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The Response of Cowpea (*Vigna unguiculata L*) Plants to Foliar Application of Sodium Selenate and Selenium Nanoparticles (SeNPs)

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Abstract

The present study describes the synthesis and rapid production of selenium nanoparticles (SeNPs) by reducing selenate in the presence of Ascorbic Acid (AA) as a reductant, coating, and stabilizing agent. The formation of nanosized selenium at 10 mm sodium selenate (Na_2SeO_4) and 1.5% AA was confirmed by the appearance of the characteristic surface plasmon absorption peak at 296 nm in UV-vis spectra. Transmission Electron Microscopy (TEM) indicates that SeNPs was mostly spherical with a mean diameter of approximately 33.4 nm. X-RAY Diffraction (XRD) pattern confirmed crystalline shape_indicating particle size of approximately 42.92 nm. The particle size distribution of SeNPs was approximately 45.9 nm by Dynamic Light Scattering (DLS). Fourier Transform Infrared (FTIR) spectroscopic analysis indicates the coating of selenium nanoparticles with ascorbic acid bonding of SeNPs with the COO^- group of ascorbic acid.

A pot experiment at the Experimental Farm of Helwan University, Cairo, Egypt during the season of 2016 was performed to evaluate the effects of foliar applications of both Na_2SeO_4 and chemically synthesized SeNPs (≈ 33.4 nm) each at 0.0, 6.25, 12.5, 25 and 50 µM on vegetative growth, yield and some physiological activities of cowpea (*Vigna unguiculata* L) plants. Foliar application of Na_2SeO_4 and SeNPs up to 25 µM significantly increased growth criteria (i.e. length of roots and stem, the root, stem and leaves fresh and dry weights, No of leaves and total leaves area cm^2/plant), weight and quality of seed compared to the corresponding untreated control plants.

Application of Na_2SeO_4 and SeNPs, especially at 6.25 µM concentration increased the Total Photosynthetic Pigments (TPP), Total Carbohydrate (TC), total soluble proteins (TSP), and different minerals in leaves accompanied by decrease in Total Soluble Sugars (TSS). SeNPs at 6.25 µM increased the levels of the growth hormones Indole Acetic Acid (IAA), Gibberellic Acid (GA_3) and Cytokinins (CKs) of cowpea leaves, with a relatively lower Abscisic Acid (ABA) content and higher GA_3/ABA ratios, followed by Na_2SeO_4 at 6.25 µM which explains the increase in growth parameters and seed weight in SeNPs and Na_2SeO_4 treated plants compared to control plants. Atomic Absorption Spectroscopy (AAS) study unveiled the residual accumulation of selenium nanoparticles in leaves and seeds of cowpea plants at 50 µM. Transmission electron microscopy showed small, dark deposits in leaf cells exposed to SeNPs, which probably originated from the nanoparticles absorbed onto the leaves and transferred to seeds.

In conclusion, application of SeNPs and Na_2SeO_4 at 6.25 µM improved vegetative growth, seed weight, nutritional value and quality of cowpea plants and seeds.

Keywords

Cowpea (*Vigna unguiculata* L); Sodium selenate; Selenium nanoparticles; Synthesis; Vegetative growth; Yield; Photosynthetic pigments; Biochemical constituents

Introduction

Cowpea (Family: Fabaceae) is one of the most ancient crops known to man. Cowpea (*Vigna ungiculata* L) is an economically important food legume in Africa [1]. Nowadays it is widely adapted and grown throughout the world. Cowpea is a versatile crop, grown as a grain legume mainly for dry beans and green pods and also as forage, green manure, and cover crop and for soil fertilization [2].

