2007). This increase at a specific thiamine concentration suggests that thiamine can modulate the accumulation of these compounds. Thiamine has been shown to increase pigment content, including total chlorophyll and anthocyanin levels, in other plants such as mathiola (Al-Sultani and AlFahham, 2021). However, at the highest concentration of 51.2 mg L^{-1}, the anthocyanin content dramatically decreased to below control levels, indicating a strong inhibitory effect at excessive concentrations. This marked reduction suggests that very high thiamine concentrations might disrupt the metabolic pathways responsible for anthocyanin synthesis or induce other stress responses that alter their accumulation. Jiang and Zhou (2019) reported an anthocyanin content of 35.2 ± 1.5 mg CGE g^{-1} in purified red radish samples extracted for 2 hours using acidified methanol. They also found that hydrolysis increases the antioxidant and antiproliferative capacities of radish anthocyanin. In contrast, the present study employed samples harvested from 15-day-old *in vitro*-grown plants, with extraction conducted for 1 hour using a non-acidified solvent. These differences in extraction methodology and the physiological age of the plant material may explain the variation among the results. Consequently, the increase in anthocyanin content in hypocotyls suggests that thiamine indirectly regulates not only primary metabolism but also secondary metabolic pathways. Specifically, thiamine treatment might alter intracellular redox balance, thereby activating gene expression in the phenylpropanoid pathway, in which anthocyanins are synthesized on the cytoplasmic face of the endoplasmic reticulum, and promoting flavonoid biosynthesis (Boubakri et al. 2013; Liu et al. 2021). The findings may benefit the optimization of *in vitro* culture media and the enhancement of secondary metabolite production in radish cultivars using plant tissue culture methods.

5 Conclusion

In conclusion, exogenous thiamine supplementation demonstrates a significant, concentration-dependent influence on the growth and anthocyanin accumulation in *R. sativus*. Thus, precise management of thiamine supplementation would be beneficial for optimizing growth in horticultural plants. Future studies should evaluate whether thiamine-induced anthocyanin accumulation improves stress tolerance. However, the effects of thiamine may differ in soil conditions, which should be studied. Also, further research is needed to elucidate the molecular mechanisms underlying these concentration-dependent effects.

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