

Research Article

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Evaluation of nano-selenium biofortification characteristics of alfalfa (*Medicago sativa* L.)

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Abstract: Selenium (Se) is indispensable for animals and humans. One option to address Se deficiency is to biofortify plants with Se. Biofortification of forage with Se nanoparticles (NPs) is gaining more attention as an efficient and safe source of Se for livestock. The purpose of this study was to investigate the effect of different concentrations of NPs-Se (0, 30, 50, 100, 150, and 250 mg·L⁻¹) on the growth of alfalfa harvested multiple times, and to provide a basis for the production of Se-enriched forages. Applying 50 mg·L⁻¹ concentration of NPs-Se had the best effect on yield over three harvests. Over three harvests, low-dose NPs-Se (30 and 50 mg·L⁻¹) application significantly increased peroxidase and superoxide dismutase activities, chlorophyll content and carotenoid content, and significantly decreased malondialdehyde content. The total Se content and Se accumulation in plants at the same harvest showed an upward trend with increasing Se concentration. At the same concentration, from first harvest to third harvest, Se content and Se accumulation showed an initially increasing and then decreasing trend. The evaluation found that foliar application of NPs-Se at

50 mg·L⁻¹ could have the greatest positive effect on the growth and yield of multiple-harvested alfalfa.

Keywords: nano-selenium, alfalfa, yield, selenium accumulation, physiological characteristics

1 Introduction

Selenium (Se) is an essential trace element for human beings. Se supplementation is of paramount importance in Se-deficient countries and regions, because it improves people's thyroid function, participates in the body's antioxidant defenses, enhances their immunity and can reduce cardiovascular disease and cancer risks [1]. Se is a component of proteins and enzymes, such as glutathione peroxidase, thyroxine 5-deiodinase, selenoprotein K, selenoprotein N, and selenoprotein P [2]. It is involved in the production of active thyroid hormones and the regulation of the immune system, while protecting cells from damage by free radicals generated during oxidative metabolism [3]. In many places, including in the United States, Australia, and China, Se is only present in small amounts in soils, resulting in a correspondingly low Se content of plant produce [4]. Recent estimates indicate that 15–20% of children and adults around the world are Se deficient [5]. Therefore, relying only on naturally occurring Se in foods is often insufficient to meet the basic needs of the human body, resulting in a low Se status which poses a potential threat to human health. Biofortification is the principal method to prevent inadequate Se intake in both humans and animals. To date, biofortification with Se has been conducted on some agricultural products, such as wheat [6], peas [7], rice [8], and vegetables [9]. Se, like several stressors, such as certain toxins, biostimulants, or non-essential elements, elicits a biphasic dose-response. In other words, low concentrations of Se will promote the growth and development of plants, while high concentrations of Se will inhibit and may even have toxic effects [10]. Nanotechnology is a rapidly developing technique for targeted and precise micronutrient fertilizing in agriculture [11].

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Se can be found in different varieties of forms, including selenite, selenate, nanoparticles of Se (NPs-Se), and selenoproteins [12]. NPs-Se is a red elemental Se with lower toxicity and higher biological activity than inorganic Se [13,14]. Therefore, NPs-Se has good prospects as a new Se source in the Se-enrichment industry.

Alfalfa (*Medicago sativa* L.) is a perennial legume forage, which is widely planted worldwide because of its adaptability, high production performance, good palatability, high protein, and high digestible fiber content [15,16]. Alfalfa has deep roots, which can enrich soil organic matter to a certain extent and prevent water erosion and wind erosion [17,18]. At the same time, with multiple harvesting during a year, high-yield alfalfa can help meet demand for forage in crop–livestock farming systems [19]. Application of Se not only increases the Se content in plants, but also increases plant yield, nutritional quality, and resistance to external environmental stressors affecting plant growth [20,21]. Therefore, the production of Se-enriched alfalfa could provide a safe source of Se combined with high yield and high-quality forage for livestock. The main methods currently used to enrich Se in alfalfa include foliar application of Se and application of Se in the soil. Studies have shown that foliar fertilization has remarkable effects compared with soil fertilization [5]. When Se is applied in soil, the concentration and form of Se, and the effects of soil microorganisms and interaction with metal ions have great influence on the absorption of Se [22,23]. However, foliar application of Se is simple, safe, and less harmful to soil [24]. The feeding value of alfalfa leaves is much greater than that of stems, so the effect of foliar application of Se content in leaves is potentially of great significance.

Previous studies on Se bioaugmentation have focused on plant biological effects over one period [25]. For forage plants such as alfalfa that are harvested several times, it is necessary to gain a deeper understanding of the effects of Se on plant physiological characteristics and Se accumulation across different harvests. Therefore, the present study aimed to clarify the effects of NPs-Se concentration (0, 30, 50, 100, 150, and 250 mg·L⁻¹) on yield, physiological characteristics, and Se accumulation during three successive alfalfa harvests. The study

aimed to explore the effects of foliar biofortification with NPs-Se on alfalfa, to clarify the suitable dose of NPs-Se in alfalfa production, to provide a safe source of Se for humans and livestock.

2 Materials and methods

2.1 Treatments and plant materials

The plant materials were the alfalfa variety “WL232HQ,” which has a fall dormancy grade of 2.6 and a cold resistance index of 1.3. Seeds were provided by Xintai Agricultural Science and Technology Co. Ltd, Baotou City, Inner Mongolia.

NPs-Se was 5% NPs-Se nutrient solution, provided by Shenzhen Zhigao Military and Civilian Integration Equipment Technology Research Institute.

