# Effects of Exogenous Selenium on Accumulations of Selenium, GABA and Antioxidant Activity of Chestnut During Germination

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*Article*

# Effects of Exogenous Selenium on Accumulations of Selenium, GABA and Antioxidant Activity of Chestnut During Germination

**Haifen Wang [1], Weiwei Liu [2], Fei Peng [1], Ziye Zhang [2,3], Jiawei Cao [2,3], Jiayu Shi [2,3], Liang He [2,3], Yunbin Jiang [2], Mengshi Wang [2] and Junwei Yuan [2,*]**

[1] Research Center of Rural Vitalization, Hebei Normal University of Science and Technology, Qinhuangdao, 066004, Hebei, China

[2] Chestnut Research Center, Hebei Normal University of Science and Technology, Qinhuangdao, 066004, Hebei, China

[3] College of Food Science and Technology, Hebei Normal University of Science and Technology, Qinhuangdao, 066004, Hebei, China

* Correspondence: junweiyuan@126.com

**Abstract**

To investigated the effect of exogenous selenium on selenium enrichment and antioxidant activity of germinated chestnut. We treated 'Zaofeng' chestnuts with 0, 20, 40, 60 and 80 mg/L concentration of $Na_2SeO_3$, and the analyses focused on total Se, $SeCys_2$, MeSecys, $Se^{IV}$, SeMet, $Se^{VI}$, γ -aminobutyric acid (GABA), antioxidant enzyme (PAL, SOD, GPX, CAT) activity, non-enzymatic antioxidant substances (total polyphenols and flavonoids) content and antioxidant capacity (DPPH, ABTS) during germination. The results indicated that low concentration of selenium (20-40 mg/L) significantly promoted the organic transformation of selenium, increased antioxidant enzyme activity and phenol accumulation, and enhanced antioxidant capacity of chestnut. High concentration of selenium (80 mg/L) induced oxidative stress, inhibited enzyme activity and reduces antioxidant capacity of chestnut. During the germination of chestnut, selenite was absorbed by embryo and subsequently transformed into organic Se in vivo, ultimately being stored in the form of $SeCys_2$. The selenium enrichment rate decreased significantly with the increase of $Na_2SeO_3$ treatment concentration. Furthermore, the treatment with 40 mg/L $Na_2SeO_3$ led to a significant increase in GABA content of germinated chestnut. Overall, 20-40 mg/L $Na_2SeO_3$ were identified as the suitable concentration for germinated chestnut. This study provides a theoretical basis for developing functional food of selenium-enriched germinated chestnut.

**Keywords:** chestnut; germination; selenium uptake; γ -aminobutyric acid; antioxidant activity

## 1. Introduction

Selenium (Se) is an essential trace element for human health, playing a vital role in various biological functions such as anti-cancer, anti-oxidation, anti-aging, heart protection, vision protection, immunity improvement, and reproductive capacity enhancement [1,2]. Prolonged Se deficiency can result in sub-health and predispose individuals to a range of associated ailments [3,4]. However, due to the variation in soil Se levels across different regions, the Se content in food sources, particularly organic Se, is generally low in many areas of world [5]. One viable approach for Se supplementation is to enhance the Se concentration in agricultural products through exogenous Se biofortification [6]. The production of Se-enriched agricultural goods relies on plants converting naturally occurring inorganic Se, which can be harmful to humans, into organic Se forms that are readily absorbed by the body [7]. Assessing the conversion efficiency of organic Se in agricultural products and ensuring balanced nutrition are crucial for the safe cultivation of Se-enriched foods.

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Common inorganic forms include $SeO^{2-}_4$ and $SeO^{2-}_3$, while major organic forms include $SeCys_2$, MeSeCys, and SeMet [8,9]. While inorganic Se is abundant and cost-effective, organic Se is primarily derived from the conversion of inorganic forms within organisms [10]. Currently, the biological enrichment of exogenous Se using plant seeds as raw materials is recognized as the convenient, efficient, and safe technology for Se-enriched agricultural production [11–13]. Seed sprouts are known to accumulate Se from various inorganic sources [14,15] and incorporate Se into newly synthesized proteins during sprout growth [16]. Studies have shown that bean sprouts germinated under inorganic Se treatment for 5 to 7 days exhibited significantly higher Se content compared to untreated bean sprouts [10]. Inorganic Se treatment has also been found to enhance Se absorption by garden cress sprouts [17]. During germination, Se accumulates in brown rice, primarily in Se-containing proteins [18]. Studies on various Se-enriched foods, including mushroom [19], ovalbumin [20], and Zea mays [21], have confirmed the potent effects of Se on specific intracellular selenoproteins and crucial antioxidant activities. Furthermore, the germination of seeds has been shown to increase the content and activity of bioactive compounds including polyphenols, flavonoids, vitamins, and free amino acids, particularly γ-aminobutyric acid (GABA) [22]. GABA, an essential inhibitory neurotransmitter in the central nervous system, plays a key role in enhancing sleep quality and reducing blood pressure [23]. With favorable water solubility, thermal stability, and safety for consumption, GABA finds wide application in food production [24].

In recent years, researchers have focused on optimizing the technological parameters for Se and GABA enrichment during crop germination [25]. In our previous study, the GABA content and antioxidant capacity of chestnut were significantly increased by the germination process [26]. However, little research related to the Se-biofortification of chestnut during germination has been reported, and the effects of Se-biofortification on the secondary metabolites of germinated chestnut remain unclear.

In this study, Se-enriched chestnut was cultivated under varying concentrations of $Na_2SeO_3$ solution to evaluate the accumulation of selenium in different parts of germinated chestnut, and the changes of antioxidant enzyme activity system. Meanwhile, the GABA, total polyphenol, flavonoid content and antioxidant capacity were assessed, aiming to clarify the optimal concentration range of selenium treatment and offer theoretical basis and technical parameters for the development of functional food of selenium-enriched germinated chestnut.