Selenium is an essential microelement for the adequate and healthy life of humans, animals, and some microorganisms. Selenium enters the food chain through the plants which take it up from soil. Selenium is a structural component of several enzymes with physiologically antioxidant properties, including glutathione peroxidases [3]. In human and animal cells, Se incorporates an antioxidative system in trace amounts, whereas the excess is harmful. In higher plants, Se has not been confirmed as an essential nutrient [4]. However, there are indications that it shows dual effect depending on Se concentration in plants, the chemical form of Se, concentration, and bioavailability in soil and soil microorganisms. Se delays plant senescence [5] increase resistance against oxidative stress [6] and improves their tolerance by enhancing their antioxidative capacity [7]. Se does appear to be a beneficial nutrient for hyper accumulator's plants, which can reach twofold higher biomass in the presence of Se [8] and also enhanced resistance to Se-sensitive herbivores and pathogens [9]. Depending on the dosage, Se has a dual effect on ryegrass; at low concentrations, it acts as an antioxidant and can stimulate the plant growth, whereas at higher concentrations it acts as a pro-oxidant reducing the yields [10].

Recently, nanoparticles of elemental selenium (Se^0) have attracted considerable attention because of their unique physical and chemical properties, which differ from the properties of the corresponding bulk materials [11]. Nano-Se, which is the bright red, soluble, highly stable and nano-defined size in the redox state of zero (Se^0), has been manufactured for use in both of the nutritional supplements and developed for applications in medical therapy [12]. Numerous important factors affect the properties and biological activities of nanoparticles, including their shape, size, surface charge and surface functional groups [13]. Se-nanoparticles could be synthesized within the reduction of a Se-salt with a reducing agent, usually in the presence of a stabilizing agent to prevent the clusters of Se atoms from growing [14]. Chemical, physical, and biological methods can be used for selenium synthesis, whereas the chemical and biological methods are common use. The chemical reduction method deals with the reduction of metal particles to nanoparticles using chemical reducing agents such as sodium borohydride or sodium citrate [15].

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Other chemical agents utilized for Se synthesis include tetra-n-tetrafluoroborate (TFATFB), hexadecyltrimethylammonium bromide (CTAB) [16], poly (N-vinyl pyrrolidine) (PVP), ethyl alcohol [17] and L-ascorbic acid [18].

Several studies have been demonstrated that Se may exert diverse beneficial effects at low concentrations as an antioxidant and a growth-promoting agent in higher plants [19]. Se at low doses increased the reproductive capacity of seeds to 43% and the concentration of selenium in seeds, tops and roots of *Brassica rapa* plants [20], significantly increased plant growth and yield components of canola under normal and salt stress conditions [21], improved grain yield and quality of wheat plants under normal and water-deficit conditions [22], increased lentil biomass and grain yield [23], maintain higher growth, fresh and dry matter drought tolerance indexes of two wheat genotypes seedlings [24] and enhanced photosynthesis, stomatal conductance, carboxylation efficiency and Rubisco content of *Nicotiana tabacum* [25]. However, at high doses, Se toxicity is supposed to be due to the replacement of S atoms by Se in S-containing amino acids; result in changes in the structure and activity of Se-substituted proteins and consequently resulting in a decrease in plant growth [26], Se acts as pro-oxidant and catalyze the oxidation of thiols and simultaneously generate superoxide that can damage cellular components [27] resulting in metabolic disturbances and a reduction in yield [28]. The inhibitory effect of the high level of Se was reported in cucumber [19] and two wheat genotypes [29].

On the other hand, Nano-Se has a higher efficiency in up-regulating selenoenzymes and exhibits less toxicity than selenite [27]. In tobacco, SeNPs significantly stimulated the organogenesis and the growth of root system while they completely inhibited by selenate [30]. In tomato, a low concentration of Nano-Se and Se, improve plant growth parameters and chlorophyll content more effectively than a higher concentration of Nano-Se/Se under high and low-temperature stress [31]. In cluster bean, fertilization with selenium nanoparticles improved the growth, yield performance and biochemical characteristics [32].

Thus Se and SeNPs are expected to influence the growth and yield of cowpea plants. A few studies have been published concerning the effect of nano-Se and other inorganic Se forms on higher plants. Therefore, the main objective of this study was to detect the effect of foliar application of sodium selenate and nano-Se on vegetative growth, yield and nutritional value and quality of seed, Se accumulation in leaves and seeds as well as some physiological activities in cowpea plants in terms of the potential use of the optimum concentrations on this species for further field applications.