The experiment was conducted at the Baotou Experimental Station for Forage Processing and High Efficient Utilization, Inner Mongolia Agricultural University. The experimental station is located in Baotou City in Inner Mongolia, China (110°37′–110°27′E; 40°05′–40°17′N). Alfalfa was sown in May 2020 using a drill, with a distance between rows of 10 cm. Because alfalfa is highly regenerative, it is generally harvested three times a year in the test area. The field experiments were conducted in 2021. After reaching a height of 20 cm, plants were sprayed with Se at a rate of 1,000 L·hm⁻². Leaf spraying concentrations were T0 = 0 mg·L⁻¹, T1 = 30 mg·L⁻¹, T2 = 50 mg·L⁻¹, T3 = 100 mg·L⁻¹, T4 = 150 mg·L⁻¹, and T5 = 250 mg·L⁻¹. The area of each test plot was 20 m² (5 m × 4 m). Each treatment was repeated three times. Alfalfa was harvested in the initial flowering stage. The specific spraying and harvest dates are shown in Table 1.

2.2 Measurements

2.2.1 Determination of yield, crude protein, and Se content

After harvesting, plants were thoroughly washed with deionized water. After oven-drying at 105°C for 0.5 h to kill bacteria, all samples were dried for 72 h at 65°C to a constant weight and the weights (DW) were recorded. For the determination of Se concentration, the dried samples were ground and cryopreserved (4°C). We weighed 2.0 g of sample (accurate to 0.0001 g), placed it in a 100 mL high-profile beaker, added 15 mL of mixed acid

Table 1: Spraying and harvest dates

	Spraying dates	Harvest times
1st harvest	May 7, 2021	June 10, 2021
2nd harvest	July 2, 2021	July 19, 2021
3rd harvest	August 16, 2021	September 13, 2021

($\text{HNO}_3\text{:HClO}_4$ ratio of 4:1) to digest overnight, then heated the solution at 160°C to clarify and cool to 50 mL, which was taken as the sample digestion solution. We then took 20 mL digestive solution and a 50 mL volume bottle, and added 8 mL hydrochloric acid and 2 mL potassium ferricyanide solution to a constant volume. Total Se contents were determined via hydride generation-atomic fluorescence spectrometry after oxidation digestion and reduction [26]. The amount of Se extracted by plants was calculated as follows [27]:

$$\text{Se extracted} = \text{Plant Se content} \times \text{Plant biomass} \quad (1)$$

2.2.2 Measurement of antioxidant enzyme activity and malondialdehyde (MDA) concentration

The fresh samples were homogenized in ice-cold phosphate buffer (0.05 M, pH 7.8). The homogenate was then centrifuged for 20 min ($11,000\times g$, 4°C). The activities of peroxidase (POD) and superoxide dismutase (SOD) enzymes were determined in the supernatant. SOD activity was detected by monitoring the photoreduction of nitroblue tetrazolium (NBT). One SOD enzyme activity unit is defined as 50% inhibition of NBT reduction at 560 nm [28]. Activity of POD was assayed by monitoring the increase in absorbance at 470 nm, as described by Zhang and Kirkham [29]. A change of 0.01 absorbance per minute corresponds to a unit of POD activity. MDA was extracted by homogenizing the fresh samples in 3 mL of 5% (w/v) trichloroacetic acid and centrifuging the homogenate for 10 min ($11,500\times g$). MDA content in the supernatant was measured using the thiobarbituric acid method and calculated using the absorption coefficient of $155 \text{ mmol}\cdot\text{L}^{-1}\cdot\text{cm}^{-1}$ [28].

2.2.3 Determination of photosynthetic pigments

Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid measurements were performed by homogenizing 0.5 g fresh leaves (samples of three plants in each replicate bulked) in 25 mL of ethanol/acetone. Absorbance of the extract at 663, 645, and 470 nm (chlorophyll a, chlorophyll b, and carotenoid) was measured with a spectrophotometer and the total chlorophyll concentration was calculated using the formulas described by Arnon [27].

2.3 Data analysis

Data analysis was performed using Microsoft Excel 2010 and SAS statistical software version 9.4. Analysis of

variance using two-way ANOVA was conducted between Se treatments and harvests, and one-way ANOVA was applied to evaluate the differences among Se treatments within the same harvest. Separation of means was performed by *post-hoc* test (Duncan's test), and significant differences were assessed at the levels $P < 0.05$ and $P < 0.01$. The data are presented as mean $\pm$ standard deviation.

In order to determine the optimum concentration, a comprehensive evaluation of the indicators of each treatment was carried out. Because of different indicator units, scoring functions were used to score the indicators. The equations of the score curves are as follows [30]:

$$f(x) = 0.1 + 0.9 \times \frac{x - L}{L - U}, L \leq x \leq U \quad (2)$$

$$f(x) = 1 - 0.9 \times \frac{x - L}{L - U}, L \leq x \leq U \quad (3)$$

where $f(x)$ is the linear score, x is the index value, and L and U are the lower and upper threshold values, respectively. Eq. 2 was used for the “more is better” scoring function, whereas Eq. 3 was used for the “less is better” function. For the “optimum” function, indicators were scored as “more is better” for the increasing part and then scored as “less is better” for the decreasing part.

On the basis of the indicator scores, the weight of each index was determined according to the results of principal component analysis (PCA), and the final comprehensive evaluation index formula for alfalfa under the treatment of NPs-Se concentration (CEI) was as follows

$$\text{CEI} = \frac{1}{3} \sum_j^m \left(\sum_i^n W_i N_i \right) \quad (4)$$

where W is the PCA weighting, N is the indicator score, i is the index for indicators, and j is the index for harvests.