## 2. Results and Discussion

### *2.1. Effects of Exogenous $Na_2SeO_3$ on Total Se Content in Germinated Chestnut*

The total selenium content of germinated chestnut treated with different concentrations of selenium is shown in Figure 1. With the increase of exogenous $Na_2SeO_3$ concentration and the prolongation of germination time, the total selenium content in germinated chestnut increased significantly ($P < 0.05$). In the control, the total Se content in chestnut remained within the range of 0.056 to 0.058 mg/kg throughout the germination process, with no significant differences observed ($P > 0.05$). The selenium of chestnut under four concentrations of exogenous $Na_2SeO_3$ did not increase significantly in the first 2 days ($P > 0.05$). This may be because the sodium selenite on the surface of chestnut was not absorbed into chestnut tissue 2 d after sowing, and was washed away during germination to prepare samples. Under 80 and 60 mg/L $Na_2SeO_3$ treatment, the total Se content in chestnut significantly increased ($P < 0.05$) from the 4th day, reached 1.049 and 0.889 mg/kg by the 8th day post-sowing respectively. Under 40 and 20 mg/L $Na_2SeO_3$ treatment, the total Se content in chestnut significantly increased ($P < 0.05$) from the 6th day. These results indicated that germination in the presence of a $Na_2SeO_3$ solution is an efficient method for Se biofortification of germinated chestnut.

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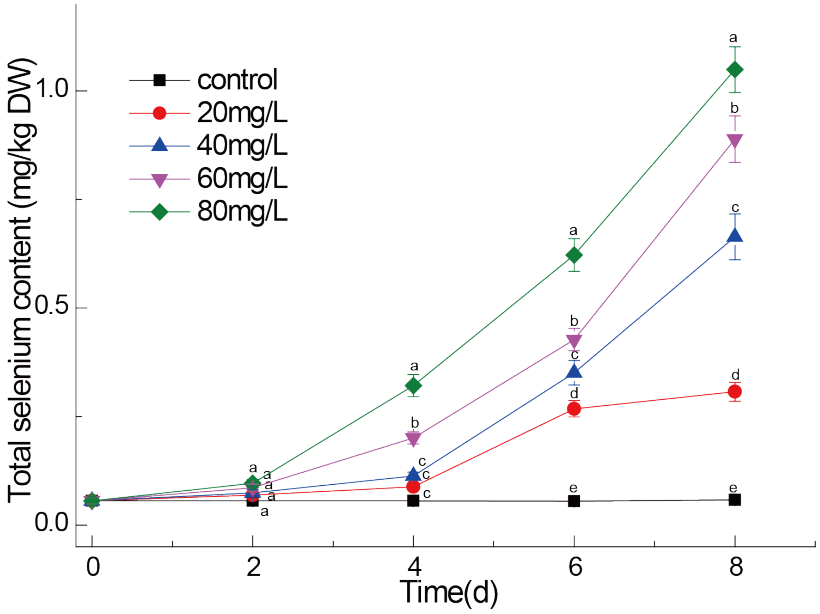


**Figure 1.** Dynamic changes of total selenium content in germinated chestnut under different concentrations of $Na_2SeO_3$ treatments. Note: Values in the figure were shown as the means ± standard error (n = 3). Vertical bars represent the standard errors of the means. Different letters represent significant differences under different $Na_2SeO_3$ treatments, *p*-value < 0.05.

In this study, the control chestnuts sourced from the primary chestnut-producing region in Qianxi county, Tangshan city, Hebei province, China exhibited a total Se content ranging from 0.056 to 0.058 mg/kg, significantly below the recommended standard for Se-enriched food by China Nutrition Society (0.1 to 1.0 mg/kg) [27]. Different plant species exhibit varying capacities for Se uptake and accumulation. For instance, rye grains treated with selenite accumulated Se up to 55 μg/g during germination [28], while alfalfa germination enriched Se concentrations up to 150 μg/g of dw [29]. The Se content accumulated by garden cress germinated with 8 mg/L selenite solution (39 μg/g) surpasses that of lupine bean sprouts grown under the same conditions (11 μg/g dm) [17]. Therefore, the appropriate Se treatment and germination duration are pivotal factors in producing high-quality Se-enriched sprouts. Germinated chestnut treated with 20 and 40 mg/L for 6-8 days, 60 mg/L for 4-8 days, 80 mg/L $Na_2SeO_3$ for 4-6 days all could serve as viable sources for Se-enriched foods, meeting the regulatory standards for Se enrichment in foods and agricultural products [6]. Se accumulation during different germination stages involves distinct processes. In the initial stage of germination (2-4 d after sowing), seeds absorb selenite and water from the environment, leading to swelling and restoration of metabolic activity [30]. During this period, selenite enters the seeds and cells through the aleurone layer with the absorbed water [31]. The total Se content in the germ experiences a rapid increase during the middle-late stage (4-8 d after sowing) of germination, a pattern similar to observations in mung beans germinated via $Na_2SeO_3$ treatment [10].

### *2.2. Effects of Exogenous $Na_2SeO_3$ on Se Speciation and Content in Chestnut*

Due to the low bioavailability and high toxicity of inorganic Se [32], efforts should be directed towards enhancing the organic speciation of Se [33]. Considering nutritional value and safety aspects, the organic Se content holds greater significance, underscoring the importance of understanding Se form and transformation during plant germination. The $Na_2SeO_3$ treatment resulted in a significant ($P < 0.05$) increase in $SeCys_2$, MeSecys, SeMet, $Se^{IV}$ and $Se^{VI}$ content of germinated chestnut, while Se-enrichment rate decreased gradually (Figure 2). On 8th days after sowing, the $SeCys_2$ content ranged from 0.056 to 0.374 mg/kg, the MeSeCys content ranged from 0 to 0.164 mg/kg, the SeMet content ranged from 0 to 0.115 mg/kg, and the Se-enrichment rate reduced from 86.42% to 62.24%. After

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treatment with sodium selenite, selenium in germinated chestnut mainly exists in organic form, which indicates that germinated chestnut can effectively transform and accumulate organic selenium. Similar results also appeared in the experiment of mung bean germination treated by $Na_2SeO_3$ soaking [34]. Plants primarily convert inorganic Se into selenomethionine and incorporate it into protein [35]. Se-Met is the main form of organic selenium in cereals and leguminous plants, while the main selenium compound in selenium-enriched plants such as garlic, onion and wild leek is SeMeCys [36]. The main forms of organic selenium in different parts of some selenium-enriched plants are also significant different. For example, in selenium-enriched broccoli, the major form of selenium in roots is Se-Met, while the main form of selenium in fruits is SeMeCys [37]. In this study, $SeCys_2$ represents the most prevalent and stable form of Se in chestnuts in their natural state.

