

BIOLOGICAL CHANGES OF GREEN PEA (*PISUM SATIVUM* L.) BY SELENIUM ENRICHMENT

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Supplement of common fertilizers with selenium (Se) for crop production will be an effective way to produce selenium-rich food and feed. The value of green pea seeds and forages as alternative protein source can be improved by using agronomic biofortification. Therefore, biological changes of green pea (*Pisum sativum* L.) and influences of inorganic forms of Se (sodium selenite and sodium selenate) at different concentrations on the accumulation of magnesium (Mg) and phosphorus (P) were investigated in greenhouse experiment. 3 mg kg⁻¹ of selenite had positive effects to enhance photosynthetic attributes and decrease lipid peroxidation significantly. At the same time, Se accumulation increased in all parts of plant by increasing Se supply. Moreover, Mg and P accumulations were significantly increased at 3 mg kg⁻¹ selenite and 1 mg kg⁻¹ selenate treatments, respectively. By contrast higher selenite concentrations (≥30 mg kg⁻¹) exerted toxic effects on plants. Relative chlorophyll content, actual photochemical efficiency of PSII (Φ_{PSII}) and Mg accumulation showed significant decrease while membrane lipid peroxidation increased. Thus, the present findings prove Se biofortification has positive effects on biological traits of green pea to provide it as a proper functional product.

Keywords: Biological changes – sodium selenit – sodium selenate – biofortification – green pea

INTRODUCTION

Deficiencies in mineral micronutrients, including iron (Fe), zinc (Zn), selenium (Se), and iodine (I), are affecting more than 50% of the world population [50]. Other minerals, such as calcium (Ca), magnesium (Mg), phosphorus (P), and copper (Cu) can also be deficient in the diets of some populations [42].

Biofortification, with aims of increasing micronutrient amounts in the edible parts of plants via breeding or the applying of biotechnology, is considered to be a cost-effective way to diminish micronutrient malnutrition in the rural populations in developing countries where the problem is most common [23, 27]. Biofortification may also include other approaches, such as the use of micronutrient fertilizers (agronomic biofortification) or enhancement of micronutrient bioavailability by manipulating the levels of pronutrient or antinutrient components in foods [10].

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Se is an essential element absorbed mostly from dietary sources in animals and humans. It is important in the prevention of several cancers, protection against viral infections oxidative stress, inflammation and suppression of HIV progression to AIDS [7]. Crop plants play an important role as a source of Se. Agronomic biofortification with Se enriched fertilizer has been widely tested based on field, pot and hydroponic experiments [3, 5, 9, 20, 40]. Currently, possible interactions and competition between Se and other major and trace elements are key scientific issues, too [13, 18, 45]. In plants, Se influences enzymatic antioxidant activities [8, 14], in which essential cations acted as enzyme co-factors. These cations play crucial roles in plant metabolism. Mg is involved in the maintenance of membrane stability and plant tissues integrity. Mg is also essential for the enzymatic activity of glutathione synthetase and ATPases [22]. In addition, phosphorus (P) is an essential macronutrient for plant growth and development [38, 44], too. As P is often deficient in soil or exists in unavailable forms that cannot be directly utilized by plants [1, 32], crops require a large amount of P fertilizer to maintain normal growth in more than 30% of the world's arable land [39]. The application of P fertilizer improves crop production, but at the expense of causing severe environmental pollution and depletion of non-renewable P rocks [38, 39].

Plant species belonging to *Fabaceae* family are the second most important crops after cereals. Legumes are known as primary plant protein source. *Pisum sativum*, the common pea plant is one of the most important legumes. Pea is valued protein source primarily for the nutritional quality of its seeds for animal feed and human consumption while its pods and shoots can be used, as forage, too. Both pea seeds and forages are rich in protein including lysine and other essential amino acids [2]. Se can easily enter into amino acids instead of sulphur hence an elevated protein content of plants can contribute to the higher accumulation of Se [29]. This fact gives green pea the potential to be used in Se biofortification programs [36].

The objective of this study was to investigate the biological changes of green pea by sodium selenite and sodium selenate enrichment at different concentrations and also to evaluate the effects of them on the uptake and accumulation of macronutrients (Mg and P) in different plant tissues.