3 Results

3.1 Yield

Treatment with Se had significant effects on alfalfa yield ($P < 0.01$, Figure 1). With increasing Se concentration, yield first increased and then decreased, reaching a maximum under T_2 , especially in the third crop. From the first harvest to the third harvest, the T_0 treatment first decreased and then tended to be constant, while yield in T_1 , T_2 , and T_3 treatments decreased first and then increased slightly. The T_4 treatment showed a downward trend and the T_5 treatment showed little change. At third harvest, the yield of the

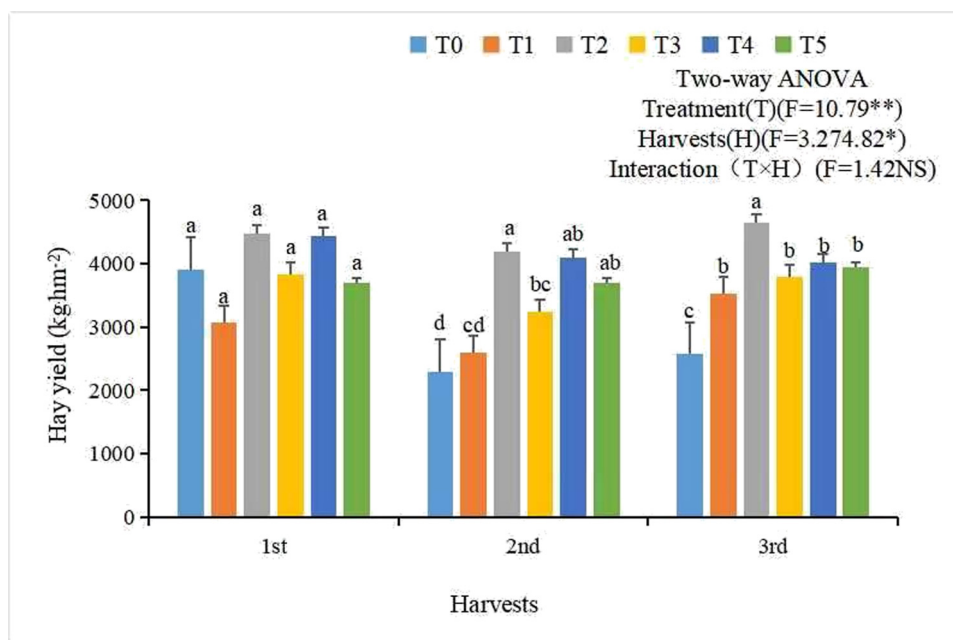


Figure 1: Effects of different Se concentrations on alfalfa yield during three consecutive harvests. $T_0 = 0 \text{ mg}\cdot\text{L}^{-1}$, $T_1 = 30 \text{ mg}\cdot\text{L}^{-1}$, $T_2 = 50 \text{ mg}\cdot\text{L}^{-1}$, $T_3 = 100 \text{ mg}\cdot\text{L}^{-1}$, $T_4 = 150 \text{ mg}\cdot\text{L}^{-1}$, and $T_5 = 250 \text{ mg}\cdot\text{L}^{-1}$. Different lowercase letters above bars indicate significant differences between different Se applications in the same harvest ($P < 0.05$). * Indicates significant according to Duncan's test ($P < 0.05$). ** Indicates significant according to Duncan's test ($P < 0.01$). NS indicates no significant according to Duncan's test ($P > 0.05$). Bars represent the standard deviation of the mean ($n = 3$).

T_1 , T_3 , T_4 , and T_5 treatments were almost the same, and the T_2 treatment was higher than the other treatments.

3.2 Total Se content

The interaction between Se concentration and harvest had significant effects on the total Se content of alfalfa ($P < 0.01$, Figure 2). Under the six concentration treatments, the changes of total Se content in alfalfa in different harvests were consistent. Total Se content increased with increasing Se dosage. From the first harvest to the third harvest, the total Se content of plants first increased and then decreased, reaching a maximum in the second harvest. In the second harvest, the total Se content of plants under the T_5 treatment reached $4.86 \text{ mg}\cdot\text{L}^{-1}$, which was 38.6 times higher than that of T_0 .

3.3 Se extracted by alfalfa

Table 2 shows that the increase in Se extraction by alfalfa was related to the trend in Se concentration. The interaction between Se concentration and harvest had significant

effects on the amount of Se extracted by alfalfa ($P < 0.01$). In each harvest, the extracted Se content in alfalfa increased with increasing Se concentration. From the first harvest to the third harvest, the extracted Se content in alfalfa in the T_0 and T_1 treatments showed an upward trend, while the T_2 , T_3 , T_4 , and T_5 treatments showed a trend of first increasing and then decreasing.

3.4 Antioxidant enzyme activities and MDA content

Se played a positive role as an antioxidant at low doses. Table 3 shows that the main effect and interactive effect of Se concentration and harvest on POD activities and MDA content in alfalfa were significant ($P < 0.01$), but the interactive effect had no significant effect on SOD activities ($P > 0.05$). The main effect of harvest was significant on SOD activities ($P < 0.01$), while the main effect of Se concentration only had a significant effect on SOD activities at the level of $P < 0.05$.

POD activity first increased and then decreased with increasing Se concentration across the three harvests (Figure 3a). In the first harvest, the Se concentrations in all treatments were significantly higher than T_0