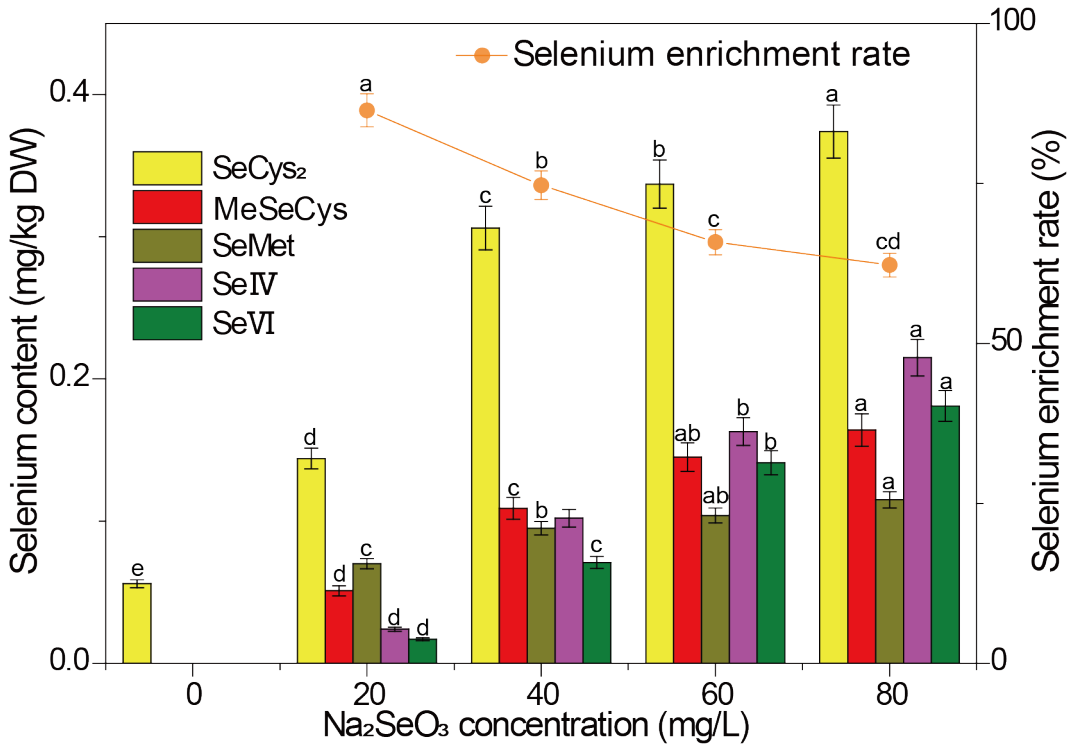


**Figure 2.** The effects of different concentrations of $Na_2SeO_3$ treatments on $SeCys_2$, MeSeCys, SeMet, $Se^{IV}$, $Se^{VI}$ and the selenium enrichment rate in germinated chestnut. Note: Values in the figure were shown as the means ± standard error (n = 3). Vertical bars represent the standard errors of the means. Different letters represent significant differences under different $Na_2SeO_3$ treatments, $p$-value < 0.05.

After being absorbed by plants, inorganic selenium will be mixed with Cys and Met to form Se-Cys and Se-Met respectively, and then further integrated into protein to form selenoprotein [17]. When cruciferous plants are cultivated in Se-rich soil, they convert less than 40% of the total Se content into selenoamino acids [38]. In contrast, when grown in Se-rich conditions, primary species can incorporate over 95% of the total Se content as selenoamino acids [39,40]. In this study, the Se-enrichment rate decreased gradually with the increase in $Na_2SeO_3$ concentration in this study. This may be because when the concentration of $Na_2SeO_3$ reaches a certain threshold, the ability of Se assimilation and transformation into organic selenium decreases. This is basically consistent with the research results of selenium-enriched soybean sprouts [41].

### *2.3. Effects of Exogenous $Na_2SeO_3$ on Phenylalanine Ammonialyase (PAL), Glutathione Peroxidase (GPX), Superoxide Dismutase (SOD) and Catalase (CAT) Activity of Germinated Chestnut*

PAL is a key enzyme in phenylpropane metabolic pathway [42]. As shown in Figure 3A, PAL activity first increased (0-4 d after sowing) and then stabilized (4-8 d after sowing) during

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germination. Low concentrations of $Na_2SeO_3$ (20 and 40 mg/L) significantly enhanced PAL activity during the whole germination period ($P < 0.05$). It is worth noting that the PAL activity reached the highest at 40 mg/L, reaching 26.11 U/g on the 8th day after sowing, which was 25.2% higher than that of the control group. This finding is consistent with previous research reports, indicating that sodium selenite can enhance PAL activity in many plant systems. For example, the PAL activity of chicory was increased by 36.4% by the combined treatment of NO and nano-Se, while the PAL activity of bitter gourd seedlings was increased by 39% by nano-Se [43]. In contrast, high concentration of $Na_2SeO_3$ (80 mg/L) inhibited PAL activity. On 8th day after sowing, it decreased to 17.92 U/g, which was 14.1% lower than that of the control group. This inhibition may be attributed to the excessive accumulation of reactive oxygen species exceeding the antioxidant capacity of cells, leading to oxidative damage of enzyme proteins and destruction of redox homeostasis of cells [44]. There was no significant difference in PAL activity between 60 mg/L treatment and control group ($P > 0.05$).