MATERIALS AND METHODS

The greenhouse pot experiments were performed with calcareous chernozem soil obtained from the Látókép Experimental Station of Debrecen University (N: 47°33', E: 21°27', 113–118 m above of sea level). The parameters of this soil (Table 1) were essentially the same as previously described by Nagy et al. [26].

Eleven kg soil was weighed into Mitscherlich type pots (50 × 50 cm²). A 100 mL additional NPK fertilization (contain 1.43 g nitrogen as KNO₃, 0.2291 g P₂O₅ as KH₂PO₄ and 0.1487 g K₂O as K₂SO₄ per pot) and 100 mL Se (as two forms of sodium selenite (Na₂SeO₃; active form: Se^{IV}) and sodium selenate (Na₂SeO₄; active form: Se^{VI}) in five and four different concentrations, respectively, as follows: 0 (con-

Table 1
Characteristics of the experimental soil

Depth	0–0.3 m
pH (KCl)	5.71
pH (H ₂ O)	6.58
Soil texture category	loamy clay
Total water-soluble salt	0.015%
CaCO ₃	0.202%
Humus (organic matter)	3.54%
KCl-soluble NO ₃ -N+NO ₂ -N	8.04 mg kg ⁻¹
AL-soluble P ₂ O ₅	199 mg kg ⁻¹
AL-soluble K ₂ O	451 mg kg ⁻¹
AL-soluble Na	332 mg kg ⁻¹
AL: 0.1 mol dm ⁻³ ammonium-lactate and 0.1 mol dm ⁻³ acetic-acid	
KCl-soluble Mg	176 mg kg ⁻¹
KCl-soluble SO ₄ ²⁻ -S	6.04 mg kg ⁻¹
KCl-EDTA-soluble Cu	5.79 mg kg ⁻¹
KCl-EDTA-soluble Zn	7.9 mg kg ⁻¹
KCl-EDTA-soluble Mn	262 mg kg ⁻¹

trol), 1, 3, 10, 30 and 90 mg kg⁻¹ and 0 (control), 1, 3, 10 and 30 mg kg⁻¹, prepared with distilled water) were mixed and manually sprayed and supplemented to the soil as an aqueous solution – as evenly as possible – using dispenser bottles of 0.5 L (nominal volume). Green peas (*Pisum sativum* L.) were sown in separate experiments with three replications and the bi-factorial trials were arranged in a randomized complete block design. Pots were weighed daily and water loss was supplemented with ion exchanged water. At the third principal stage of growth; Stem elongation (the third true leaf has unfolded at the third node), immature plants were removed so that eight intact and mature plants were remained in every pot. Growing period lasted 50 days in May and June and the plants were harvested at the end of the seventh principal stage of growth; Development of fruit [Pods have reached typical size (green ripe)-peas fully formed].

Three mg kg⁻¹ Se^{VI} treatments stayed at flowering stage (sixth principal stage of growth) and did not grow more; 10 and 30 mg kg⁻¹ Se^{VI} treatments did not even grow and stayed at the end of 0 principal stage of growth; Germination (Emergence: shoot breaks through soil surface).

Relative chlorophyll content (SPAD value)

SPAD values as a relative chlorophyll content in the leaf were measured under natural conditions by using the SPAD chlorophyll meter (SPAD-502; Konica Minolta Sensing, Inc., Japan). The last fully developed leaves were measured at the end of growing period in 5 repetitions per pot.

Chlorophyll fluorescence parameters

Based on fluorescence induction kinetics, fluorescence parameters and ratios have been established to assess photochemical activity of plants. The parameters of *in vivo* chlorophyll fluorescence were detected with a PAM 2100 (Heinz Walz GmbH, Germany) modulated light fluorometer as described [33]. Samples were dark-adapted for 20 minutes. After dark adaptation, the initial fluorescence (F_0) was excited by weak light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) and the maximal fluorescence (F_m) was induced by white saturating flash light ($8000 \mu\text{mol m}^{-2} \text{s}^{-1}$). The actual photochemical efficiency of PSII as yield ($\Delta F/F_m = (F_m' - F_0)/F_m'$) was measured on the last fully developed leaves – at the end of growing period – on light-acclimated conditions under natural light between 11:00–12:00 h. The photosynthetically active radiation was around $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ and the temperature was around $30 \text{ }^\circ\text{C}$.