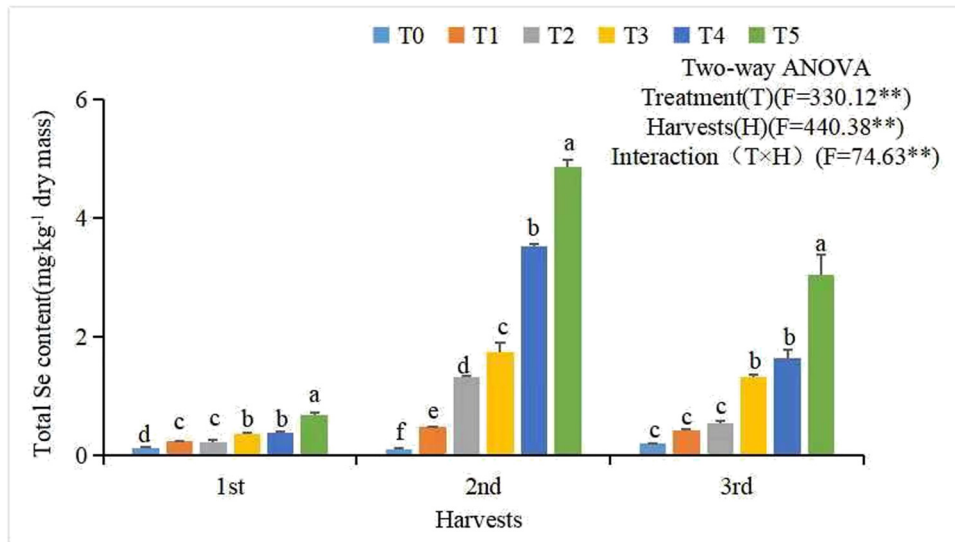


Figure 2: Effects of different Se concentrations on total Se content of alfalfa during three consecutive harvests. $T_0 = 0 \text{ mg}\cdot\text{L}^{-1}$, $T_1 = 30 \text{ mg}\cdot\text{L}^{-1}$, $T_2 = 50 \text{ mg}\cdot\text{L}^{-1}$, $T_3 = 100 \text{ mg}\cdot\text{L}^{-1}$, $T_4 = 150 \text{ mg}\cdot\text{L}^{-1}$, and $T_5 = 250 \text{ mg}\cdot\text{L}^{-1}$. Different lowercase letters above bars indicate significant differences between different Se applications in the same harvest ($P < 0.05$). **Indicates significant according to Duncan's test ($P < 0.01$). Bars represent the standard deviation of the mean ($n = 3$).

Table 2: Effects of different Se concentrations on total amount of Se extracted by alfalfa during three consecutive harvests ($\text{g}\cdot\text{hm}^{-2}$)

	T_0	T_1	T_2	T_3	T_4	T_5
1st Harvest	0.39 ± 0.14^d	0.70 ± 0.08^d	0.97 ± 0.10^{cd}	1.38 ± 0.22^{bc}	1.67 ± 0.51^b	2.48 ± 0.63^a
2nd Harvest	0.24 ± 0.09^d	1.22 ± 0.33^d	5.54 ± 0.68^c	5.60 ± 0.46^c	14.46 ± 1.81^b	17.94 ± 1.41^a
3rd Harvest	0.48 ± 0.03^c	1.48 ± 0.24^c	2.50 ± 0.21^{bc}	4.99 ± 0.70^b	6.59 ± 0.91^b	12.11 ± 3.04^a
F-test (two-ways)						
Treatment	**					
Harvest	**					
Treatment × harvest	**					

Note: Data are mean $\pm$ SD around the mean ($n = 3$). $T_0 = 0 \text{ mg}\cdot\text{L}^{-1}$, $T_1 = 30 \text{ mg}\cdot\text{L}^{-1}$, $T_2 = 50 \text{ mg}\cdot\text{L}^{-1}$, $T_3 = 100 \text{ mg}\cdot\text{L}^{-1}$, $T_4 = 150 \text{ mg}\cdot\text{L}^{-1}$, and $T_5 = 250 \text{ mg}\cdot\text{L}^{-1}$. Different lowercase letters in superscripts indicate significant differences between different Se applications in the same harvest ($P < 0.05$). ** Indicates significant according to Duncan's test ($P < 0.01$).

Table 3: Variance analysis results of antioxidant enzyme activity and MDA of alfalfa under different Se concentrations and harvests

Sources of changes	df	F (POD)	F (SOD)	F (MDA)
Treatment	5	105.9**	3.44*	24.93**
Harvest	2	494.92**	26.93**	69.17**
Treatment × harvest	10	24.31**	2.04	6.08**

Note: POD: peroxidase; SOD: superoxide dismutase; MDA: malondialdehyde. * Indicates significant according to Duncan's test ($P < 0.05$). ** Indicates significant according to Duncan's test ($P < 0.01$).

($P < 0.05$), and POD activity reached a maximum under T_1 , which was 1.65 times higher than that under T_0 . In the second harvest, POD activity reached a maximum under the T_2 treatment, which was significantly increased by

0.54 times compared with T_0 ($P < 0.05$), while POD activity under T_3 , T_5 , and T_0 treatments had no significant effect ($P > 0.05$). In the third harvest, POD activity reached a maximum under the T_1 treatment, which was significantly increased by 0.63 times compared with T_0 ($P < 0.05$) but was significantly lower than T_0 under the T_5 treatment ($P > 0.05$), indicating that POD activity began to be inhibited under high Se concentration.

The effect of Se concentration on SOD activity in alfalfa varied between harvests (Figure 3b). In the first harvest, with increasing Se concentration, SOD activity first increased and then remained stable, and T_2 treatment was a turning point. SOD activity under T_2 to T_5 concentration treatments was significantly higher than in the T_0 treatment ($P < 0.05$). In the second harvest, SOD