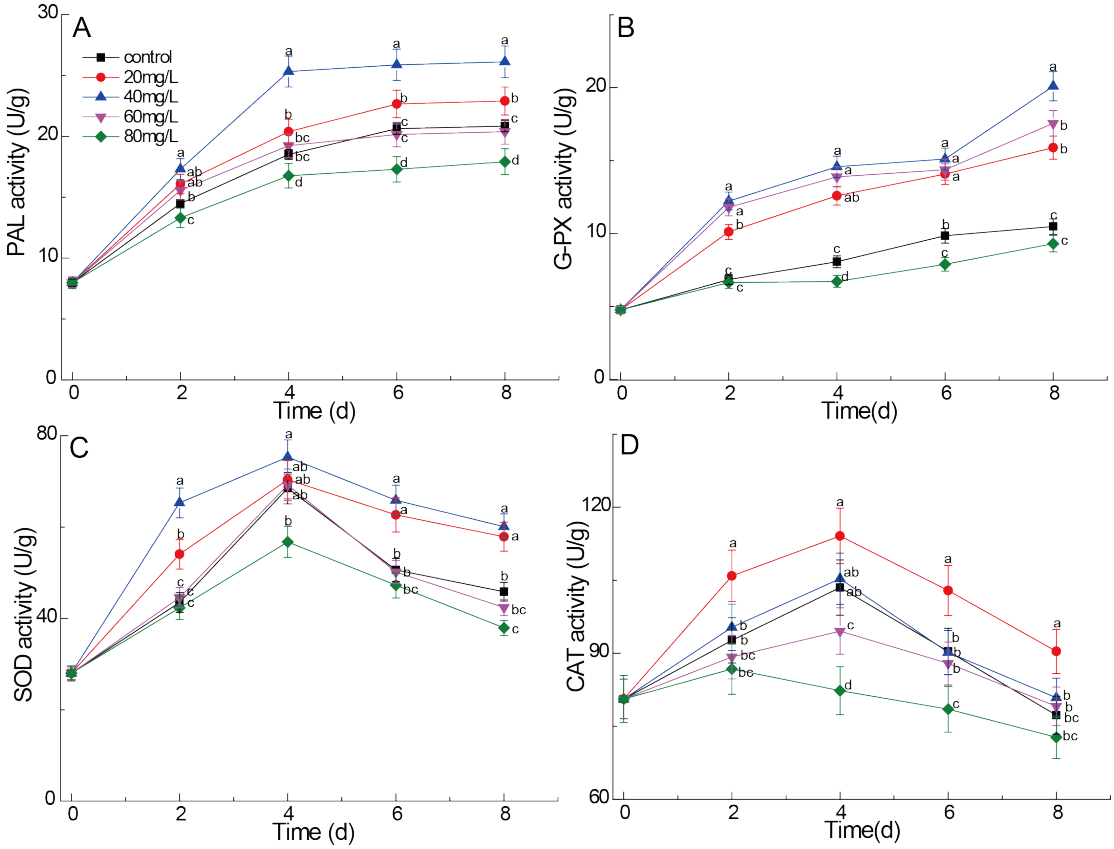


**Figure 3.** Dynamic changes of PAL (A), SOD (B), GSH-Px (C) and CAT (D) of germinated chestnut under different concentrations of $Na_2SeO_3$ treatments. Note: Values in the figures were shown as the means ± standard error (n = 3). Vertical bars represent the standard errors of the means. Different letters represent significant differences under different $Na_2SeO_3$ treatments, *p*-value < 0.05.

In all selenium treatment groups, GPX activity increased continuously during the whole germination period, and reached the maximum on the 8th day (Figure 3B). GPX activity in the control group increased moderately from 4.77 U/g on day 0 to 10.48 U/g on day 8. GPX, as a selenase containing selenium in the form of selenocysteine in its active center, is the most sensitive to selenium treatment among all the detected enzymes. The activity of GPX increased significantly under the treatment of 40 mg/L selenium (1.92 times that of the control group), which highlighted the efficiency of selenium bioaugmentation in germinated chestnut. 20 and 60 mg/L treatments also significantly increased GPX activity, reaching 15.90 and 17.56 U/g respectively. This result confirmed earlier studies, and showed that trace selenium could enhance GPX activity in germinated seeds and seedlings of wheat, rice and various vegetables [45]. However, 80 mg/L treatment reduced GPX

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activity to 9.31 U/g on the 8th day, which was only slightly lower than that of the control group ($P > 0.05$), indicating that it was inhibited under excessive selenium concentration. This shows that excessive selenium interferes with the synthesis or stability of selenase [46]. This inhibition may be due to selenium-induced oxidative stress interfering with protein biosynthesis.

SOD serves as the first line of defense against superoxide anions, catalyzing the dismutation of $O_2^{-\cdot}$ into $H_2O_2$ and $O_2$ [48]. Unlike PAL and GPX, SOD activity rapidly increased from day 0 to day 4 across all groups, peaked on day 4, and then gradually declined by day 8 (Figure 3C), consistent with the typical unimodal curve of SOD during seed germination. At the peak (day 4), SOD activity in the control group was 68.51 U/g. Treatment with 40 mg/L Se significantly increased SOD activity to 75.32 U/g, which was 9.9% higher than the control. The 40 mg/L treatment maintained the highest SOD activity throughout germination. In contrast, the 80 mg/L treatment resulted in SOD activity of 56.76 U/g on day 4, 17.1% lower than the control. The promoting effect of low Se concentration (20-40 mg/L) on SOD activity is consistent with previous findings in alfalfa seeds [29]. The mechanistic basis of this activation involves Se-induced mild oxidative stress, which upregulates the expression of antioxidant enzyme-encoding genes, including SOD, via ROS-mediated signaling pathways involving transcription factors such as WRKY and DREB [49].

With the prolongation of germination time, CAT activity in all treatment groups showed a trend of first increasing and then decreasing, peaking on day 4 (Figure 3D). The effect of different selenium concentrations on CAT activity was concentration-dependent: low-concentration (20 mg/L) treatment significantly increased CAT activity at all time points ($P < 0.05$), reaching a maximum of 114.09 on day 4, which was 10.2% higher than the control. The 40 mg/L $Na_2SeO_3$ treatment resulted in slightly higher CAT activity than CK, but the difference was not significant ($P > 0.05$). 60 and 80 mg/L $Na_2SeO_3$ treatments significantly inhibited CAT activity, with the 80 mg/L $Na_2SeO_3$ showing a 20.5% reduction compared to the control on day 4, and remaining at the lowest level throughout germination. Low-concentration Se significantly increased CAT activity during chestnut germination, consistent with the antioxidant effects of selenium in other plants [50,51]. When Se concentration reached 60 mg/L or higher, CAT activity was markedly inhibited. High Se concentration causes excessive selenium accumulation in plants, inducing bursts of reactive oxygen species (ROS) such as superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$). Additionally, selenium binds to sulfhydryl (-SH) groups, disrupting the structure of enzyme proteins and inactivating the CAT active center [52]. Of note, although CAT activity was inhibited under high Se treatment, a small increase followed by a rapid decline was still observed in the early germination stage (0-2 d after sowing), indicating that seeds can still initiate a limited antioxidant response at the beginning of germination, but this response is quickly suppressed as selenium toxicity intensifies.

In a word, $Na_2SeO_3$ synergistically regulates the antioxidant enzyme network in germinating chestnuts. At 20-40 mg/L, selenium enhances the activities of PAL, SOD, GPX, and CAT, thereby strengthening $H_2O_2$ scavenging and phenolic biosynthesis pathways, whereas a high concentration (80 mg/L) causes irreversible oxidative damage.