Materials and Methods

Chemicals

All biochemicals used in this study were of high purity, purchased from Sigma-Aldrich Chemical Co., Germany and Merck (Rio de Janeiro, RJ, Brazil) and used without further purification. Distilled and deionized water was used in all experimental work.

Synthesis of selenium nanoparticles (SeNPs)

Synthesis of selenium nanoparticles (SeNPs) were carried out by the reduction of sodium selenate according to [18] method using ascorbic acid as a reducing and stabilizing agent with slight modification, wherein sodium selenate was used instead of selenite. A stock of aq. solution of 10 mM sodium selenate [33] and ascorbic acid powder 1.5% (w/v) were reacted. The ascorbic acid powder was added to sodium selenate solution under magnetic stirring at room temperature for 15 min. The mixture was allowed to react till the color change was observed from colorless to light orange, then absorbance was measured spectrophotometrically at wavelength 300 nm each 15min for 1hr to ensure reaction complete and no significant change in absorbance.

After the color change was observed the mixture was diluted directly to ≈ 6.25, 12.5, 25, and 50 µM using deionized water for direct application on plants, the pH of the diluted solutions was 3.4. Selenium nanoparticles were characterized using various analytical techniques.

Characterization of selenium nanoparticles

The characterization of SeNPs was performed by UV-Vis spectroscopy, Transmission Electron Microscopy (TEM) and the Fourier Transform Infrared (FT-IR) spectroscopy at National Research Center. Dynamic Light Scattering (DLS) and X-Ray Diffraction (XRD) at Atomic Energy Authority, Cairo, Egypt.

UV-Vis spectrum of SeNPs solution was recorded in the range of 290-350 nm at a resolution of 1 nm using a T80+UV/VIS Spectrometer PG Instruments Ltd. The exact size and morphology of the synthesized SeNPs was seen under TEM model JEOL electron microscope JEM-100 CX (JEM-2100 HR). DLS measurements were performed to determine the average particle size and distribution of SeNPs by using PSS-NICOMP 380-ZLS particle sizing system, St. Barbara, California, USA. The nature and size of SeNPs was analyzed by X-Ray Diffraction (XRD) using XRD-6000 series, including stress analysis, residual austenite quantitation, crystallite size/lattice strain, crystallinity calculation, materials analysis via overlaid X-ray diffraction patterns Shimadzu apparatus using nickel-filter and Cu-Ka target, Shimadzu Scientific Instruments (SSI), Kyoto, Japan. The diffracted intensities were recorded from 0° to 90° 2θ angles. FT-IR measurements were carried out in order to obtain information about presence and chemical structure of coating around the SeNPs for their stabilization and to understand the transformation of functional groups due to the reduction process. FTIR spectra of SeNPs, ascorbic acid and Na_2SeO_4 (control) were recorded from 400-4000 cm^{-1} wavenumbers at a resolution of 2 cm^{-1} using FT/IR-6100 spectrometer by employing KBr Pellet technique.

Time course experiment and treatments

A pot experiment was conducted at the Experimental Farm of Helwan University, Cairo, Egypt, from March to July 2016. A homogenous lot of seeds of cowpea (*Vigna unguiculata* L) were provided by the Horticulture Research Institute, Agriculture Research Center, Ministry of Agriculture, Giza, Egypt. Ten seeds of cowpea were sown in each earthenware pots (40 cm diameter and 30 cm in depth), filled with 15 kg of clay loamy soil in texture (consists of clay 50.04%, silt 28.96%, fine sand 15.86%, and coarse sand 5.14%). The well-established plants were thinned to 5 plants in each pot. The pots were divided into two groups; each was subdivided into 5 subgroups, including thirty earthenware pots. A foliar spray with 500 ml was applied twice to cowpea plants at 45 and 52 Days After Sowing (DAS), with sodium selenate (Na_2SeO_4) and selenate nanoparticles (SeNPs), each at 0.0, 6.25, 12.5, 25 and 50 µM. Control plants were sprayed with distilled water. The pots were arranged in complete randomized block designs with different treatments.