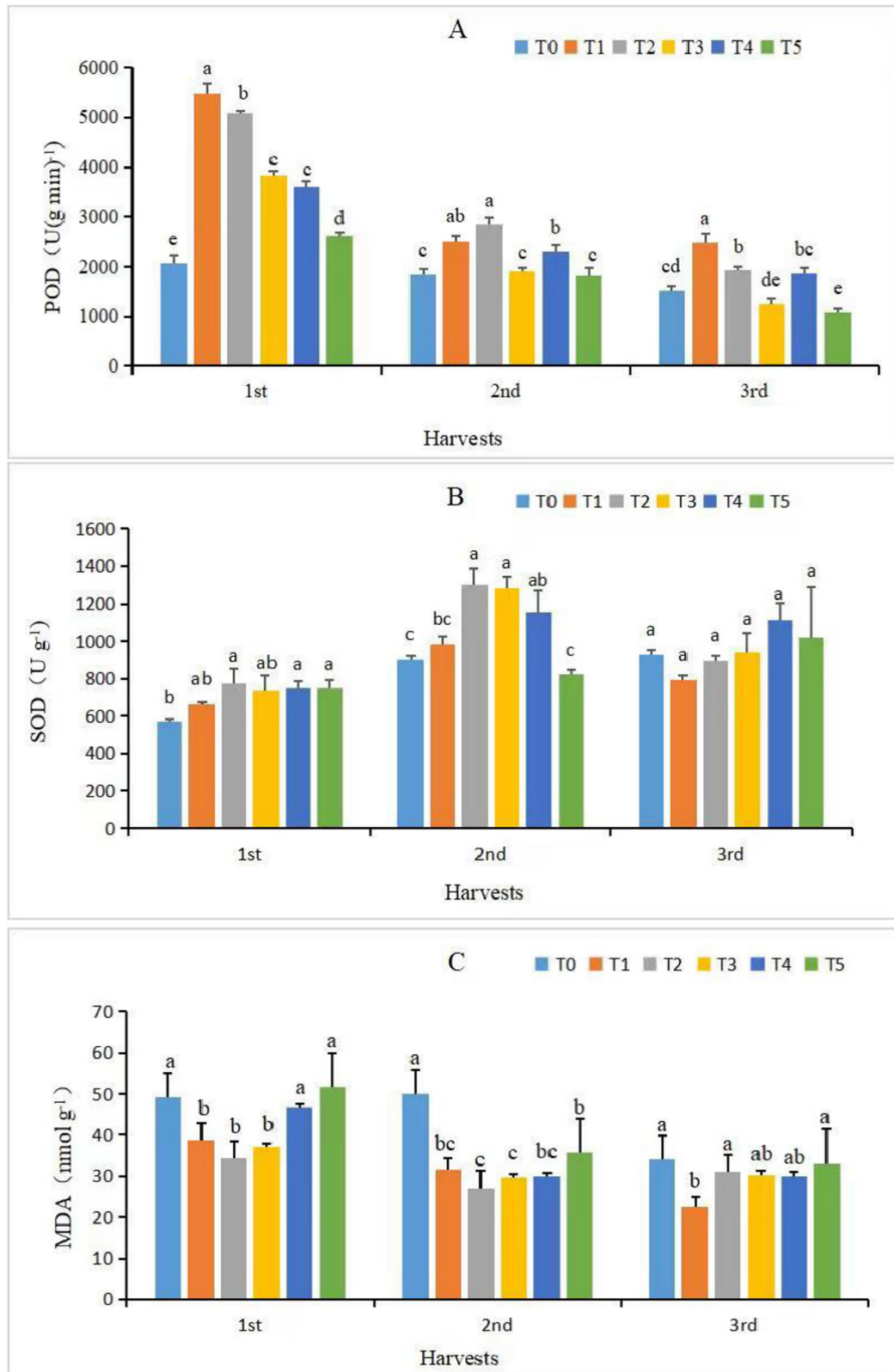


Figure 3: Effects of different Se concentrations on antioxidant enzyme activity and lipid peroxidation products in alfalfa during three consecutive harvests: (a) POD, (b) SOD, and (c) MDA. $T_0 = 0 \text{ mg}\cdot\text{L}^{-1}$, $T_1 = 30 \text{ mg}\cdot\text{L}^{-1}$, $T_2 = 50 \text{ mg}\cdot\text{L}^{-1}$, $T_3 = 100 \text{ mg}\cdot\text{L}^{-1}$, $T_4 = 150 \text{ mg}\cdot\text{L}^{-1}$, and $T_5 = 250 \text{ mg}\cdot\text{L}^{-1}$. Different lowercase letters above bars indicate significant differences between different Se applications in the same harvest ($P < 0.05$). Bars represent the standard deviation of the mean ($n = 3$).

activity first increased and then decreased with increasing Se concentration, and reached a maximum value in the T2 treatment, which was significantly higher than under T0 ($P < 0.05$). In the first and second harvests, the SOD activity of the T2 treatment was 0.35 and 0.44 times higher than that of T0, respectively. In the third harvest, there was no significant difference in SOD activity between treatments ($P > 0.05$).

The response of MDA content in alfalfa to the six Se concentration treatments was consistent among the three harvests (Figure 3c). In the first and second harvests, with increasing Se concentration, MDA content first decreased and then increased, and both harvests had the lowest value in the T2 treatment, which was significantly reduced by 30% and 46% compared with T0, respectively ($P < 0.05$). In the third harvest, the T1 treatment was significantly lower than the other treatments ($P < 0.05$), with a significant reduction of 34% compared to T0 ($P < 0.05$).

3.5 Photosynthetic pigments

As shown in Table 4, the interaction of Se concentration and harvest had no significant effect on chlorophyll a, chlorophyll b, or total chlorophyll content ($P > 0.05$), but there was a significant effect on carotenoids ($P < 0.01$). The main effect of harvest on photosynthetic pigments was significant ($P < 0.01$). Se concentration had a significant effect on chlorophyll a, total chlorophyll content, and carotenoids ($P < 0.05$) but had no significant effect on chlorophyll b ($P > 0.05$). Chlorophyll a content was observed in the first harvest, but Se concentration treatment had no significant effect on it ($P > 0.05$). In the second and third harvests, chlorophyll a first increased and then decreased with increasing Se concentration, and reached its highest point under the T1 and T2 treatments, respectively, which were significantly higher than those under T0 ($P < 0.05$). Chlorophyll b content first increased and then decreased with increasing Se concentration in the first and second harvests, reaching maximum values in the T3 and T1 treatments, respectively, which were significantly higher than those under T0 ($P < 0.05$). In the third harvest, Se concentration treatment had no significant effects ($P > 0.05$). Across the three harvests, total chlorophyll content first increased and then decreased with increasing Se concentration. On the whole, total chlorophyll content was significantly higher than T0 under low Se concentration treatments ($P < 0.05$), and there was no significant effect under high Se concentration treatments ($P > 0.05$). In the

