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Selenium biofortification of green spinach with optimum phosphorus fertilization and selenium application timing

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Abstract

A selenium (Se) biofortification program for vegetables has never been attempted in Malaysia. This study was performed to determine the optimum Se fertilization rates for high yield and Se uptake by green spinach (*Amaranthus* spp.) through a glasshouse experiment and to evaluate the effect of Se application timing on dry matter, growth, and Se accumulation in green spinach through field experiments. Glasshouse experiment conducted with factorial randomized complete block design showed that applying P fertilizer as recommended for green spinach increased yield and Se uptake by the leaves and stems of green spinach. Selenium application in the form of Se(IV) solution at 120 g ha^{-1} to sandy clay soil was determined to be the optimum application rate, as higher rates reduced plant yield and decreased Se uptake by green spinach. Three cycles of field experiments showed that a single application of 120 g ha^{-1} Se at 14 d after planting (DAP) produced the optimum results in terms of green spinach growth and dry matter. Although the highest Se accumulation in leaves was observed when Se was applied at 7 DAP (26.04–29.20 μg 10 plants^{-1}), its detrimental effect on yield caused it to be considered an inappropriate time of application. Therefore, with optimum Se accumulation in leaves (21.16–22.38 μg 10 plants^{-1}) and stems (5.89–5.98 μg 10 plants^{-1}), application of Se as Se(IV) solution at 14 DAP at a rate of 120 g ha^{-1} has been identified as an effective Se fertilization management strategy for producing Se-biofortified green spinach.

1 | INTRODUCTION

Selenium vegetable biofortification programs have never been studied in Malaysia. Biofortification, a process used to increase the concentration of essential nutrients beneficial to human health in the consumable parts of plants, can be achieved through either fertilizer application (agronomic biofortification) or crop selection and breeding (genetic

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Abbreviations: AAS, atomic absorption spectrophotometer; CEC, cation exchange capacity; DAP, days after planting; DOA, Department of Agriculture; GF-AAS, graphite furnace–atomic absorption spectrophotometer; LAI, leaf area index; NIST, National Institute of Standard & Technology; OC, organic carbon; OM, organic matter; PAR, photosynthetically active radiation; RCBD, randomized complete block design.

biofortification). Agronomic biofortification through the use of inorganic Se as fertilizer has been shown to be a successful strategy for increasing the Se content in food crops (Premarathna et al., 2012).

Selenium fertilization of food crops is an interesting topic of study due to the controversies that have arisen from the narrow gap between its nutritional and toxicological values. According to Krystyna (2009), frequent consumption of food with less than 0.1 mg kg^{-1} Se will lead to Se deficiency in humans, whereas frequent intake of food containing more than 1 mg kg^{-1} Se will cause Se toxicity. Thus, biofortification programs using inorganic Se must be performed carefully, since an over-accumulation of Se in food crops might cause toxicity for consumers as well as phytotoxicity to the plants themselves, which might reduce the yield.

Although Se has been classified as a non-essential nutrient for plants, the beneficial effects of Se as a plant growth-promoting agent have been reported in several studies, such as in Hartikainen (2005), where a 13% increase in ryegrass (*Lolium perenne* L.) yield was observed during the second harvest. This growth-promoting effect might be due to decreased lipid peroxidation (TBARS) rates in plants. This decrease coincides with an increase in GSH-Px activity and a higher concentration of tocopherols (vitamin E), which act as scavengers of lipid peroxide radicals and singlet oxygen. This finding suggests that, in addition to promoting increased plant yield, Se application might increase the nutritional value of the forage. In addition, Qiuhui, Xu, and Pang (2003) found that Se application increased the number of sprouts and yield of the green tea plant, significantly enhanced the sweetness and aroma of their leaves, and significantly decreased leaf bitterness. Hence, the statement that Se has no role in supporting plant growth needs to be reviewed.

The amount of Se accumulated in plants primarily depends on Se availability in the soil, which is affected by many soil processes. One of the main processes occurring in soil is the sorption of Se on the soil surface. Anions such as phosphate (PO_4^{3-}) compete with Se for fixation sites in the soil. As mentioned by Fordyce (2005), the addition of PO_4^{3-} can displace selenite from being fixed on the soil surface and facilitate its uptake by plants. Further, a study by Carter, Robbins, and Brown (1972)) showed that P fertilization of soil raised Se concentrations in alfalfa (*Medicago sativa* L.) grown in soils of the United States. In addition, P application was found to increase Se uptake by berseem (*Trifolium alexandrinum* L.) (Liu, Wang, Jiang, & Cao, 2004).

However, several studies have also indicated that P may decrease the Se uptake by plants. As reported by Yong-Guan, Elizabeth, Fang-Jie, Paul, and Andrew (2009), selenite uptake was suppressed by the presence of phosphate ions in the nutrient solution but increased in the absence of P. A study of winter wheat (*Triticum aestivum* L.) hydroponics performed by Liu et al. (2018) also found that increased P decreased Se accumulation in every part of the winter wheat plant. These findings suggest that an increase in P supply significantly suppresses root-to-shoot transport of Se. As discussed in Li, McGrath, and Zhao (2008), the phosphate transport pathway in plants plays a role in selenite uptake. In a short-term (30-min) experiment, phosphate competitively inhibited selenite influx into wheat roots and caused a marked decrease in selenite uptake. Lenny et al. (2015) further reported that in soils with low P concentration, plants have a mechanism to increase the expression of phosphate transporter genes to balance out the lower phyto-availability of that nutrient. Therefore, Se is readily taken up by plants in the absence of P.

Core Ideas

- Applying P fertilizer as recommended increased green spinach yield and Se uptake.
- The optimum rate for selenite application to sandy clay soil is 120 g ha^{-1} Se.
- Selenium application at 7 DAP was detrimental to yield.
- Selenium application at 14 DAP is an effective strategy.

Conflicting results have been reported regarding the relationship between Se and P. Conventional vegetable cultivation practices in Malaysia include application of macronutrient-rich fertilizers (e.g., N:P:K 15:15:15) at high rates, which is a vital requirement in vegetable production. Therefore, this practice has been thought to affect plant Se accumulation, though no comprehensive study has been done. To produce a plan for Se fertilization management to produce selected vegetables in Malaysia, then, the effect of the application of these macronutrients, especially P, on Se uptake by vegetables needs to be studied.

Additionally, one drawback of agronomic biofortification via fertilization is the practice of repeated nutrient application, which increases the cost and logistical difficulty of this approach and may cause adverse environmental effects (Carvalho & Vasconcelos, 2013). A meta-analysis by Ros, Rotterdam, Bussink, and Bindraban (2016) found that application time was the main factor affecting the crop response to Se fertilizer. Green spinach (*Amaranthus* spp.) has been identified as a Se accumulator and is an excellent candidate for development as a functional food (Mabeyo et al., 2015). Based on area planted, green spinach is one of the most highly produced vegetables in Malaysia after mustard (*Brassica* spp.). In 2018, an area of 6,248 ha of

TABLE 1 Initial physico-chemical properties of the soil media used in the glasshouse experiment

Parameters[a]	
pH (H_2O)	6.05 ± 0.1
Cation exchange capacity, cmol$_c$ kg^{-1}	9.44 ± 0.6
Exchangeable bases, cmol$_c$ kg^{-1}	
Ca	0.4 ± 0.1
Mg	0.17 ± 0.02
K	0.02 ± 0.004
Organic C, %	1.61 ± 0.05
Total Se, mg kg^{-1}	0.13 ± 0.02
Available P, mg kg^{-1}	7.10 ± 0.3
Soil texture	Sandy clay (37% clay, 15% silt, 48% sand)

[a] Mean ± standard error ($n = 4$).

TABLE 2 Initial physico-chemical properties of the soil used in the field experiment

Parameters[a]	
pH (H_2O)	5.4 ± 0.2
Cation exchange capacity, cmol$_c$ kg^{-1}	8.7 ± 0.2
Exchangeable bases, cmol$_c$ kg^{-1}	
Ca	0.43 ± 0.03
Mg	0.18 ± 0.02
K	0.03 ± 0.01
Organic C, %	1.8 ± 0.1
Total Se, mg kg^{-1}	0.11 ± 0.01
Soil texture	Sandy clay loam (clay 34%, silt 19%, sand 47%)

[a] Mean ± standard error ($n = 4$).

green spinach planted in Malaysia produced 75,220 Mg of crop (Department of Agriculture Malaysia, 2018).