### *2.4. Effects of Exogenous $Na_2SeO_3$ on GABA Content in Chestnut*

The changes in GABA content during chestnut germination under different $Na_2SeO_3$ treatments are shown in Figure 4. In all treatment groups, GABA content gradually increased from day 0 to day 6, reflecting the natural activation of GABA biosynthesis during germination, peaked at day 6, and then decreased from day 6 to day 8. The 40 mg/L $Na_2SeO_3$ treatment consistently maintained the highest GABA content throughout germination, reaching 164.87 mg/g DW at day 6, which was 26.2% higher than the control ($P < 0.05$). In contrast, the 80 mg/L treatment resulted in GABA levels 20.9% lower than the control ($P < 0.05$), indicating that the inhibitory effect of high Se concentration becomes more pronounced during the late germination stage when GAD activity is naturally elevated. Low Se concentrations (20 and 40 mg/L) significantly promoted GABA accumulation throughout the germination period, whereas the high Se concentration (80 mg/L) inhibited GABA accumulation.

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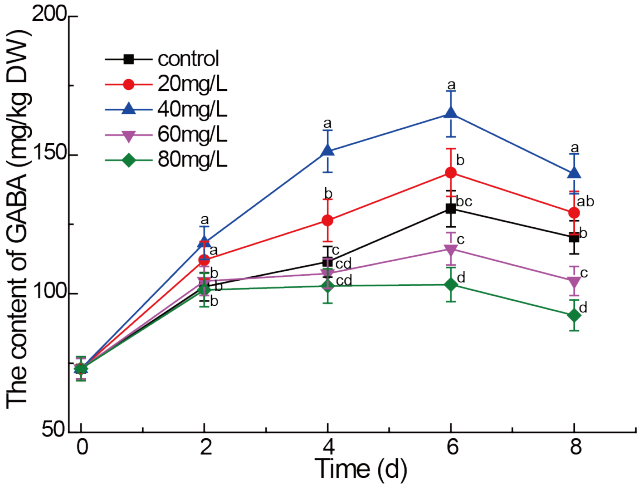


**Figure 4.** Comparison of γ - aminobutyric acid (GABA) content in chestnut under different concentrations of $Na_2SeO_3$ treatments. Note: Values in the figure were shown as the means ± standard error (n = 3). Vertical bars represent the standard errors of the means. Different letters represent significant differences under different $Na_2SeO_3$ treatments, *p*-value < 0.05.

The stimulatory effect at low Se concentrations (20-40 mg/L) can be attributed to the induction of mild oxidative stress. Sub-toxic levels of selenium generate moderate amounts of reactive oxygen species (ROS), which act as signaling molecules to activate stress-responsive transcription factors and upregulate the expression of GAD and other genes involved in GABA metabolism [53]. This interpretation is consistent with the recognition of GABA as a novel protective agent capable of mitigating oxidative stress [54]. In study of cut lily flowers, exogenous GABA treatment elevated antioxidant enzyme activities, prevented hydrogen peroxide accumulation, and maintained higher levels of total phenols and soluble proteins, thereby delaying senescence [55]. Conversely, the increase in endogenous GABA accumulation under mild selenium-induced stress may similarly contribute to the antioxidant defense system of germinating chestnuts. At high Se concentration (80 mg/L), the capacity of the cellular antioxidant system is overwhelmed, leading to excessive ROS accumulation, which causes oxidative damage to the GAD enzyme protein, disruption of cellular redox homeostasis, and inhibition of GABA biosynthesis [56]. The reduction in GABA content under the 80 mg/L Se treatment (20.9% lower than the control) is largely consistent with results observed in highland barley seeds [57].

The optimal Se concentration for GABA enhancement identified in this study (40 mg/L $Na_2SeO_3$) differs from the concentrations reported for other crop species. In foxtail millet, the appropriate concentrations were 60 mg/L for soaking and 2 mg/L for spraying [25]. These differences may reflect species-specific variations in Se sensitivity, as well as differences in experimental conditions including germination duration, temperature, seed size, and Se application method. Chestnut, as a large-seeded species with substantial nutrient reserves, may exhibit distinct patterns of GABA accumulation and Se responsiveness compared to small-seeded cereals or legumes. The observation that GABA is the major amino compound during chestnut germination and early seedling growth highlights the particular importance of this metabolite in chestnut's nitrogen metabolism and stress adaptation.

### *2.5. Effects of Exogenous $Na_2SeO_3$ on Total Polyphenols and Flavonoids Content in Chestnut*

Under all treatments, the total polyphenols content (TPC) in germinating chestnuts gradually increased throughout the 8-day germination period (Figure 5A), reflecting the natural activation of phenylpropanoid metabolism during seed germination. On day 8, the TPC in the control group (0 mg/L) was 28.92 mg GAE/g DW. Low selenium concentrations (20 and 40 mg/L) significantly increased TPC, with the highest value (33.11 mg GAE/g DW) observed at 40 mg/L, which was 14.5%

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higher than the control. The TPC at 20 mg/L (31.42 mg GAE/g DW) was also slightly higher than the control, but the difference was not significant ($P > 0.05$). These findings are generally consistent with observations in other germinated seeds [58]. In contrast, the 80 mg/L $Na_2SeO_3$ treatment reduced TPC to 25.72 mg GAE/g DW, which was 11.1% lower than the control, indicating that phenolic biosynthesis is inhibited under excessive selenium stress.

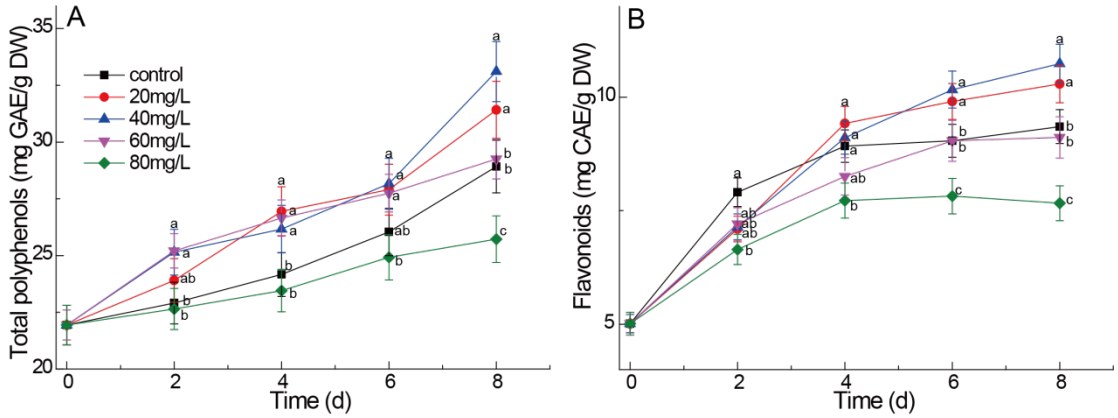


**Figure 5.** Dynamic changes of total polyphenols (A) and flavonoids (B) content of germinated chestnut under different concentrations of $Na_2SeO_3$ treatments. Note: Values in the figures were shown as the means ± standard error (n = 3). Vertical bars represent the standard errors of the means. Different letters represent significant differences under different $Na_2SeO_3$ treatments, *p*-value < 0.05.