Table 4: Effects of different Se concentrations on photosynthetic pigments in alfalfa leaf during three consecutive harvests

	Treatment						F-test (two-ways)		
	T0	T1	T2	T3	T4	T5	Treatment	Harvest	Treatment × harvest
Chlorophyll ^a (mg·g ⁻¹)									
1st harvest	1.86 ± 0.52 ^a	2.27 ± 0.09 ^a	2.40 ± 0.02 ^a	2.48 ± 0.17 ^a	2.51 ± 0.09 ^a	2.32 ± 0.09 ^a	*	**	NS
2nd harvest	2.59 ± 0.10 ^c	2.97 ± 0.24 ^a	2.91 ± 0.18 ^{ab}	2.68 ± 0.06 ^{bc}	2.70 ± 0.06 ^{bc}	2.53 ± 0.09 ^c			
3rd harvest	2.52 ± 0.12 ^b	2.45 ± 0.11 ^b	2.81 ± 0.02 ^a	2.49 ± 0.16 ^b	2.44 ± 0.15 ^b	2.50 ± 0.03 ^b			
Chlorophyll ^b (mg·g ⁻¹)									
1st harvest	1.07 ± 0.04 ^b	1.2 ± 0.01 ^{ab}	1.19 ± 0.02 ^{ab}	1.40 ± 0.24 ^a	1.22 ± 0.09 ^{ab}	1.17 ± 0.06 ^b	NS	**	NS
2nd harvest	1.33 ± 0.07 ^b	1.47 ± 0.09 ^a	1.42 ± 0.09 ^{ab}	1.37 ± 0.06 ^{ab}	1.42 ± 0.07 ^{ab}	1.42 ± 0.02 ^{ab}			
3rd harvest	0.84 ± 0.05 ^a	0.83 ± 0.05 ^a	0.97 ± 0.01 ^a	0.86 ± 0.08 ^a	0.85 ± 0.01 ^a	0.90 ± 0.05 ^a	**	**	**
Carotenoid (mg·g ⁻¹)									
1st harvest	0.63 ± 0.04 ^b	0.71 ± 0.03 ^a	0.72 ± 0.01 ^a	0.73 ± 0.04 ^a	0.71 ± 0.02 ^a	0.67 ± 0.02 ^{ab}	**	**	**
2nd harvest	0.78 ± 0.05 ^b	0.96 ± 0.05 ^a	0.93 ± 0.05 ^a	0.89 ± 0.05 ^a	0.90 ± 0.04 ^a	0.95 ± 0.01 ^a			
3rd harvest	0.81 ± 0.05 ^{ab}	0.87 ± 0.01 ^a	0.8 ± 0.03 ^{ab}	0.78 ± 0.07 ^b	0.75 ± 0.05 ^b	0.82 ± 0.02 ^{ab}	*	**	NS
Total chlorophyll (mg·g ⁻¹)									
1st harvest	2.93 ± 0.89 ^b	3.47 ± 0.15 ^{ab}	3.59 ± 0.05 ^{ab}	3.88 ± 0.10 ^a	3.73 ± 0.24 ^a	3.49 ± 0.19 ^{ab}			
2nd harvest	3.92 ± 0.17 ^c	4.44 ± 0.33 ^a	4.33 ± 0.27 ^{ab}	4.05 ± 0.08 ^{bc}	4.13 ± 0.12 ^{abc}	3.95 ± 0.09 ^c			
3rd harvest	3.36 ± 0.19 ^b	3.28 ± 0.21 ^b	3.78 ± 0.02 ^a	3.36 ± 0.30 ^b	3.30 ± 0.17 ^b	3.40 ± 0.05 ^b			

Note: Data are mean ± SD around the mean ($n = 3$). T0 = 0 mg·L⁻¹, T1 = 30 mg·L⁻¹, T2 = 50 mg·L⁻¹, T3 = 100 mg·L⁻¹, T4 = 150 mg·L⁻¹, and T5 = 250 mg·L⁻¹. Different lowercase letters in superscripts indicate significant differences between different Se applications in the same harvest ($P < 0.05$). * Indicates significant according to Duncan's test ($P < 0.05$). ** Indicates significant according to Duncan's test ($P < 0.01$). NS indicates no significant according to Duncan's test ($P > 0.05$).

first harvest, compared with T_0 , carotenoid contents in T_1 – T_4 treatments were significantly increased ($P < 0.05$), while carotenoid content in T_5 treatment was not significantly affected ($P > 0.05$). In the second harvest, compared with T_0 , the carotenoid content of the T_1 – T_5 treatments increased significantly ($P < 0.05$). In the third harvest, compared with T_0 , there was no significant difference in the carotenoid contents in T_1 – T_5 treatments ($P > 0.05$).

3.6 Comprehensive evaluation

The comprehensive score of the effect of Se concentration on various indicators of alfalfa is shown in Table 5. The variables were transformed using linear scoring functions. After deciding the shape of the anticipated response (“more is better,” “less is better”), the limits or threshold values were assigned for each indicator. Yield, total Se content, POD, SOD, and photosynthetic pigments were evaluated using “more is better” curves. MDA was evaluated using a “less is better” curve, because the lower the MDA content, the lower the cell membrane damage. The scores were then calculated by using Eqs. 2–4. The final ranking was $T_2 > T_4 > T_3 > T_1 > T_5 > T_0$.