Thus, the specific objectives of this study were to (a) determine the effect of P fertilization on Se uptake by green spinach; (b) determine the optimum Se fertilization rates for high yield and Se uptake by green spinach through a glasshouse experiment; and (c) evaluate the effect of Se application timing on green spinach dry matter, growth, and Se accumulation through field experiments.

2 | MATERIALS AND METHODS

2.1 | Glasshouse experiment

One species of Se, selenite [Se(IV)], was tested in this experiment. The experiment was conducted under glasshouse conditions using a factorial randomized complete block design (RCBD) that included three rates of P fertilizer application and four rates of Se fertilizer application. There were a total of 12 (4 × 3) treatments, and each treatment was replicated three times. Green spinach was grown in pots containing soil with a sandy clay texture mixed with compost and sand at a ratio of 3:2:1.

TABLE 3 Dry matter of biofortified green spinach with various rates of Se at three levels of P fertilizer

Treatment	Dry matter (g 10 plants^{-1})
P fertilizer, %; $n = 12$	
0	50.08 ± 1.2c[a]
50	60.00 ± 1.9b
100	77.42 ± 6.8a
Selenium rates, g Se ha^{-1}; $n = 9$	
0	53.44 ± 1.6c
60	67.78 ± 6.4b
120	75.33 ± 8.2a
180	53.44 ± 1.6c
Significance level	
P fertilizer	***
Se rates	***
P × Se	***
Mean	62.50
CV	6.36

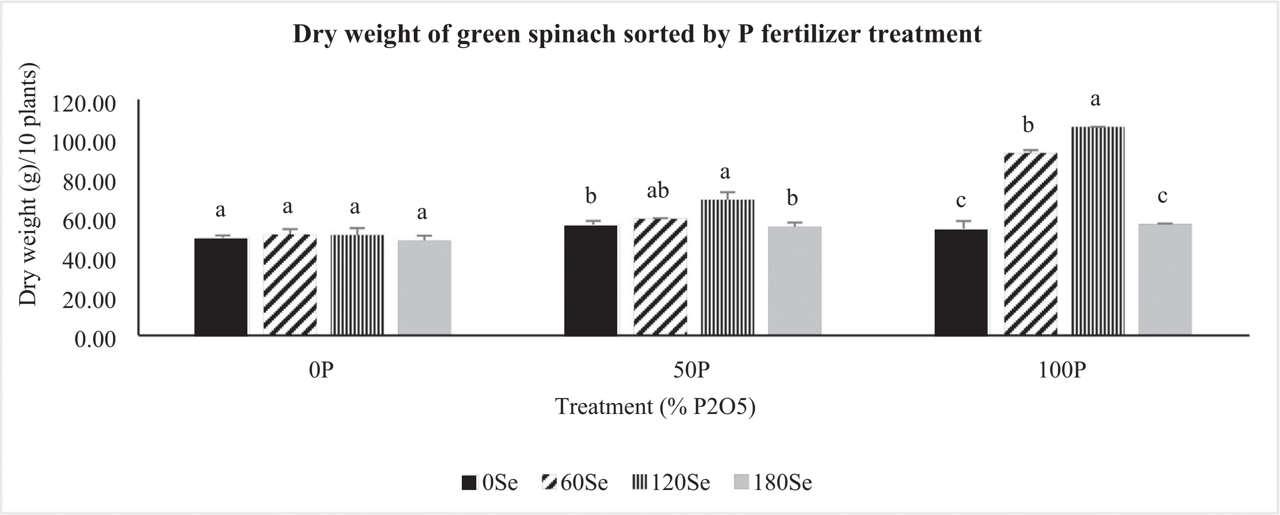
[a] Means (± standard error) with different letters for each cycle are significantly different ($P \leq .05$) using LSD ($n = 4$).

***$P \leq .001$.

Each experimental unit consisted of 10 plants per pot and was treated with soil application of Se(IV) in the form of sodium selenite solution at rates of 0, 60, 120 and 180 g ha^{-1}. Phosphorus fertilizer was applied at 0, 50, and 100% of the recommended amount (23 kg ha^{-1} P_2O_5) as diammonium phosphate. Macronutrient fertilizers were applied following the recommendation of the Department of Agriculture (DOA) at 7 and 14 DAP together with sodium selenite solution. There was no treatment for application timing, as every pot received P and Se fertilizer at both application times. Nitrogen and K fertilizer were uniformly given to each pot at 100 kg ha^{-1} N and 144 kg ha^{-1} K_2O in the forms of urea and muriate of potash, respectively. Phosphorus fertilizer was given according to treatment. Urea was added after taking into consideration the amount of N present in the diammonium phosphate (18% N).

2.1.1 | Soil media analysis

The initial properties of the soil media were determined prior to the experiment. Soil pH was determined in a



Means with different letters for each P treatment are significantly different (P≤0.05) using Tukey's HSD test.

FIGURE 1 Dry weight of green spinach sorted by P fertilizer treatment

TABLE 4 Selenium uptake by biofortified green spinach at various Se application rates and at three levels of P fertilizer

	Selenium uptake		
Treatment	**Leaves**	**Stems**	**Roots**
	μg 10 plants^{-1}, based on dry wt.		
P fertilizer, %; *n* = 12			
0	13.06 ± 2.2a[a]	3.40 ± 0.6b	1.18 ± 0.2a
50	13.01 ± 2.0a	3.27 ± 0.5b	1.24 ± 0.2a
100	14.14 ± 2.3a	4.48 ± 0.8a	1.31 ± 0.3a
Selenium rates, g ha^{-1} Se; *n* = 9			
0	2.02 ± 0.2c	0.54 ± 0.1c	0.14 ± 0.02c
60	15.42 ± 0.8b	4.51 ± 0.4b	1.06 ± 0.1b
120	18.65 ± 1.3a	5.56 ± 0.5a	1.73 ±0.1a
180	17.54 ± 1.2ab	4.24 ± 0.2b	2.04 ± 0.1a
Significance level			
P fertilizer	ns	**	ns
Se rates	***	***	***
P × Se	***	**	ns
Mean	13.41	3.72	1.24
CV	12.97	19.12	19.55

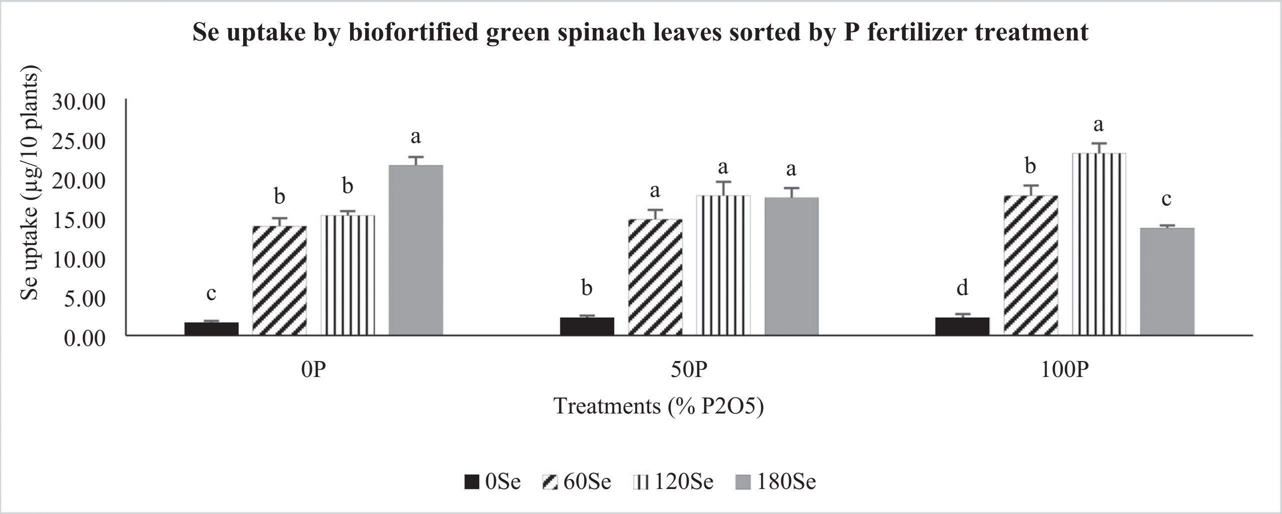
Note. ns, not significant.

[a] Means (± standard error) with different letters for each cycle are significantly different ($P \leq .05$) using LSD ($n = 4$).