Across all treatments, total flavonoids content (TFC) in germinating chestnuts accumulated rapidly during the first 4 days, and the accumulation rate slowed down during the later period (4-8 days) (Figure 5B). Low selenium concentrations (20 and 40 mg/L) showed no significant difference in TFC from the control during the first 4 days ($P > 0.05$), but TFC was significantly higher than the control from day 6 to day 8 ($P < 0.05$). These findings are generally consistent with increased flavonoid accumulation observed in other selenium-biofortified germinated seeds [59,60]. In contrast, the 80 mg/L treatment reduced TFC to 7.66 mg CAE/g DW, which was 18.1% lower than the control.

Low concentrations of $Na_2SeO_3$ (20-40 mg/L) significantly promoted the accumulation of total polyphenols and flavonoids in germinated chestnuts, indicating that appropriate selenium biofortification during seed germination can effectively enhance the accumulation of phenolic compounds in various crops. For example, in black soybeans [58], selenium biofortification significantly increased total phenolic and total flavonoid contents in germinated seeds. Similarly, selenium-enriched germination significantly increased total phenolic and total flavonol contents in hulless barley seeds [57] and soybeans [41]. It is speculated that the underlying mechanism is the activation of the phenylpropanoid pathway in germinating chestnuts, which is generally consistent with the changes in PAL activity described above (Figure 3A).

### *2.6. Effects of Exogenous $Na_2SeO_3$ on Antioxidant Capacity of Chestnut*

DPPH radical scavenging activity, a widely used indicator of antioxidant capacity based on hydrogen atom transfer, showed a gradual increase throughout the germination period across all treatments (Figure 6A). This reflected the natural accumulation of phenolic compounds and other antioxidants during seed germination, consistent with the enhanced PAL activity and increased phenolic content observed in this study. On day 8, the DPPH scavenging activity in the control (0 mg/L) was 0.353 mg TE/g DW. Low selenium concentrations (20 and 40 mg/L) significantly increased DPPH scavenging activity, with the highest value (0.396 mg TE/g DW) observed at 20 mg/L, which was 12.2% higher than the control. These results are generally consistent with findings in foxtail millet sprouts [25]. In contrast, the effect of high selenium concentration on DPPH scavenging activity gradually weakened. The DPPH scavenging activity at 80 mg/L on day 8 was 0.286 mg TE/g DW,

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which was 19.0% higher than the control, indicating that hydrogen atom transfer-based antioxidant capacity is inhibited under excessive selenium stress.

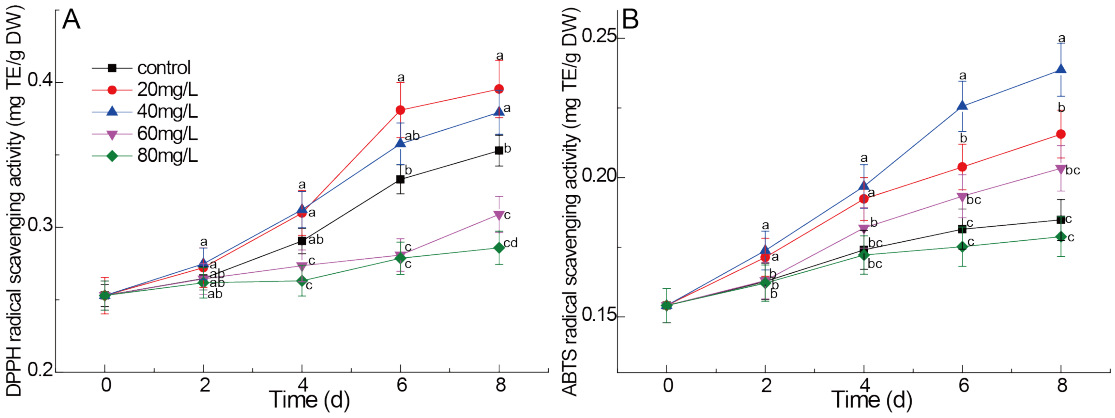


**Figure 6.** Dynamic changes of DPPH (A) and ABTS (B) radical scavenging activity of germinated chestnut under different concentrations of $Na_2SeO_3$ treatments. Note: Values in the figures were shown as the means ± standard error (n = 3). Vertical bars represent the standard errors of the means. Different letters represent significant differences under different $Na_2SeO_3$ treatments, *p*-value < 0.05.

ABTS radical scavenging activity reflects antioxidant capacity based on electron transfer. Similar to DPPH scavenging activity, ABTS radical scavenging activity increased steadily throughout the germination period, reaching a maximum on day 8 across all treatments (Figure 6B). The ABTS scavenging activity in the control group on day 8 was 0.185 mg TE/g DW. Low selenium concentrations significantly increased ABTS scavenging activity, with the highest value (0.239 mg TE/g DW) observed at 40 mg/L, which was 29.2% higher than the control. The 20 mg/L treatment also significantly increased ABTS scavenging activity to 0.216 mg TE/g DW (16.5% higher than the control). These findings are consistent with increased ABTS radical scavenging activity observed in germinated seeds of mung bean [61]. The ABTS scavenging activity of germinated chestnuts under 80 mg/L treatment was 0.179 mg TE/g DW (slightly lower than the control), but the difference was not significant ($P > 0.05$).