Malondialdehyde content

Lipid peroxidation (LPO) was determined – at the end of growing period – from leaf blade by the method of Zhang and Huang [49], by measuring the amount of malondialdehyde (MDA). The leaf tissues (~100 mg) were homogenized in 1 mL 0.1% (w/v) trichloroacetic acid (TCA) solution using cold mortar and pestle. The homogenates were centrifuged at $10,000 \times g$ for 10 min. And then 4 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA solution was added into 1 mL of supernatant and incubated at $96 \text{ }^\circ\text{C}$ for 30 min. The tubes were cooled by transferring into an ice bath. The absorbance of the supernatant was recorded at 532 nm. Standard curve was generated from MDA standard. The concentration of MDA in samples was calculated from absorbance calibration curve.

Peroxidase (POX) activity

Peroxidase activity of leaves was assayed by the method followed by Sanchez et al. [31]. The activity of peroxidase was expressed as:

Specific activity ($\text{UA mg}^{-1} \text{ protein}$) = Unit activity ($\text{U min}^{-1} \text{ g}^{-1} \text{ FM}$)/Protein content ($\text{mg g}^{-1} \text{ FM}$).

Quantification of total Se, Mg and P

Element analysis was carried out by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Perkin Elmer OPTIMA 3300 DV) and inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Elemental X7). Dried samples (1 ± 0.01 g) were homogenized and decomposed by $\text{HNO}_3\text{-H}_2\text{O}_2$ treatment as previously described [16]. Briefly, samples were kept in 10 mL concentrated HNO_3 overnight, then heated to 60°C for 45 min in a LABOR MIM OE 718/A block digestion apparatus. Following the first digestion step, 3 mL 30% H_2O_2 was added to the samples and digestion was continued at 120°C for another 90 min. After cooling the samples to room temperature, volume was adjusted to 50 mL with deionized water. Samples were then mixed by shaking and filtered through FILTRAK 388 filters.

Data analysis

Data were statistically analyzed using SPSS, 19.0 software (2010). Standard error was calculated and analysis of variance (ANOVA) was performed on the data to determine the least significance difference (Tukey test) between treatment means with the level of significance at $P \leq 0.05$.

RESULTS

Photosynthetic parameters

Although chlorophyll content is sensitive to environmental changes, no significant difference in relative chlorophyll content was shown in green pea leaves treated with Se^{IV} in 1–30 mg kg^{-1} concentration range (Fig. 1A). In contrast, 90 mg kg^{-1} Se^{IV} significantly decreased the relative chlorophyll content by 34.7% in comparison to control.

The maximal photochemical efficiency of PSII (Fv/Fm) is derived parameter of chlorophyll *a* fluorescence transient, point out to maximal quantum yield of PSII did not change significantly in case of Se^{IV} 0–90 mg kg^{-1} and Se^{VI} 1 mg kg^{-1} concentration range, respectively (Fig. 1B). On the other hand, 3 mg kg^{-1} Se^{IV} increased the actual photochemical efficiency of PSII (Φ_{PSII}) significantly, whereas; 30 and 90 mg kg^{-1} Se^{IV} treatments decreased this value (Fig. 1C).

Malondialdehyde content

The concentration of MDA in the shoot tissues can indicate the level of oxidative damage caused by Se added to the soil. The accumulation of MDA in the green pea leaves was stimulated after the Se treatment, by 13% in the presence of 90 mg kg^{-1}

Se^{IV}, as compared to the control plants (Fig. 1D). On the other hand, in plants supplied individually with 3 mg kg⁻¹ Se^{IV}, the MDA concentration significantly decreased by 18.4% in comparison to the control plants.