** $P \leq .01$.

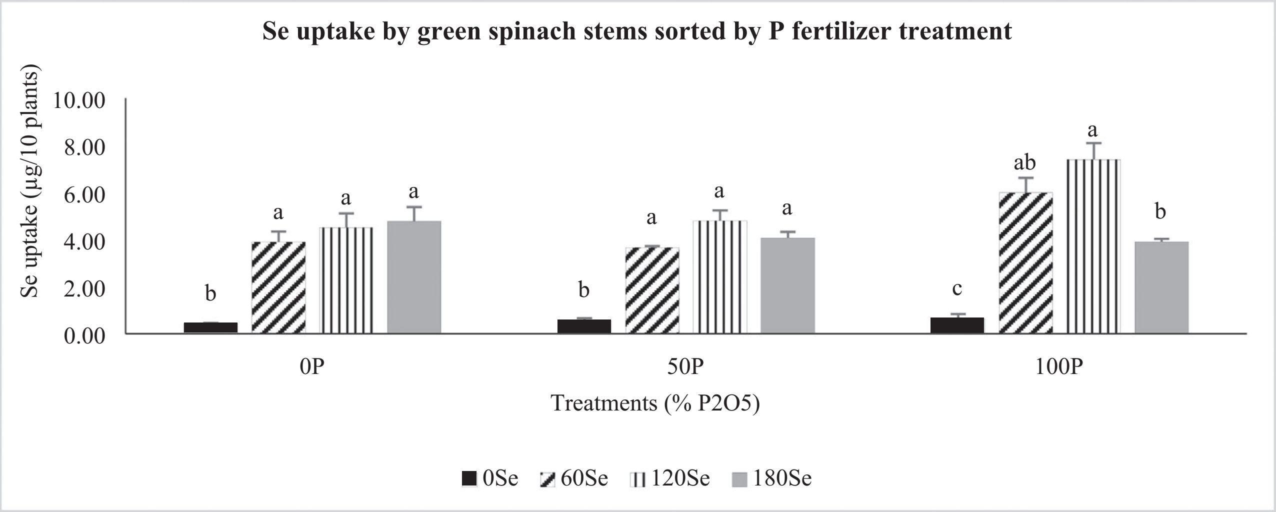
*** $P \leq .001$.

water suspension of soil using a soil/solution ratio of 1:2.5. Cation exchange capacity (CEC) was determined by the leaching method with 1 M ammonium acetate (NH_4OAc, pH 7.0). Organic carbon (OC) was determined using the Walkley and Black method, available P by the Bray–Kurtz no. 2 method and total Se concentration by the aqua-regia (3:1 HCl/HNO_3) method. The initial total Se concentration of the soil media was 0.13 mg kg^{-1}. According to Gupta and Gupta (2017), a total soil Se concentration of less than 0.6 mg kg^{-1} is considered deficient, especially for growing food crops for human and animal consumption. The properties of the soil media used in this experiment are presented in Table 1.



Means with different letters for each P treatment are significantly different (P≤0.05) using Tukey's HSD test.

FIGURE 2 Selenium uptake by leaves of green spinach sorted by P fertilizer treatment

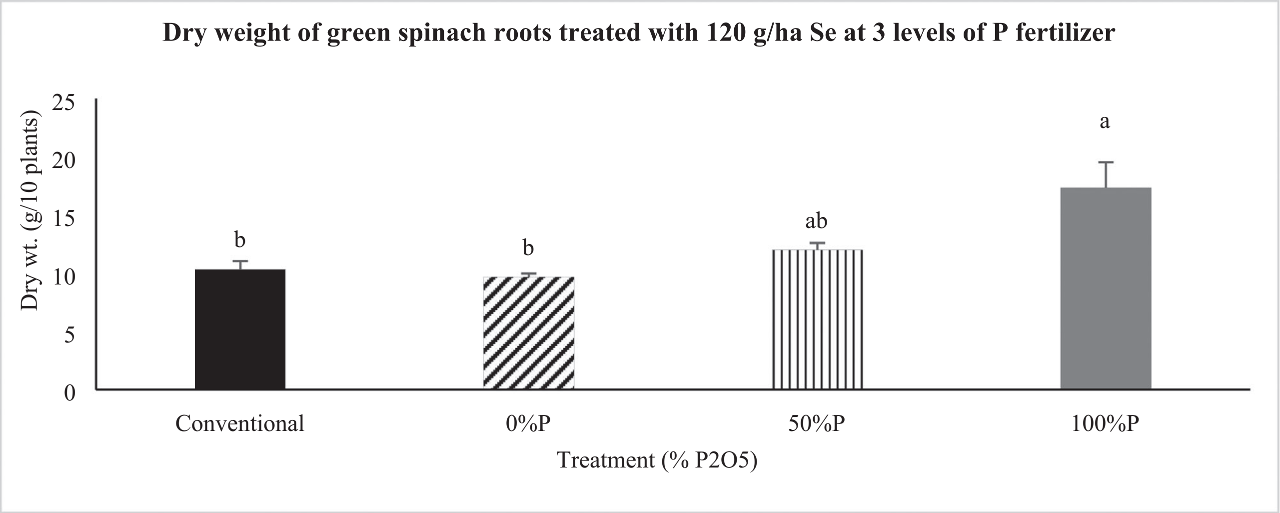


Means with different letters for each P treatment are significantly different (P≤0.05) using Tukey's HSD test.

FIGURE 3 Selenium uptake by stems of green spinach sorted by P fertilizer treatment

2.1.2 | Elemental analysis in plant samples

For plant Se and P elemental analysis, destructive plant samples were taken at harvest (30 DAP) from each replicate of each treatment. Green spinach was separated into three parts (roots, stem, and leaves), and plant samples were dried at 60 °C until a constant weight was obtained. Finally, samples were weighed to determine their dry mass and ground to a fine powder. Dried plant samples were digested with nitric acid as described by Ali et al. (2014), heated for 2 h at 60 °C and 6 h at 125 °C, and diluted to 50 ml with distilled water. The acid digests were analyzed for Se and P using Perkin Elmer Analyst graphite furnace-atomic absorption spectrophotometer (GF-AAS) and AAS, respectively. Certified reference material (tomato [*Lycopersicon esculentum* Mill.] leaves, NIST 1573a) was included for quality assurance. Nutrient uptake was calculated by multiplying the Se concentration by the dry weight of each plant part.



Means with different letters are significantly different (P≤0.05) using Tukey's HSD test.

FIGURE 4 Dry weight of green spinach roots treated with 120 g ha^{-1} Se at three levels of P fertilizer

TABLE 5 Phosphorus uptake by biofortified green spinach at various Se application rates and at 3 levels of P fertilizer

	Phosphorus uptake		
Treatment	**Leaves**	**Stems**	**Roots**
	g 10 plants^{-1}, based on dry wt.		
P fertilizer, %, $n = 12$			
0	0.04 ± 0.002c[a]	0.01 ± 0.001c	0.003 ± 0.0003c
50	0.20 ± 0.01b	0.07 ± 0.004b	0.03 ± 0.001b
100	0.44 ± 0.04a	0.21 ± 0.02a	0.08 ± 0.005a
Selenium rates, g ha^{-1} Se; $n = 9$			
0	0.18 ± 0.04c	0.07 ± 0.02b	0.03 ± 0.009b
60	0.26 ± 0.07ab	0.13 ± 0.05a	0.04 ± 0.01ab
120	0.28 ± 0.08a	0.11 ± 0.04a	0.05 ± 0.01a
180	0.19 ± 0.05bc	0.08 ± 0.07b	0.04 ± 0.06ab
Significance level			
P fertilizer	[***]	[***]	[***]
Se rates	[***]	[***]	[*]
P × Se	[**]	[***]	ns
Mean	0.23	0.10	0.04
CV	24.43	17.33	24.12

Note. ns, not significant.

[a] Means (± standard error) with different letters for each cycle are significantly different ($P \leq .05$) using LSD ($n = 4$).