4 Discussion

Alfalfa is an important forage plant in agricultural and animal husbandry production systems, and yield is a strongly prioritized indicator. At an ideal concentration, Se can promote plant growth, but excessive amounts of Se can be toxic to some plant species, including wheat [6], rice [8], and vegetables [9]. In this study, the application of NPs-Se promoted the yield of alfalfa harvested three times but did not inhibit the yield. Especially in the third harvest, a low concentration of NPs-Se was found to significantly improve yield, and the best effect was obtained when the concentration was T_2 (i.e., $50 \text{ mg}\cdot\text{L}^{-1}$). When concentrations were T_4 ($150 \text{ mg}\cdot\text{L}^{-1}$) and T_5 ($250 \text{ mg}\cdot\text{L}^{-1}$), the yield of alfalfa showed a downward trend in the third harvest compared with the first two harvests. This finding is inconsistent with the findings of Bai *et al.* [31], whose study showed that the application of sodium selenite in soil inhibits antioxidant enzyme activities and photosynthetic parameters of alfalfa when Se concentration exceeded $20 \text{ mg}\cdot\text{L}^{-1}$, resulting in the inhibition of alfalfa growth. The reasons for the lack of inhibition in our study may be that, first the Se application method was different, as foliar spraying was

used in this study, and second it may be that the highest concentration used in our study did not reach the threshold for toxicity for alfalfa. We found that there was no significant effect of NPs-Se on the yield of the first harvest, which was consistent with the results of Xia *et al.* [32]. However, there was a significant difference between the effects of NPs-Se concentrations on the yield of the second and third harvest. Previous studies only investigated the effects on one harvest and did not explore the effect of Se on multiple harvests. Therefore, the biological effects of Se NPs on multiple harvests are different and further studies are needed to elucidate the underlying mechanisms.

The Se content in common plants is 0.02 – $1.5 \text{ mg}\cdot\text{L}^{-1}$ [33]. In the present study, the Se content in alfalfa increased with increasing NPs-Se concentration. Moreover, the amount of Se extracted from plants also increased. These results were similar to those of previous studies [28,34]. In this study, in the three harvests, both the Se content and the extraction amount reached a maximum in the second harvest. The reason may be that at the second harvest, when temperature was high, alfalfa grew vigorously and had a strong ability to absorb nutrients such as Se. There was a clear downward trend in the third harvest, especially at high concentrations (150 and $250 \text{ mg}\cdot\text{L}^{-1}$). The results of this study were consistent with those of Di *et al.* [35]. This indicates that the high concentration of NPs-Se produced an inhibitory effect on Se content and extraction amount in the third harvest compared with the first two harvests. It is possible that foliar sprays cause the transfer of Se from leaves to roots and that multiple sprays cause the roots to accumulate Se up to the domain value of alfalfa [32]. This finding provides new insights for the development of organic Se-enriched products by studying alfalfa harvested several times a year.

Reactive oxygen species (ROS) results from electron leakage to O_2 during photosynthetic electron transport, and a certain concentration of ROS (at a level that does not induce harmful effects) is necessary for plants to maintain their normal physiological processes [36]. However, excessive ROS damages plants and suppresses growth when plants are exposed to stress [37]. The accumulation of excess ROS would cause membranous peroxidation in the cell. MDA is the final soluble product in the process of membrane lipid peroxidation. The higher the content of MDA, the more severe the damage to the cell membrane system, so the content of MDA is usually used to measure the degree of oxidative stress [38,39]. SOD and POD are the main antioxidant enzymes involved in scavenging ROS and reducing damage to the membrane system. In the current study, after low-dose NPs-Se treatment, the activities of SOD and POD were significantly enhanced, while the content of

Table 5: Comprehensive evaluation of the effect of Se concentration on alfalfa in three harvests

Treatment	Harvest	Yield	Total Se content	POD	SOD	MDA	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid	CEI	Ranking
T0	1st harvest	0.4304	0.1038	0.3027	0.1000	0.1742	0.1000	0.4375	0.1000	0.1000	0.3505	6
	2nd harvest	0.1000	0.1000	0.2565	0.5110	0.1536	0.6919	0.8031	0.6901	0.5091		
	3rd harvest	0.2033	0.1170	0.1911	0.5424	0.6449	0.6351	0.1141	0.3563	0.5909		
T1	1st harvest	0.3932	0.1246	1.0000	0.2158	0.4970	0.4324	0.6203	0.4219	0.3182	0.5548	4
	2nd harvest	0.2141	0.1700	0.3932	0.6098	0.7231	1.0000	1.0000	1.0000	1.0000		
	3rd harvest	0.5694	0.1605	0.3857	0.3739	1.0000	0.5784	0.1000	0.3086	0.7545		
T2	1st harvest	0.9338	0.1227	0.9171	0.3504	0.6384	0.5378	0.6063	0.4934	0.3455	0.6624	1
	2nd harvest	0.8265	0.3307	0.4610	1.0000	0.8623	0.9514	0.9297	0.9344	0.9182		
	3rd harvest	1.0000	0.1832	0.2763	0.5034	0.7376	0.8703	0.2969	0.6066	0.5636		
T3	1st harvest	0.6890	0.1492	0.6615	0.3027	0.5549	0.6027	0.9016	0.6662	0.3727	0.5723	3
	2nd harvest	0.4623	0.4101	0.2677	0.9811	0.7829	0.7649	0.8594	0.7675	0.8091		
	3rd harvest	0.6686	0.3307	0.1353	0.5506	0.7588	0.6108	0.1422	0.3563	0.5091		
T4	1st harvest	0.9203	0.1511	0.6165	0.3202	0.2577	0.6270	0.6484	0.5768	0.3182	0.5885	2
	2nd harvest	0.7877	0.7485	0.3495	0.8162	0.7733	0.7811	0.9297	0.8152	0.8364		
	3rd harvest	0.7596	0.3912	0.2594	0.7688	0.7671	0.5703	0.1281	0.3205	0.4273		
T5	1st harvest	0.6335	0.2097	0.4125	0.3169	0.1000	0.4730	0.5781	0.4338	0.2091	0.5203	5
	2nd harvest	0.6339	1.0000	0.2509	0.4070	0.5925	0.6432	0.9297	0.7079	0.9727		
	3rd harvest	0.7270	0.6578	0.1000	0.6527	0.6741	0.6189	0.1984	0.3801	0.6182		
Weight		0.1211	0.0560	0.1169	0.1050	0.1003	0.1136	0.1239	0.1380	0.1252		

Note: T0 = 0 mg·L⁻¹, T1 = 30 mg·L⁻¹, T2 = 50 mg·L⁻¹, T3 = 100 mg·L⁻¹, T4 = 150 mg·L⁻¹, and T5 = 250 mg·L⁻¹. POD: peroxidase; SOD: superoxide dismutase; MDA: malondialdehyde; CEI: comprehensive evaluation index.