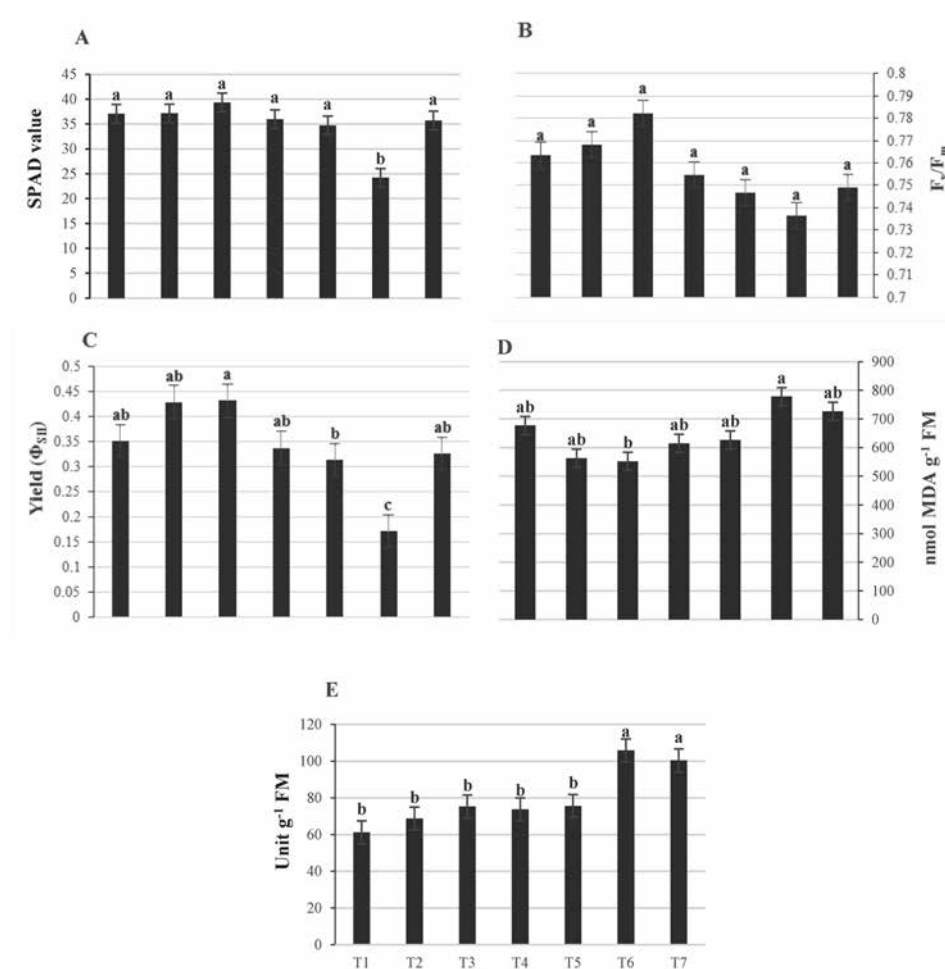


Fig. 1. Effect of different concentration of applied Se forms induced changes in (A) SPAD chlorophyll content, (B) maximal photochemical efficiency of PSII (F_v/F_m) (C) actual photochemical efficiency of PSII (Φ_{PSII}), (D) concentration of MDA in leaves (E) activity of peroxidase in leaves at 50 days stage of growth. T1 = control; T2 = Se^{IV} (1 mg kg⁻¹); T3 = Se^{IV} (3 mg kg⁻¹); T4 = Se^{IV} (10 mg kg⁻¹); T5 = Se^{IV} (30 mg kg⁻¹); T6 = Se^{IV} (90 mg kg⁻¹); T7 = Se^{VI} (1 mg kg⁻¹). Significant differences in the mean value of each treatment group are indicated by different lower case letter based on Tukey test ($p < 0.05$, $n = 5 \pm s.e.$) and the same lower case letters shows no significant difference between the treatments

Peroxidase (POX) activity

The treatment of plants with various concentration of Se^{IV} (1, 3, 10, 30, 90 mg kg⁻¹) and 1 mg kg⁻¹ Se^{VI} increased the POX activity of leaves by 10.9%, 18.6%, 16.9%, 18.8% 42.1% and 39% over the control (Fig. 1E).

Quantification of total Se

The total Se content in all of the green pea organs increased when enhancing both Se^{IV} and Se^{VI} concentrations in the soil (Table 2). The relationship between the total Se content and the Se^{VI} dose (0 and 1 mg kg⁻¹) was linear, and in the 1 mg kg⁻¹ Se^{VI}-exposed green pea the total Se content in roots was 1.3-, 6.8- and 5.7-fold higher than in shoots, pods and seeds, respectively. A 30 and 90 mg kg⁻¹ Se^{IV} treated samples displayed significant differences at lower concentrations of Se^{IV} in all of the organs and roots, seeds, shoots and pods had the order of the most to the least total Se content.