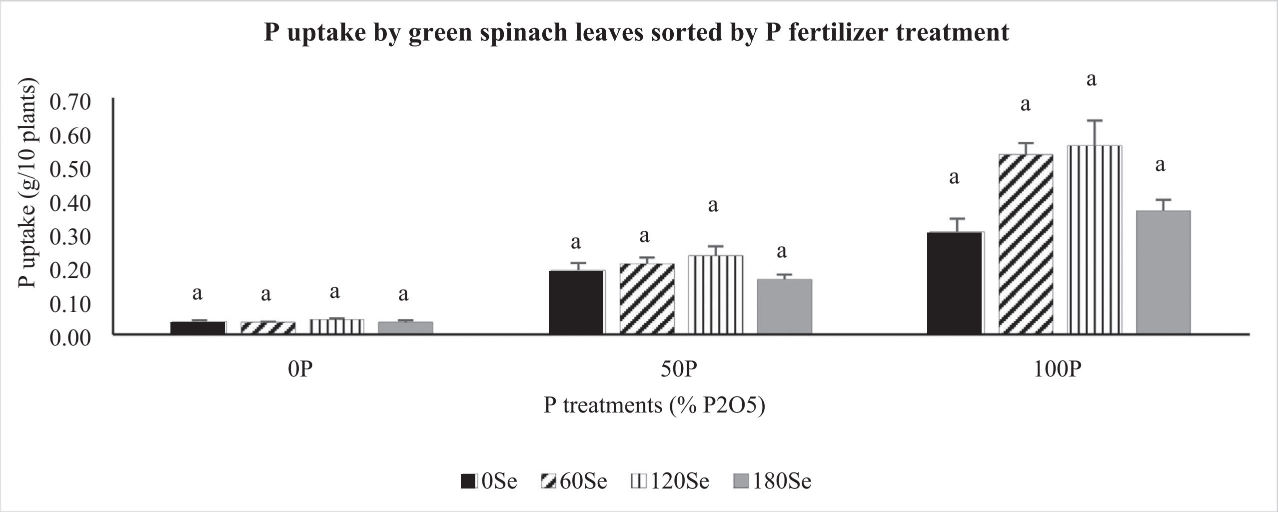
[*] $P \leq .05$.

[**] $P \leq .01$.

[***] $P \leq .001$.

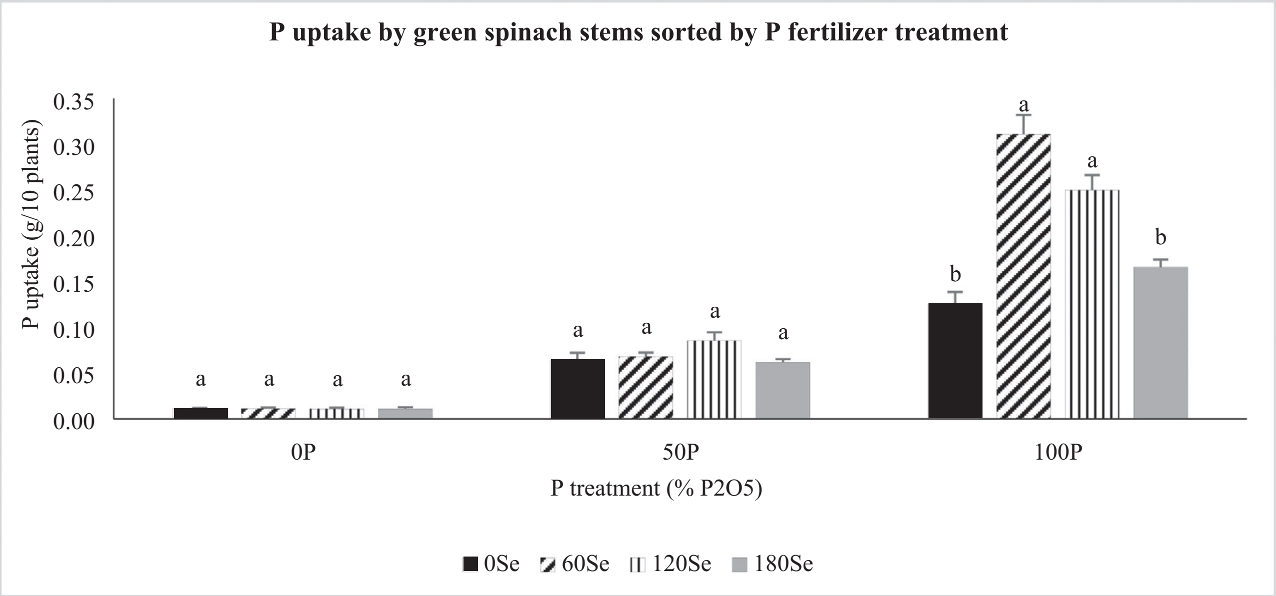
2.1.3 | Statistical analysis

Data on the dry matter and Se and P uptake by green spinach were statistically analyzed using SAS 9.4 software. The significance of treatment effects was determined by ANOVA, and the mean value of each treatment was subjected to multiple comparison analysis using Tukey's honestly significant difference (HSD) at a significance level of $P \leq .05$. Polynomial regression analysis was used to determine the optimum Se fertilization rates for high yield and Se uptake by green spinach.



Means with different letters for each P treatment are significantly different ($P \leq 0.05$) using Tukey's HSD test.

FIGURE 5 Phosphorus uptake by leaves of green spinach sorted by P fertilizer treatment



Means with different letters for each P treatment are significantly different ($P \leq 0.05$) using Tukey's HSD test.

FIGURE 6 Phosphorus uptake by stems of green spinach sorted by P fertilizer treatment

2.2 | Field experiment

The field study was performed for three cycles in an uncultivated open field area in Serdang, Selangor, using the optimum Se fertilization rates obtained from the glasshouse experiment. The soil of the experimental area had a sandy clay loam texture and had not been previously fertilized with Se; therefore, a residual effect from previous Se application in the soil is considered nil. The initial properties of the soil used for the field experiments are shown in Table 2. These experiments were conducted in a randomized complete block design (RCBD).

There were four treatments, and each treatment was replicated four times. Each treatment received the same amount of sodium selenite solution, which was applied to the soil at a rate of 120 g ha^{-1}. However, application of sodium selenite was performed at different times. Control (T1), which without any Se application, was included for comparison to other Se-fertilized treatments. For Se-fertilized plants, Se was applied as follows: (a) at both 7

TABLE 6 Leaf P and Se uptake stoichiometry

Treatment	Leaf P/Se ratio
	μg 10 plants^{-1}
P fertilizer, %; ($n = 12$)	
0	7,790 ± 2,897.8b[a]
50	30,852 ± 9,985.3b
100	59,436 ± 20,412.5a
Selenium rates, g ha^{-1} Se	
0	88,987 ± 24,793.2a
60	15,762 ± 4,114.3b
120	13,322 ± 3,116b
180	12,700 ± 3,878.2b
Significance level	
P fertilizer	***
Se rates	***
P × Se	*
Mean	32,692.61
CV	83.33

[a] Means (± standard error) with different letters for each cycle are significantly different ($P \leq .05$) using LSD ($n = 4$).

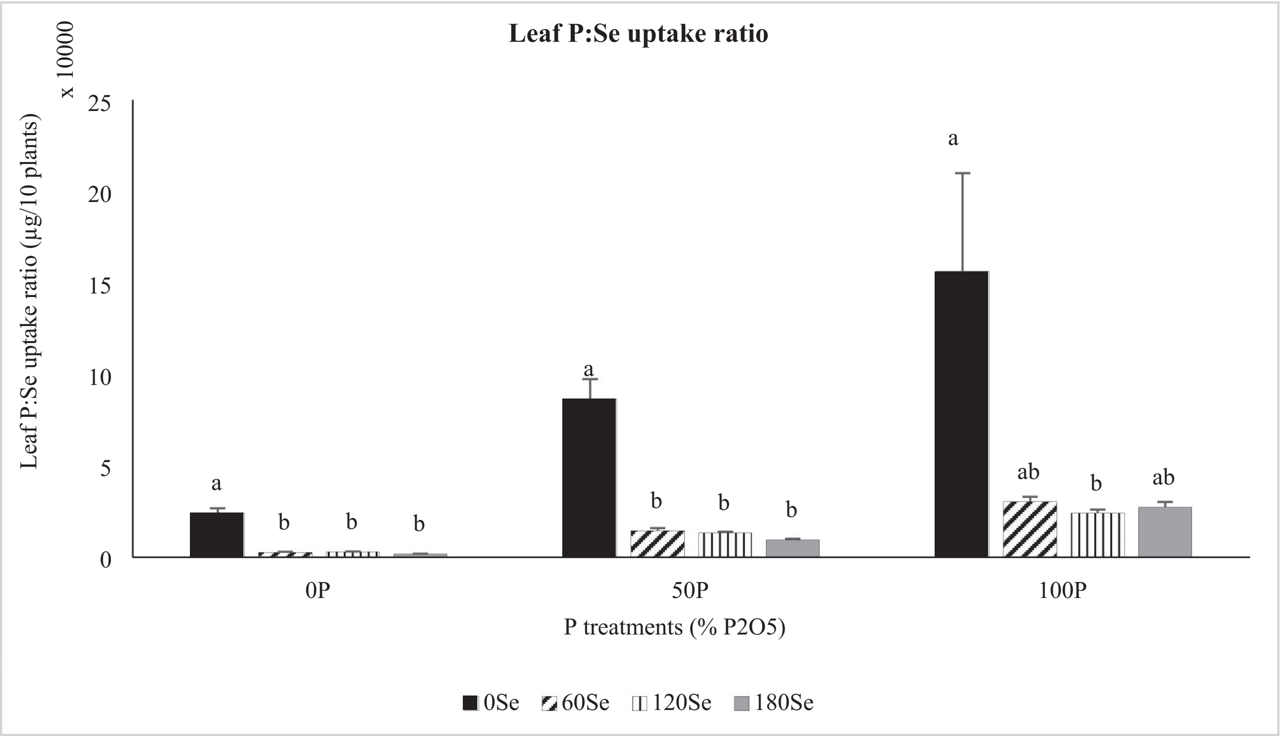
* $P \leq .05$.