MDA was significantly reduced, especially in the first and second harvests. However, in the third harvest, SOD activity was not significantly affected compared to T_0 , while POD activity was significantly increased at lower doses. Under the high concentration treatment ($250 \text{ mg}\cdot\text{L}^{-1}$), POD activity was inhibited. This shows that POD activity is more sensitive than SOD activity to NPs-Se treatment. In the third harvest, POD activity was significantly increased after Se concentration treatment in T_1 ($30 \text{ mg}\cdot\text{L}^{-1}$) compared with T_0 ($0 \text{ mg}\cdot\text{L}^{-1}$), while MDA content was significantly decreased. This shows that POD is more effective than SOD in scavenging ROS and reducing MDA content. This finding is consistent with the studies by Bai *et al.* [31] and Duan *et al.* [33]. The response of SOD activity to Se may be related to the change of SOD gene transcription level. Studies have shown that Se can significantly affect the transcription level of CuZn-SOD in chloroplast of potato plants under light stress [40]. When the Se application level was higher than the toxicity threshold, POD activity decreased significantly, which may be due to the stress caused by high Se on leaves beyond the regulation of antioxidant system and the accumulation of ROS [31,41]. In short, it is not suitable to apply high concentration of NPs-Se to alfalfa harvested more than once a year. At the same time, the application of an appropriate amount of NPs-Se can improve the antioxidant capacity, which also provides a new insight for alfalfa cultivation in adverse environments, such as saline and alkali soils.

Plants may contain selenoprotein structures similar to thioredoxin and iron-sulfur protein involved in the regulation of chlorophyll synthesis and photosynthesis [42,43]. In this study, photosynthetic pigments first increased and then decreased with increasing Se concentration, which was consistent with the results of Lin *et al.* [27] and Hawrylak-Nowak [44]. Over three harvests, low concentrations of NPs-Se significantly increased chlorophyll a, chlorophyll b, carotenoids, and total chlorophyll content, especially at the T_2 ($50 \text{ mg}\cdot\text{L}^{-1}$) concentration. This also explains why the yield under T_2 ($50 \text{ mg}\cdot\text{L}^{-1}$) concentration was higher than that under other treatments. It may be that low concentrations of Se can protect chlorophyll and PS II functions from oxidative damage through up-regulation of antioxidants [45,46]. However, high Se has adverse effects on porphobilinogen synthase required for chlorophyll biosynthesis and inhibits biosynthesis by lipid peroxidation [47]. In the first harvest, the concentration of NPs-Se had the greatest effect on carotenoids, followed by effects on chlorophyll b, showing a contrasting trend to that for MDA content. This result indicates that carotenoids are efficient antioxidants that scavenge peroxyl radicals and singlet molecular oxygen in plants, thus protecting photosynthetic membranes from photooxidation

[48,49]. Wang *et al.* found no significant effect of foliar spraying of higher concentrations of Se on the carotenoid content of alfalfa, a result similar to the present study in the first harvest and different from our results in the second harvest [50]. The reason for this may be that in the second harvest, the high concentration of Se damaged the plant and stimulated increased carotenoid content to mitigate this damage. In the third harvest, T_2 ($50 \text{ mg}\cdot\text{L}^{-1}$) concentration had insignificant effects on carotenoid and chlorophyll b content compared with T_0 but had a significant effect on chlorophyll a and total chlorophyll content. This shows that the response of chlorophyll b to NPs-Se was initially stronger than that of chlorophyll a. It may be that chlorophyll b is a branch of the synthesis pathway of chlorophyll a. An appropriate Se concentration in the early stage will promote chlorophyll an oxygenase to synthesize chlorophyll b, and oxygen will be involved at the same time [51,52]. It could also be that Se increases stomatal conductance, facilitates the entry of oxygen, and speeds up the process [21,53]. However, this effect may gradually weaken with repeated application of Se, thereby promoting the synthesis of chlorophyll a.

Due to the different units of each index assessed, in order to facilitate the comprehensive evaluation of each treatment, it is common to quantify each index by using a function. Andrews *et al.* compared index methods composed of different indicator selection methods with scoring functions (linear and non-linear) for vegetable production systems [54]. PCA is a common method of multivariate statistical analysis [55]. Our study suggests that it was reasonable to combine PCA with the curve function to comprehensively evaluate alfalfa after each treatment. In this study, the indicators of alfalfa treated with NPs-Se concentration were comprehensively evaluated to determine the optimal Se concentration. T_2 scored the highest, indicating the best overall effect after treatment.

5 Conclusions

The purpose of this study was to investigate the biofortification effect of different concentrations of NPs-Se on multiple harvested alfalfa. The results showed that under long-term bioaugmentation, the application of low concentration of NPs-Se could increase chlorophyll content, carotenoid content, SOD activity, and POD activity of alfalfa, and reduce MDA content, thereby limiting the damage to plants from external factors and achieving the purpose of increasing yield. The positive effects of the T_2 ($50 \text{ mg}\cdot\text{L}^{-1}$) concentration treatment were the

most obvious. Our results further suggest that the application concentration of NPs-Se can be appropriately increased in the second harvest, thereby increasing the cumulative amount of Se. However, prior to the realization of large-scale practical projects, further research on the utilization of NPs-Se and its existing forms in plants is needed to determine the effectiveness of NPs-Se.

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