Total Se contents of green pea plants in different parts of roots, shoots, pods, and seeds showed an significant increase after biofortification, with respect to the initial concentrations of Se in plants not supplemented with Se (controls); without supplementation only traces of Se could be detected (Table 2).

Table 2

The accumulation of Se in the green pea plant's organs (mg kg⁻¹ DM) cultivated with different concentration of applied Se forms (selenite: Se^{IV} and selenate: Se^{VI}) for a growing period of 50 days

Applied Se (mg kg ⁻¹)	Root	Shoot	Pod	Seed
Control	0.84±0.23 ^c	0.32±0.03 ^d	0.19±0.01 ^d	0.25±0.1 ^c
1 Se ^{IV}	3.87±0.00 ^c	0.36±0.01 ^d	0.23±0.02 ^d	0.26±0.3 ^c
3 Se ^{IV}	49.35±4.88 ^c	3.83±0.35 ^d	2.80±0.10 ^d	7.39±1.13 ^c
10 Se ^{IV}	147±64 ^c	10.02±0.36 ^c	7.85±0.22 ^c	16.5±0.62 ^c
30 Se ^{IV}	541±81 ^b	20.97±0.93 ^b	18.90±0.56 ^b	41.5±5.17 ^b
90 Se ^{IV}	1401±64 ^a	56.37±4.61 ^a	54.07±3.52 ^a	343±16 ^a
Applied Se (mg kg ⁻¹)	Root	Shoot	Pod	Seed
Control	0.84±0.23	0.32±0.03	0.19±0.01	0.25±0.1
1 Se ^{IV}	1220±0	886±6.08	178±4	214±16

Significant differences in the mean value of each treatment group are indicated by different lower case letter based on Tukey test ($p < 0.05$, $n = 3 \pm s.e.$).

Table 3
The accumulation of Mg and S in the aboveground organs of green pea plant (mg kg^{-1} DM) cultivated with different concentration of applied Se (selenite: Se^{IV} and selenate: Se^{VI}) forms for a growing period of 50 days

Element	Plant part	Applied Se (mg kg^{-1})							
		Control	1 Se^{IV}	3 Se^{IV}	10 Se^{IV}	30 Se^{IV}	90 Se^{IV}	1 Se^{VI}	
Mg	Shoot	36±5 ^b	2337±367 ^a	2470±259 ^a	2049±80 ^a	2033±154 ^a	2002±53 ^a	2046±680 ^a	
	Pod	26±3 ^c	1174±134 ^{ab}	1365±162 ^a	1103±206 ^{ab}	962±42 ^{ab}	504±50 ^{bc}	1023±197 ^{ab}	
	Seed	10±3 ^b	448±114 ^a	516±95 ^a	373±85 ^{ab}	360±78 ^{ab}	11±0 ^b	361±50 ^{ab}	
P	Shoot	19±2 ^d	666±127 ^c	753±61 ^c	948±37 ^b	814±56 ^{bc}	661±22 ^c	1205±58 ^a	
	Pod	35±2 ^c	950±59 ^{abc}	678±50 ^{abc}	1212±94 ^{ab}	1118±100 ^{ab}	878±70 ^{bc}	1948±698 ^a	
	Seed	85±0 ^b	3432±62 ^a	3609±614 ^a	4564±492 ^a	3831±123 ^a	1044±108 ^b	5037±438 ^a	

Significant differences in the mean value of each treatment group are indicated by different lower case letter based on Tukey test ($p < 0.05$, $n = 3 \pm s.e.$).

Quantification of total Mg and P in the aboveground organs

The total Mg content in the 3 mg kg⁻¹ of Se^{IV} treated green pea was the highest and 98% increase was observed in all of the aboveground organs, in comparison to the controls (Table 3). Moreover, in all concentrations, the order according to the highest amounts was shoot, pod and seed. Whereas the total P content in the 1 mg kg⁻¹ of Se^{VI} was the highest in all of the organs and by 98% increase was seen comparing with the controls. In contrast, total amount of P accumulated in the highest degree, into the seed, followed by pod and shoot, respectively.