*** $P \leq .001$.

and 14 DAP (T2), (b) once at 7 DAP (T3), and (c) once at 14 DAP (T4).

The planting area for each treatment was 1 by 3 m. Green spinach seeds were sown in a sowing tray and transplanted to a bed 1 wk after sowing. The planting distance between plants was kept at 20 by 10 cm. Sodium selenite solution was sprayed manually onto the soil surface according to treatment. No P fertilizer treatment was done in this field experiment because the optimum rates were obtained from the previous glasshouse experiment. Thus, NPK fertilizer was given to all treatment groups at the same rate (100 kg ha^{-1} N, 23 kg ha^{-1} P_2O_5, 144 kg ha^{-1} K_2O).

Plant growth data, such as the plant height and number of leaves, were recorded at 28 DAP. Ten plants were randomly selected from each replicate treatment for the measurement of plant height and number of leaves. Data on the leaf area index (LAI) and light interception were also recorded and measured using an AccuPAR PAR/LAI Ceptometer Model LP-80. The ceptometer is a portable device used to determine the light intercepted by plant canopies as well as to calculate the LAI. The main components of this device are an integrated microprocessor-driven data logger and a probe with 80 sensors. Data were collected by inserting the probe under the canopy (Hossain, Wang, Chen, & Li, 2017). Photosynthetically active radiation (PAR) measurements were recorded from each treatment at 10-min intervals above and below the canopy during days with a



Means with different letters for each P treatment are significantly different (P≤0.05) using Tukey's HSD test.

FIGURE 7 Leaf P/Se ratios of green spinach sorted by P fertilizer treatment

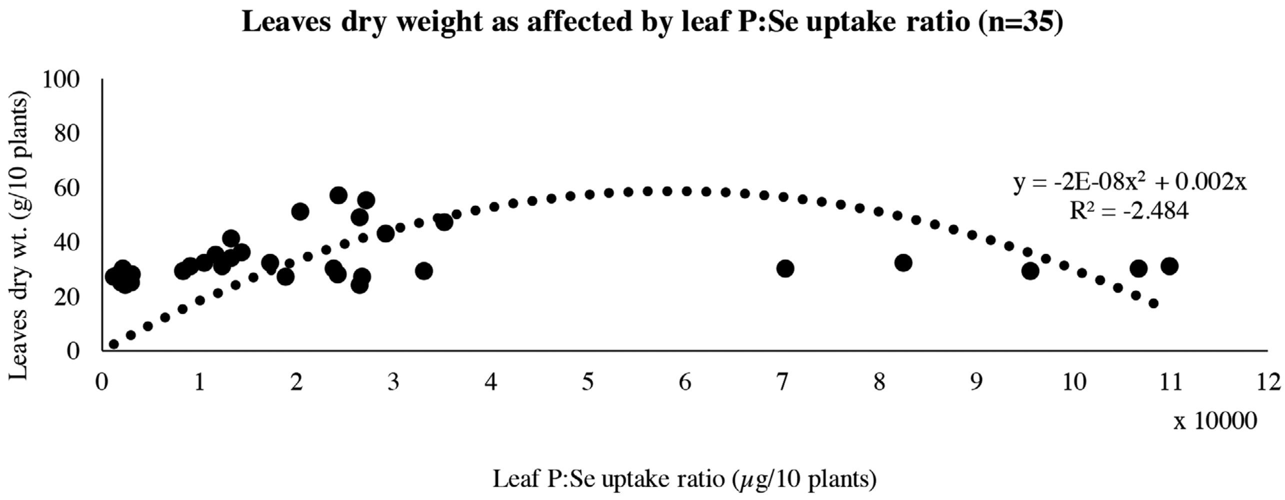


FIGURE 8 Relationship between the stoichiometry of leaf P/Se and the dry weight of leaves

clear sky. The measurements were taken between 1100 and 1300 h. Light interception was calculated using the equation below:

$$\text{Light interception} = 100 - [(\text{Below PAR}/\text{Above PAR}) \times 100]$$

2.2.1 | Plant analysis

At 30 DAP, 10 randomly selected plant samples from each replicate treatment were harvested and divided into roots, stems, and leaves. The plant samples were dried at 60 °C until a constant weight was obtained. The samples were weighed to determine their dry mass and ground to a fine powder. Dried plant samples were digested with nitric acid as described by Ali et al. (2014). Samples were then heated for 2 h at 60 °C and 6 h at 125 °C and diluted to 50 ml with distilled water. The acid digests were analyzed for Se using Perkin Elmer Analyst GF-AAS. Certified reference material (tomato leaves, NIST 1573a) was included for quality assurance. Nutrient uptake was calculated by multiplying the Se concentration by the dry weight of each plant part.

2.2.2 | Statistical analysis

Data on the dry matter and growth of green spinach for all three cycles were statistically analyzed using SAS 9.4 software. The significance of treatment effects was determined by ANOVA, and the mean value of each treatment was subjected to multiple comparison analysis using the LSD test at a significance level of $p \leq .05$. The relationship between dry weight and Se concentration in leaves was determined by regression analysis. Data regarding the Se concentration in leaves and the dry matter of leaves were compiled from glasshouse and field experiments (three cycles).

3 | RESULTS AND DISCUSSION

3.1 | Effect of phosphate on the dry matter and selenium uptake by green spinach

The dry matter of green spinach biofortified with various rates of Se and levels of P fertilizer is shown in Table 3.

The results from the glasshouse study show that the dry matter of green spinach was affected by the P fertilizer level at different Se application rates. As shown in Table 3, there is an interaction between the P and Se treatments. Hence, the dry weights of green spinach sorted by P fertilizer treatment are presented in Figure 1. Based on Figure 1, 100% P fertilizer with application of 120 g ha^{-1} Se produced the highest dry matter, which was significantly greater than the other treatments.

Meanwhile, at 50% P fertilizer, plants treated with 120 g ha^{-1} Se show dry matter that is high but is not significantly different from plants treated with 60 g ha^{-1} Se. For 0% P fertilizer, no significant differences were observed among the treatments. Reducing the P fertilizer to 0 and 50% clearly reduced the dry matter of green spinach.

In terms of Se accumulation, green spinach leaves accumulated the highest amounts of Se, followed by their stems and roots. Selenium uptake by biofortified green spinach is shown in Table 4. Based on the table, the Se uptake by the leaves and stems of green spinach is affected by the P fertilizer level at different Se application rates since there are interactions between the P and Se treatments. Meanwhile, Se intake by roots was not affected by the P