Notably, ABTS scavenging activity was consistently higher than DPPH scavenging activity across all treatments, reflecting the differential sensitivity of these two radicals to antioxidant compounds in germinating chestnuts. This study demonstrates that low concentrations of $Na_2SeO_3$ (20-40 mg/L) significantly enhance both DPPH and ABTS radical scavenging activities in germinating chestnuts, indicating that selenium biofortification during seed germination can effectively improve the total antioxidant capacity of various crops. In foxtail millet sprouts, soaking with 60 mg/L $Na_2SeO_3$ combined with spraying 2 mg/L $Na_2SeO_3$ significantly increased free and bound phenolic contents, thereby enhancing in vitro antioxidant activity [25]. Similarly, mung bean seeds and sprouts treated with 30 mg/L $Na_2SeO_3$ exhibited significantly elevated ABTS and DPPH radical scavenging activities, accompanied by specific enrichment of benzoic acid, rutin, and luteolin glycoside [62]. Wheat sprouts also showed increased ABTS, DPPH, and SOD activities under moderate selenium biofortification (0.25-0.50 mg/L) [63]. Peanut seeds soaked with 7.5 μmol/L $Na_2SeO_3$ exhibited significantly improved antioxidant capacity and sprout quality [64]. Furthermore, chickpea sprouts treated with 2 mg/100g seeds $Na_2SeO_3$ showed a significant 33% increase in antioxidant capacity [65].

## 3. Materials and methods

### *3.1. Plant Materials and Treatments*

The seeds of chestnut (*Castanea mollissima*) variety "Zaofeng" were harvested from healthy trees in the primary chestnut production region of Qinglong County, Qinhuangdao City, Hebei Province, China. The seeds were stored at -3 °C for preservation. Following selection of uniform seeds, they

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were washed with sterile water, soaked for 12 h, and then evenly distributed on trays (50 cm × 30 cm × 15 cm) filled with sterilized sand mixed with sterile water (control) or varying concentrations (20, 40, 60, and 80 mg/L) of $Na_2SeO_3$ (sourced from MedChem Express, Taian Havay Chemicals CO., LTD., Shandong, China). The trays were maintained at 22 °C and 85% relative humidity for 8 days [26]. Ten chestnut seeds per replicate were collected at 0, 2, 4, 6 and 8 days post-sowing, with ground into powder after seed coat removal and stored at -40 °C for subsequent physiological and biochemical analysis. Each treatment was replicated three times, with each replicate consisting of 30 seeds.

### *3.2. Determination of Se Content*

#### 3.2.1. Total Se Content

The total selenium content was determined using fluorescence spectrophotometry [66]. The procedure was as follows: 0.5 g of dried chestnut powder was digested overnight by heating with 10 mL of a mixed solution of $HNO_3$ and $HClO_4$ (9:1, v/v). Subsequently, 5 mL of HCl (6 mol/L) was added to the digest. The heat-digested sample was then reacted with 2,3-diaminonaphthalene, and after extraction with cyclohexane, the fluorescence intensity was measured. A calibration curve was prepared using a selenium standard solution, and the total selenium content was calculated and expressed as mg/kg DW.

#### 3.2.2. Five Se Speciations Content

0.3 g of the dried chestnut sample was taken and placed in a digestion vessel, followed by the addition of 5 mL of $HNO_3$ and 1 mL of $H_2O_2$ for microwave-assisted digestion. Upon completion, the solution was cooled, the digestion vessel was opened, and the sample solution was transferred to a 10 mL volumetric flask. The vessel was rinsed three times with ultrapure water, the wash solutions were combined, and the final volume was adjusted with ultrapure water. Subsequently, 0.5 g digestive solution was transferred to a 20 mL centrifuge tube and supplemented with 60 mg of protease XIV, 30 mg of lipase, and 10 mL of Tris-HCl. The mixture was thoroughly mixed, subjected to ultrasonicate for 30 min, placed horizontally in a constant temperature water bath oscillator at 37 °C and 12000 r/min. The remaining residue was added to 5 mL of hydrolysis solution containing 100 mg of catabolic enzyme, and the above steps were repeated. The two supernatants were combined and filtered through a 0.22 μm water system filter membrane [67]. A standard curve was constructed using a mixed standard series solution with Se concentrations plotted against the chromatographic peak area. The speciation content of Se in the sample was determined using the provided formula (1):

$$X=\frac{(C-C_0)\times V}{m}\times 1000 \quad (1)$$

Where: X——Speciation content of Se in the sample (mg/kg); C——Determination of concentration of sample extract (μg/L); $C_0$——Concentration of sample blank determination (μg/L); V——Total volume of sample extract at constant volume (L); m——Weight of sample (g).

#### 3.2.3. Calculation of Selenium Enrichment Rate

The enrichment rate of selenium were calculated by Equations(2) and (3), respectively [68].

$$O_{se}=C_{SeCys2}+C_{MeSeCys}+C_{SeMet} \quad (2)$$

$$R_{se}\ (\%)= O_{se}/T_{se} \times 100 \quad (3)$$

where $C_{SeCys2}$ represents the $SeCys_2$ content of germinated chestnut, $C_{MeSeCys}$ represents the MeSeCys content of germinated chestnut, $C_{SeMet}$ represents the SeMet content of germinated chestnut, $T_{se}$ represents the total selenium content of germinated chestnut, $O_{se}$ represents the organic selenium content, and $R_{se}$ represents the selenium enrichment rate.

### *3.3. Determination of PAL, GPX, SOD and CAT Activity*

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The extraction and activity assays of PAL, GPX, SOD, and CAT were performed following Du et al. (2024) [69]. Fresh chestnut samples (3 g) were homogenized with 6 mL of ice-cold PBS (50 mmol/L, pH 7.8) containing 1 mmol/L EDTA, 2% (m/v) PVP-K30, and 0.3% Triton X-100. After grinding for 60 s, the mixture was centrifuged at 10,000 × g for 5 min at 4 ° C. The resulting supernatant served as the crude enzyme extract for all assays, with activity determination based on Zhang et al. (2021). PAL assay: The reaction mixture contained 1 mL of 0.02 M L-phenylalanine, 2 mL of 0.1 M sodium borate buffer (pH 8.8), and 0.1 mL of enzyme extract. After 30 min, absorbance was measured at 290 nm. One unit (U) is defined as an absorbance increase of 0.01 per 30 min under assay conditions. SOD assay: The reaction system comprised 1.7 mL PBS (50 mM, pH 7.8), 0.3 mL methionine (130 mM), 0.3 mL NBT (750 μM), 0.3 mL EDTA-$Na_2$ (100 μM), 0.1 mL enzyme extract, and 0.3 mL riboflavin (20 μM). After 15 min under fluorescent light, absorbance of reduced NBT was read at 560 nm. One unit of SOD is defined as 50% inhibition of NBT reduction. GPX assay: The mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM GSH, and 1 mM sodium azide. After pre-incubation at 37 °C for 5 min, absorbance was measured at 412 nm. One unit is defined as an absorbance change of 0.005 per min at 412 nm. CAT assay: Activity was determined by monitoring the decrease in absorbance at 240 nm in a mixture of 0.1 mL enzyme extract and 2.9 mL PBS (50 mM, pH 7.0) containing 20 mM $H_2O_2$. One unit is defined as an absorbance decrease of 0.01 per min at 240 nm. All absorbance values were measured by a UV-Vis spectrophotometer (UT-1901; METASH, Shanghai, China), and all enzyme activities are expressed as U/g.