Treatments with 90 mg kg⁻¹ of Se^{IV} caused a significant decrease in both Mg and S levels in different parts of the plant. In addition, the total Mg and S contents in different parts of green pea situated above the ground such as shoots, pods, and seeds showed a significant increase after biofortification, with respect to the initial concentrations of them in plants not supplemented with Se (controls) (Table 3).

DISCUSSIONS

There is increasing evidence that Se can have beneficial effects on the growth, yield formation and stress tolerance of plants [11]. The physiological, biochemical or molecular mechanisms behind the stimulated growth and improved tolerance have not yet been determined completely. Nevertheless, enhanced antioxidant capacity (reviewed in 11) and more efficient accumulation of carbohydrates [37] are thought to be contributing factors in the better performance of the plants.

The response of green pea plants to Se exposure has been previously described by only a few authors [29, 35], hence the literature in this field is quite limited.

It is believed that improved growth is the result of efficient chlorophyll fluorescence parameters and enhanced chlorophyll synthesis. The findings of the present study revealed that effective quantum yield of PSII photochemistry (Φ_{PSII}) increased significantly in the presence of Se^{IV} (3 mg kg⁻¹) whereas, increasing the concentration of Se lowered this parameter and also chlorophyll content. Our findings are in line with work that corroborated low doses of Se enhanced photosynthesis in rice seedlings [41]. However, Se toxicity induces the damage of photosynthetic apparatus, inhibits photosynthesis, and results in the over-production of starch [41].

MDA formation in plants exposed to adverse environmental conditions is a consequence of lipid peroxidation caused by oxidative stress [17]. In green pea plants supplied with 3 mg kg⁻¹ Se^{IV}, MDA concentrations in the leaf tissues decreased significantly, as compared to the control plants. In the plants supplied with the higher dose of Se^{IV} (90 mg kg⁻¹), the level of MDA was the highest. Consistent with our results, several studies have also shown that Se supplementation may counteract the accumulation of harmful lipid peroxides in the plant cells [28, 30, 46]. These results can be attributed to the anti-oxidative effects of Se on plants reported previously [11, 12, 28].

POX is a type of antioxidant enzymes that is triggered in plants to balance the excess of reactive oxygen species (ROS) [4]. This antioxidant can react with ROS directly or indirectly via enzyme catalysis to counteract the production of ROS, under stress conditions, as Mittler [24] believed that ROS, under control conditions, act as signals for the activation of the stress response and defence pathways. In the present investigation, under excess of Se, enzymatic POX antioxidant system increased to scavenge the Se induced excess ROS. It has been shown that excess Se gives rise to the robust accumulation of ROS in plants, although the actual role of Se in plants has not yet been resolved [25]. Feng et al. [8] proposed that the increased production of ROS at high Se levels may be partially related to an imbalance in the levels of glutathione (GSH), thiols (SH), ferredoxins (Fdred) and/or NADPH, which can play vital roles in the assimilation of Se. If these substances are not sufficient to simultaneously meet the needs of Se-assimilation and ROS quenching, the addition of Se may lead to a ROS burst and the inhibition of plant growth. However, in the present study, treatment of plants with 90 mg kg⁻¹ Se^{IV} enhanced the participation of antioxidant POX at protective mechanisms.

Different parts of green pea plants exposed to (treated with) Se^{VI} presented higher concentrations of total Se, as a likely consequence of selenite using the sulphate path through plants [51], other than its generally higher uptake/retention and translocation efficiencies [13, 15]. Also, Se levels in the root tissues were much higher than in the aboveground parts in case of all Se^{IV} concentrations applied and in case of 1 mg kg⁻¹ Se^{VI} as well.

Based on this study we can conclude that 3 mg kg⁻¹ of Se^{IV} increases the accumulation of Mg in green pea plants and especially in its shoots but high concentration of Se^{IV} (≥ 30 mg kg⁻¹) inhibits this process. A number of literature data [6, 21, 43, 47, 48] confirm these findings. Also, 1 mg kg⁻¹ of Se^{VI} caused the highest accumulation of P in all the aboveground parts of green pea plants and particulares in the seeds. On the other hand, excessive uptake of Se (90 mg kg⁻¹ Se^{IV}) by the plant made a reverse condition. Earlier results of Sing [34] and Liu and Gu [19] are in agreement with our findings, too.

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