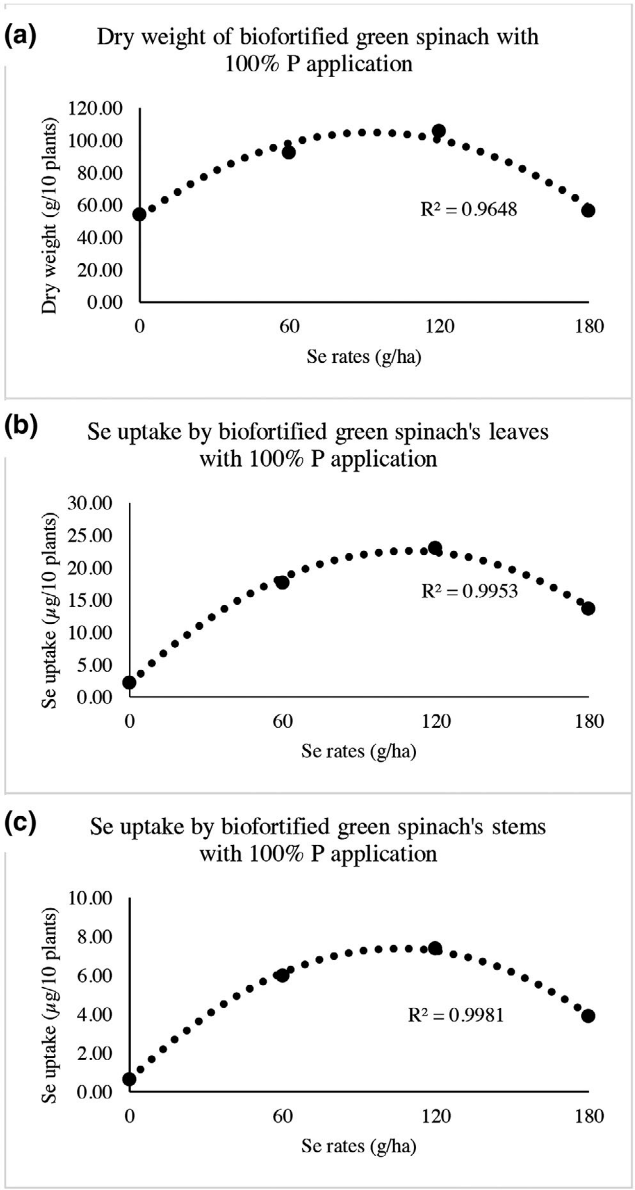


FIGURE 9 Effect of Se fertilization rates on (a) the dry matter and Se uptake by (b) leaves and (c) stems of green spinach

fertilizer level at different rates of Se application. At various P levels, no significant difference was observed in the Se intake by roots, whereas a higher Se intake by roots was observed at Se application rates of 120 and 180 g ha^{-1}, and the lowest Se intake was observed in the untreated control (0 g ha^{-1} Se).

Selenium uptake by leaves sorted by P fertilizer level is shown in Figure 2. In leaves, when 100% P fertilizer was applied, the highest Se accumulation was observed at a Se application rate of 120 g ha^{-1}. Decreased Se uptake by leaves was observed when plants were treated with a higher rate of Se (180 g ha^{-1} Se). Meanwhile, when 50% P fertilizer was applied, no significant differences were observed in the Se uptake by leaves among Se-treated plants. When 0% P fertilizer was applied, the highest Se accumulation was observed in leaves at a Se application rate of 180 g ha^{-1}, whereas control (0 g ha^{-1} Se) plants showed the lowest Se uptake by leaves for each P treatment.

In stems (Figure 3), when 100% P fertilizer was applied, the highest Se accumulation was observed at a Se application rate of 120 g ha^{-1}.

However, no significant differences were observed in the Se uptake by stems between plants subjected to 60 or 120 g ha^{-1} Se treatment. A decrease in Se uptake by stems was observed when plants were treated with a higher rate of Se (180 g ha^{-1} Se). When 50 and 0% P fertilizer were applied, no significant differences were observed in the Se uptake by stems among Se-treated plants. As observed for leaves, control plants (0 g ha^{-1} Se) showed the lowest Se intake by stems for each P treatment.

Earlier studies pointed out that P can reduce the uptake and accumulation of Se in plants (Li et al., 2008; Liu et al., 2018; Mora, Pinilla, Rosas, & Cartes, 2008). However, the results shown in Figures 2 and 3 indicate a higher Se uptake in leaves (23 µg 10 plants^{-1}) and stems (7 µg 10 plants^{-1}) upon application of 100% P fertilizer and 120 g ha^{-1} Se. A lower production of dry matter occurred when no P fertilizer and 180 g ha^{-1} Se were applied. These application rates can therefore be regarded as an inefficient fertilization strategy despite the observation of even higher Se uptake by leaves (21 µg 10 plants^{-1}).

The results of the present study agree with a rice (*Oryza sativa* L.) study by Liu et al. (2004) in which the accumulation of Se in plants was increased by P application according to the plant's requirements. However, if more P was applied than the plant required, Se uptake was inhibited.

There are two possible explanations for this phenomenon. First, as Carter et al. (1972) reported, P addition replaces adsorbed Se in soil due to its higher adsorption capacity, thus releasing Se from the exchange site and eventually increasing the level of Se available in soil for plant use. Second, P application may promote increased plant Se uptake through the improvement in growth of root proliferation.

The effect of P fertilizer application on dry matter of roots has been analyzed and is shown in Figure 4. The results showed that higher root dry matter was observed with application of 100% P and 120 g ha^{-1} Se. According to a study by Goodson, Parker, Amrhein, and Zhang (2003) on root growth in rhizoboxes using Se-enriched soil, there is a tendency for the roots of Se accumulator plants to proliferate in soils where Se is present. In fact, Saffaryazdi, Lahouti, Ganjeali, and Bayat (2012) also found that Se supplementation significantly increased root length by 18–34% compared to untreated plants. Since green spinach

TABLE 7 Plant height and number of leaves of biofortified green spinach with different timings of Se application

	Plant height			Number of leaves		
Treatments	**1st cycle**	**2nd cycle**	**3rd cycle**	**1st cycle**	**2nd cycle**	**3rd cycle**
	cm					
T1 (control)	35.3 ± 1.6b[a]	32.00 ± 0.4c	37.75 ± 0.5b	42.8 ± 0.9a	38.75 ± 0.5b	40.50 ± 1.3b
T2 (7 and 14 DAP)	34.3 ± 2.5b	36.25 ± 0.3b	40.50 ± 0.3a	42.5 ±1.2a	46.50 ± 1.3a	44.75 ± 0.9b
T3 (7 DAP)	27.8 ± 1.0c	30.50 ± 0.3c	34.00 ± 0.7c	34.0 ± 1.7b	38.50 ± 0.6b	42.25 ± 1.7b
T4 (14 DAP)	39.0 ± 1.7a	40.75 ± 1.1a	43.00 ± 1.6a	46.0 ± 1.6a	49.75 ± 1.5a	51.75 ± 1.8a
LSD value ($P \leq .05$; $n = 4$)	3.64	1.79	2.75	3.88	3.89	5.32

[a] Means (± standard error) with different letters for each cycle are significantly different ($P \leq .05$) using LSD ($n = 4$).

TABLE 8 Leaf area index (LAI) and light interception of green spinach biofortified with Se applied at different times

	LAI			Light interception		
Treatments	**1st cycle**	**2nd cycle**	**3rd cycle**	**1st cycle**	**2nd cycle**	**3rd cycle**
				%		
T1 (control)	1.8 ± 0.05b[a]	1.75 ± 0.03b	1.93 ± 0.03b	97.2 ± 0.1b	96.59 ± 0.3b	96.05 ± 0.6b
T2 (7 and 14 DAP)	1.8 ± 0.02b	2.09 ± 0.03a	2.12 ± 0.03a	97.4 ± 0.1b	97.96 ± 0.1a	98.37 ± 0.1a
T3 (7 DAP)	1.6 ± 0.08c	1.70 ± 0.01b	1.90 ± 0.02b	96.6 ± 0.1c	96.51 ± 0.2b	96.74 ± 0.1b
T4 (14 DAP)	2.0 ± 0.03a	2.13 ± 0.03a	2.21 ± 0.08a	98.4 ±0.1a	98.07 ± 0.1a	98.84 ± 0.1a
LSD value ($P \leq .05$; $n = 4$)	0.15	0.09	0.16	0.43	0.58	1.00

[a] Means (± standard error) with different letters for each cycle are significantly different ($P \leq .05$) using LSD ($n = 4$).

is a Se accumulator, an increase in root proliferation was expected, and this increase might account for the plant's increased Se accumulation.