### *3.4. Determination of GABA Content*

The procedures were conducted following the methodology outlined by Jannoey, P., et al. [70] with slight modification. Briefly, 0.2 g of dried chestnut sample flour was extracted using 1 mL of 0.1 mol/L hydrochloric acid solution at 70 °C for 1 h, followed by centrifugation at 12000 rpm for 10 min. Subsequently, Transfer 10 μL of the supernatant to a derivatization tube, followed by the addition of 70 μL of accqtag ultraborate buffer and 20 μL of accqtag reagent. After vortexing, the mixture is heated at 55 °C for 10 minutes and then cooled in preparation for measurement. The flow rate of HPLC system was 0.5 mL/min, the column temperature was 55 °C and the sample volume was 1 μL. GABA quantification was calculated using the GABA standard curve, and the results were expressed as mg/kg DW.

### *3.5. Determination of Total Polyphenols and Flavonoids Content*

The extraction process for total polyphenols content (TPC) and total flavonoid content (TFC) was similar to that of the GABA. The Folin–Ciocalteu spectrophotometric method was used to measure total phenolic content [71]. In brief, 500 μL of each sample was placed into a 10 mL centrifuge tube, mixed with 3.00 mL of distilled water and 250 μL of Folin-Ciocalteu reagent, and allowed to react for 5 minutes. Then, 750 μL of 7% (w/v) $Na_2CO_3$ was added. After incubating at 20 °C for 1 h, the absorbance was recorded at 765 nm using a UV-Vis spectrophotometer (UT-1901; METASH, Shanghai, China). Total phenolic content was quantified by reference to a calibration curve prepared with gallic acid (GAE) solution. The results are expressed as mg GAE/g DW. The total flavonoid content were determined by method the of Ren et al. (2024) [72]. In brief, 250 μL of sample extract was mixed with 1250 μL of distilled water, followed by addition of 75 μL of 5% $NaNO_2$ solution. After 5 minutes of incubation, 150 μL of 10% $AlCl_3 \cdot 6H_2O$ was added. Then, 500 μL of 1 mol/L NaOH and 275 μL of distilled water were introduced. The absorbance of the mixture was measured at 510 nm using a UV-Vis spectrophotometer (UT-1901; METASH, Shanghai, China). Total flavonoid content (TFC) was calculated using a calibration curve prepared from a catechin (CAE) standard solution. The results were expressed as mg CAE/g DW.

### *3.6. Determination of DPPH Radical Scavenging Activity*

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DPPH radical scavenging activity was evaluated using a modified version of the method described by Farhadi et al.(2016) [73]. Briefly, 100 μL of methanol extract from each sample was mixed with 3.9 mL of 0.1 mmol/L DPPH in methanol in a 10 mL centrifuge tube. After thorough mixing, the reaction mixture was kept in the dark for 30 minutes. Absorbance was then measured at 517 nm using a UV-Vis spectrophotometer (UT-1901; METASH, Shanghai, China). The scavenging activity was quantified by reference to a Trolox standard curve, and results are expressed as milligrams of Trolox equivalents per gram of dry weight (mg TE/g DW).

### *3.7. Determination of ABTS Radical Scavenging Activity*

ABTS radical cation scavenging activity was assessed using a modified version of the method described by Chang et al. (2007) [74]. In brief, 10 mL of ABTS stock solution was diluted with 25 mL of 0.325 mol/L phosphate buffer and 65 mL of MilliQ water. Then, 100 μL of methanol extract from each sample was mixed with 1.9 mL of 0.325 mol/L phosphate buffer and 2.0 mL of the diluted ABTS radical solution. After thorough mixing, the reaction was kept in the dark for 1 hour. Absorbance was recorded at 734 nm using a UV-Vis spectrophotometer (UT-1901; METASH, Shanghai, China). Scavenging activity was quantified by a Trolox standard curve, and results are expressed as milligrams of Trolox equivalents per gram of dry weight (mg TE/g DW).

### *3.8. Statistical Analysis*

All experiments were performed in triplicates, and the results are reported as mean values with standard errors. Statistical analyses were carried out using SPSS 22.0 (SPSS Inc. Chicago, IL, USA), while graphical analysis was conducted using Origin 2023 (Microcal Software, Northampton, MA). Duncan's multiple range tests were used for analysis of variance (ANOVA), with statistical significance set at $P < 0.05$.

## 4. Conclusion

In summary, exogenous $Na_2SeO_3$ treatment exerts dose-dependent effects on selenium biofortification, phenolic metabolism, antioxidant enzyme activities, and total antioxidant capacity in germinating chestnuts. Low selenium concentrations (20-40 mg/L) significantly promoted total selenium accumulation and the conversion of organic selenium (SeMet, SeCys), with the highest proportion of organic selenium (81.3%) observed at 20 mg/L. Concurrently, low selenium treatment upregulated the activities of PAL, GPX, SOD, and CAT, promoted the accumulation of γ-aminobutyric acid (GABA), total polyphenols and flavonoids, and consequently enhanced DPPH and ABTS radical scavenging activities. The 40 mg/L $Na_2SeO_3$ treatment was identified as the optimal concentration, yielding the greatest improvements across all measured parameters. In contrast, high selenium concentration (80 mg/L) gradually inhibited these beneficial effects, leading to a decreased proportion of organic selenium, reduced enzyme activities, diminished accumulation of phenolics and GABA, and weakened antioxidant capacity, indicating oxidative stress and metabolic disorder. These findings reveal that the functional activity of germinating chestnuts exhibits a "low-dose promotion, high-dose inhibition" response to selenium treatment, and establish 40 mg/L $Na_2SeO_3$ as the optimal concentration for producing selenium-enriched germinated chestnuts with improved nutritional quality and antioxidant potential. This study provides a theoretical basis and technical parameters for the development of functional foods from selenium-enriched germinated chestnuts.

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**Data Availability Statement:** The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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