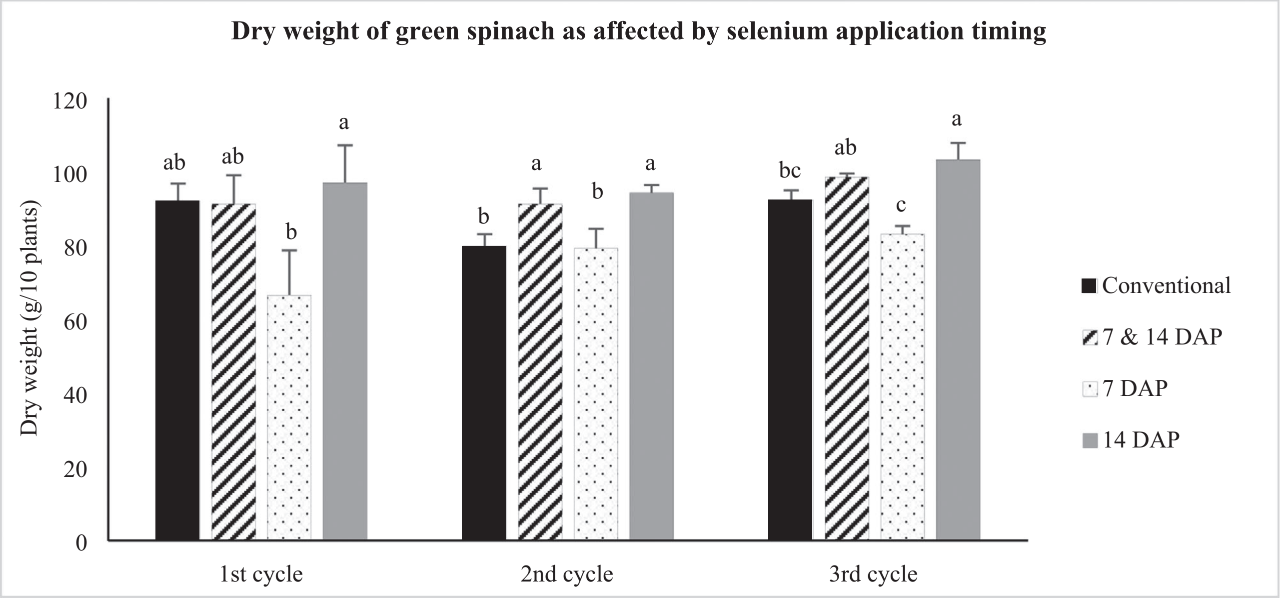
3.2 | Effect of selenium application on phosphorus uptake by green spinach

The supply of selenite enhanced Se accumulation in green spinach in addition to its dry matter. However, the effect of Se application on plant P uptake needed to be determined due to the importance of P in plant health. Table 5 shows the P uptake by biofortified green spinach. Based on the table, P uptake by green spinach leaves and stems is affected by P fertilizer levels at different Se application rates since there are interactions between the P and Se treatments. Meanwhile, the P content in roots was not affected by the P fertilizer level at various Se application rates. At various P rates, there was significant difference observed in the P intake by roots at each P treatment. Higher Se intake by roots was observed at a Se application rate of 120 g ha^{-1}, whereas the lowest Se intake was observed in the untreated control (0 g ha^{-1} Se).

The P uptake by green spinach leaves sorted by P fertilizer level is shown in Figure 5. When 0, 50, or 100% P fertilizer was applied, no significant differences were observed in P uptake by leaves among all treatments. This result indicates that the Se supply did not affect P uptake by green spinach leaves. However, different results were observed for stems. Figure 6 shows the P uptake by green spinach stems sorted by P fertilizer level. Based on the figure, when 50 and 0% P fertilizer was applied, no significant difference was observed for the P uptake by green spinach stems among all treatments. However, when 100% P fertilizer was applied, a higher P uptake by the stems of green spinach was observed at Se application rates of 60 and 120 g ha^{-1}, whereas Se addition at 180 g ha^{-1} led to reduced P uptake by stems, but significant differences from the control were not observed.

3.3 | Leaf phosphorus and selenium uptake stoichiometry for green spinach

Interactions between P and micronutrients have been reported in a wide variety of plants. Hence, the stoichiometric approach of determining the ratio between leaf P and Se uptake (the leaf P/Se ratio) was used to evaluate whether P uptake is limited during Se application. Table 6 shows leaf P/Se uptake for green spinach. The leaf P/Se ratio was found to be affected by the P fertilizer level at



Means with different letters for each cycle are significantly different at $P \leq 0.05$ using LSD (n=4)

FIGURE 10 Dry matter of green spinach biofortified with Se at different application times

different Se application rates, which indicates the presence of an interaction between P and Se treatments.

Figure 7 shows the leaf P/Se ratios of green spinach sorted by P fertilizer treatment. Based on the figure, it was found that the leaf P/Se ratios for Se-treated plants were significantly lower than those in plants without Se application at the 0 and 50% P fertilizer levels. With 100% P fertilizer application, plants without Se application showed the highest leaf P/Se ratios, and these ratios were significantly different from those of plants treated with 120 g ha^{-1} Se. Meanwhile, no significant differences were observed for leaf P/Se uptake with other Se-treated plants.

The generally high values of the leaf P/Se ratios indicated that Se application did not suppress P uptake. A higher P content was observed in leaves compared to the content of Se. The lower leaf P/Se ratios in Se-treated plants compared to untreated plants results from the denominator value of Se. No comparisons could be made with leaf P/Se ratios from previous studies. However, the value of the leaf P/Se ratio can be attributed to the growth of the green spinach.

Figure 8 shows the relationship between the stoichiometry of leaf P/Se and the dry weight of leaves. The data indicate that the leaf P/Se ratios directly affect plant dry matter. Decreased dry matter was observed when the leaf P/Se ratio exceeded 5×10^4 µg 10 plants^{-1}. The optimum leaf P/Se ratio for producing higher dry matter was approximately 4×10^4 µg 10 plants^{-1}, whereas ratios above or below this value resulted in a lower dry weight.

3.4 | Optimum selenium fertilization rates for high yield and selenium uptake by green spinach

Dry matter and Se uptake by green spinach leaves and stems following application of 100% P fertilizer are shown in Figure 9. The data show that the optimum rate for applying Se to sandy clay soil as Se(IV) solution is 120 g ha^{-1}. Higher Se application rates led to an increased Se concentration in green spinach leaves and stems but also reduced plant yield and decreased Se uptake by green spinach.

3.5 | Effect of selenium application timing on growth, dry matter, and selenium accumulation by green spinach

The three cycles of field experiments showed that the time of Se application affected green spinach growth when the same amount of Se (120 g ha^{-1}) was applied. In agreement with the meta-analysis by Ros et al. (2016), it was found that application time was the main factor controlling crop response to Se fertilizer. The plant height and number of leaves of green spinach with different timings of Se application are shown in Table 7.

Selenium application at 14 DAP (T4) resulted in the highest plant height at maturity (28 DAP), and the differences in plant height between Se-treated and non-treated (control) plants were statistically significant for all three cycles. Regarding leaf number, no significant differences were observed between plants treated with Se at 14 DAP (T4) and control plants (T1) during the first cycle. However, during the second and third cycles, significant differences were observed in leaf number between T4 and control (T1) plants.

In addition to the plant height and number of leaves, the leaf area index (LAI) and light interception were also measured. Table 8 shows the LAI and light interception measurements of green spinach biofortified with Se applied at different times.

From the results, it was found that plants treated with Se at 14 DAP (T4) exhibited high LAI and light interception and that these values differed significantly from those of control (T1) plants for all three cycles. According to Rodrigo, Santamaria, and Poblaciones (2014), many authors have recommended a single application of Se for wheat (*Triticum* spp.) during either the stem elongation stage or during the emergence of the flag leaf ligule/collar (GS-39 according to the Zadocks scale). A single application of Se during this stage of growth is considered advantageous compared to several Se applications at different times.

The data shown in Tables 7 and 8 indicate that the active growth stage in green spinach occurs after 14 DAP. Hence, Se application at 14 DAP effectively facilitated plant growth. Although no significant differences were observed in leaf number between the two times split application (T2) and the single application (T4) in the first and second cycles and, further, no significant differences in plant LAI and light interception between plants in the T2 and T4 groups during the second and third cycles, the minimum number of applications was preferable because it requires less labor and is more economical.

In terms of yield, Figure 10 shows the dry matter of green spinach biofortified with Se at different application times. The data indicate that Se application timing affected dry matter. Plants treated with Se at 14 DAP (T4) showed higher dry matter for all three cycles than plants treated with Se at 7 DAP (T3). No significant differences were observed between plants receiving the two times split application (T2) and the single application (T4).

Selenium application did not increase plant dry matter during the first cycle of field experiments, as no significant differences were observed between control (T1) and Se-treated (T2, T3, T4) plants. However, during the second and third cycles of planting, increases of 18 and 12%, respectively, were observed in dry matter for plants treated with Se at 14 DAP (T4) compared to control (T1) plants. According to Ros et al. (2016), application of Se fertilizer during the vegetative stage of crops enables quick Se uptake and might also stimulate plant growth. However, in the present study, plants treated with Se at 14 DAP showed the highest dry matter, and plants treated with Se at 7 DAP exhibited

TABLE 9 Selenium uptake by green spinach biofortified with Se at different application times

Treatments	Leaves			Stems			Roots		
	1st cycle	2nd cycle	3rd cycle	1st cycle	2nd cycle	3rd cycle	1st cycle	2nd cycle	3rd cycle
T1 (Control)	2.22 ± 0.1c[a]	1.73 ± 0.1c	1.82 ± 0.1c	0.53 ± 0.1b	0.65 ± 0.03c	0.51 ±0.1c	0.20 ± 0.03b	0.19 ± 0.03b	0.21 ± 0.02b
T2 (7 and 14 DAP)	21.14 ± 1.0b	20.72 ± 1.0b	21.43 ± 1.3b	3.45 ± 0.5b	4.15 ±0.2b	4.66 ± 0.2b	1.48 ± 0.1a	1.63 ±0.1a	1.42 ± 0.1a
T3 (7 DAP)	29.20 ±0.7a	26.04 ± 0.8a	29.18 ± 0.5a	4.48 ± 0.2b	5.35 ± 0.3a	5.12 ± 0.4b	1.80 ± 0.3a	1.85 ± 0.2a	1.37 ± 0.1a
T4 (14 DAP)	21.16 ± 0.6b	21.54 ± 0.3b	22.38 ± 1.0b	5.89 ± 0.4a	5.95 ± 0.5a	5.98 ± 0.3a	1.60 ± 0.2a	1.81 ± 0.1a	1.55 ± 0.1a
LSD value ($P \leq .05$; $n = 4$)	1.60	2.13	2.37	1.13	0.89	0.82	0.52	0.43	0.31

[a] Means (± standard error) with different letters for each cycle are significantly different ($P \leq .05$) using LSD ($n = 4$).

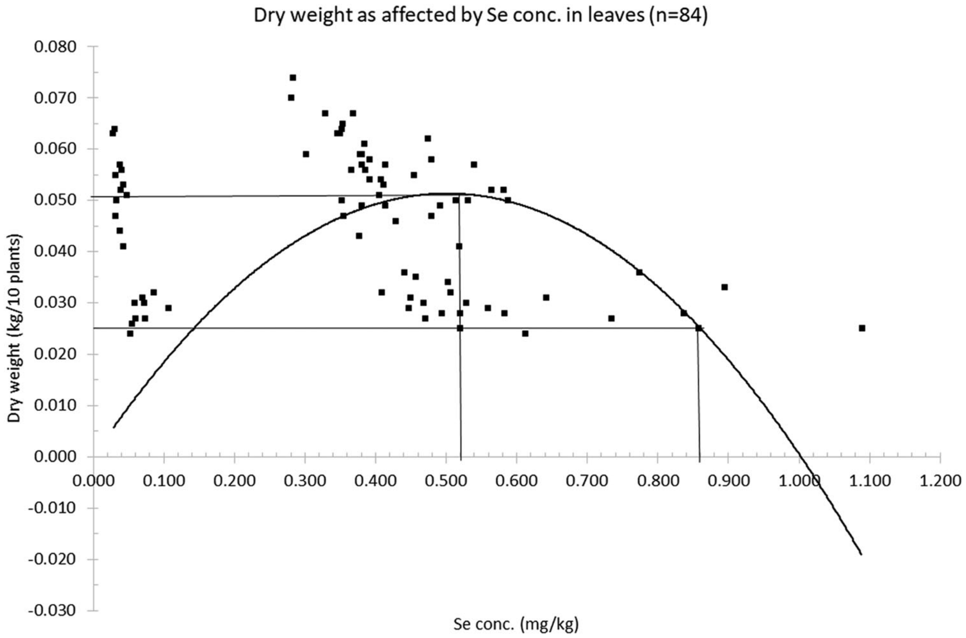


FIGURE 11 Effect of the Se concentration in leaves on the dry matter of leaves

the lowest dry matter, though the difference from control (T1) plants was not statistically significant.

In terms of accumulation, Table 9 shows the Se uptake by green spinach. Leaves accumulated the highest amounts of Se, followed by stems and roots.

Although same Se was applied at the same rate, green spinach leaves exhibited higher Se uptake (26.04–29.20 μg 10 plants^{-1}) when the whole amount of 120 g ha^{-1} Se was applied at 7 DAP (T3). Leaf uptake of Se was statistically significantly higher in T3 plants than in the other treatment groups for all three cycles.

In stems, although no significant differences were observed in the Se uptake between Se application at 7 DAP (T3) and 14 DAP (T4) during the second cycle of the field experiment, during the first and third cycles, higher stem Se accumulation (5.89–5.98 μg 10 plants^{-1}) was observed when Se was applied at 14 DAP (T4). For roots, the Se uptake was unaffected by Se application timing, as there was no significant difference observed in Se-treated plants (T2, T3, T4) for all three cycles.

According to Loganathan and Hedley (2006), the accessibility of soil-applied Se tends to decline over time. A single application of Se has been shown to be inadequate to maintain Se levels in pastures for the entire growing season. Soil reactions and interactions involving selenite may have depleted the availability of Se. However, the results from the T3 treatment group show that Se fertilizer applied during the early growth stage increased the plant uptake of applied Se. This finding is in agreement with Ros et al. (2016), who showed that early application of Se can stimulate rapid uptake of Se but not plant yield.

Although a higher Se uptake was observed when Se was applied at 7 DAP, Se application at 14 DAP could also be considered an appropriate time of application of this element because it results in higher dry matter values (94.25–103.25 g 10 plants^{-1}). Treatment at 14 DAP also leads to the highest plant height, LAI, and light interception at maturity (Tables 5 and 6). In fact, treatment at 14 DAP produces the highest Se accumulation in stems (5.89–5.98 μg 10 plants^{-1}). Although no significant differences were observed in dry matter (Figure 6) and leaf Se uptake (Table 7) between the two times split application at 7 and 14 DAP (T2) and the single application at 14 DAP (T4), the minimum number of applications was preferable because it requires less labor and is more economical.

3.6 | Relationship between the selenium concentration in leaves and dry weight

In the T3 treatment group, which received Se treatment at 7 DAP, the high concentration of Se in leaves (avg. 0.63 mg kg^{-1}, dry wt.) and the small plant size may cause Se intolerance and reduce leaf dry weight. Figure 11 shows the dry weight of leaves as affected by the Se concentration in leaves.

Data on the dry matter and Se concentration were compiled from both glasshouse and field experiments with Se application rates between 0 and 180 g ha^{-1}. Based on the plot, a higher dry matter of leaves (50 g 10 plants^{-1}) was observed when the Se concentration was approximately 0.52 mg kg^{-1}. There was a reduction in dry matter of approximately 50% when the Se concentration in leaves reached 0.87 mg kg^{-1}. As Xue et al. (2000) mentioned, based on the study on ryegrass and lettuce (*Lactuca sativa* L.), at low Se concentrations (5.64–6.43 mg kg^{-1}), Se acts as an antioxidant and nourishes plant growth, whereas at higher Se concentrations (71.9–270 mg kg^{-1}), oxidative stress occurs in which Se acts as a pro-oxidant and reduces crop yield (Saha, Fayiga, & Sonon, 2017). A study by Rani, Dhillon, and Dhillon (2005) found that the critical level of Se in plants treated with Se(IV) leading to a significant decrease in yield was 104.8 mg kg^{-1} in raya (*Brassica juncea* Czern L.), 76.9 mg kg^{-1} in maize (*Zea mays* L.), 41.5 mg kg^{-1} in rice, and 18.9 mg kg^{-1} in wheat shoots. The data suggest that the toxic level of Se in green spinach at which yield declines by 50% (TL-50) is 0.86 mg kg^{-1}, as shown in Figure 11.

4 | CONCLUSION

In conclusion, the glasshouse experiment showed that applying P fertilizer as recommended for green spinach increases the yield and Se uptake by leaves and stems of green spinach. Application of Se to sandy clay soil in the form of Se(IV) solution at 120 g ha^{-1} can be considered the optimum application rate for green spinach as higher rates reduce plant yield and decrease Se uptake. In terms of the time of application, in three cycles of field experiments, a single application of Se at 14 DAP produced the best results in terms of growth and dry matter of green spinach. Although the highest Se accumulation in leaves was observed when Se was applied at 7 DAP (26.04–29.20 μg 10 plants^{-1}), this treatment time has a detrimental effect on yield (13% lower than control) and is thus regarded as an inappropriate time of application. Therefore, with optimum Se accumulation in leaves (21.16–22.38 μg 10 plants^{-1}) and stems (5.89–5.98 μg 10 plants^{-1}), Se application in the form of Se(IV) solution at 14 DAP at a rate of 120 g ha^{-1} has been identified as an effective Se fertilization management strategy for producing Se-biofortified green spinach.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest regarding this manuscript.

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