Fertilization was carried out for each pot at a proportion of 2 g

calcium superphosphate (15.5% P_2O_5) pre-sowing; similarly, nitrogen in the form of ammonium nitrate (33.0% N) was applied at the rate of 1 g/ pot before the first irrigation. Potassium sulfate (48% K_2O) was added at the rate of 1 g/ pot to the soil in two equal doses at 21 and 35 days after sowing. Irrigation was done regularly to maintain soil field capacity during the experiment.

Growth criteria

At pre flowing stage after two weeks from the second foliar spray (75 days). Ten plants (5 replicates) were randomly collected early in the morning from each of the experimental groups, carefully washed with distilled water and then different growth parameters (i.e. stem length, root length, leaf number, fresh and dry weights of stem, root, and leaves (g)/plant and total leaves area (cm^2 $plant^{-1}$) were recorded. Dry weights were obtained by drying plant samples in an oven with drift fan at 70°C until constant weights. Representative fresh samples were taken from each treatment for determination of photosynthetic pigments and phytohormones contents.

At the fruiting stage, harvested seeds were taken from different groups to determine the dry weight of seeds, total carbohydrates, crude protein and selenium content in the seeds and leaves.

Photosynthetic pigments

Photosynthetic pigments including chlorophylls (a and b) and carotenoids were determined in fresh leaves of cowpea (*Vigna unguiculata* L) plants at 75 DAS [34]. The concentration of chlorophyll a, b and carotenoids were calculated as mg/g fresh weight equivalent.

Total soluble sugars

Total soluble sugars (TSS) were determined in the dry powdered cowpea leaves using anthrone technique [35]. Two ml anthrone solution (2 g L^{-1} H_2SO_4 95%) were added to 1 ml sample and maintained on a boiling water-bath for 3 min. After cooling, the developed color was measured spectrophotometrically at 620 nm using spectrophotometer (Cecil CE. 1010). Standard curve of glucose was prepared and used for calculating the content of TSS in samples.

Total carbohydrates

Dry samples of leaves and seeds were used to determine the total carbohydrate (TC). Briefly, 30 mg of dry powdered samples were hydrolyzed in 10 ml of 1N H_2SO_4, in digestion tubes, (80-90°C) for 8 hr. This was made up to a definite volume. Then the total soluble saccharides were determined as described above.

Total soluble proteins

Total soluble proteins in the dry powdered cowpea leaves were determined following the procedure [36]. One ml cowpea extract was mixed with 5 ml freshly mixed solution (50:1 v/v) of 2% sodium carbonate in 0.4% sodium hydroxide and 0.5% copper sulphate in 1% sodium tartrate. The mixture was left 10 minutes before addition of 0.5 ml Folin and made up to a definite volume. The optical density of the mixture was measured spectrophotometrically after 30 minutes at 750 nm using Cecil CE 1010 spectrophotometer. Standard curve of bovine serum albumin was prepared and used for the determination of the protein content in cowpea samples.

Crude protein

Crude protein percentage (CP) in the dry samples of seeds was calculated by multiplying the values of total N by 6.25 [37].

Proline content

The method [38] was used to estimate free proline in approximately 0.5 g air-dry leaves. The absorbance was measured at 520 nm using toluene as a blank. Proline concentration was determined from a standard curve and calculated as mg proline/g dry weight.

Minerals and total Se concentration

Mineral ion contents in air-dry leaves and seeds of cowpea plants were estimated at the soil, water and Environmental Research Institute (SWERI), Agriculture Research Center, Giza. After wet digestion [39] the acid digest of the plant matter was analyzed for determination of nitrogen, phosphorus, potassium, calcium, sulphur, magnesium and selenium according to the following methods. Total nitrogen was determined using the modified Micro-Kjeldahl method [37], phosphorus was determined colorimetrically at wavelength 660 nm using the vanadate molybdate method [39] and calculated using a standard curve of dihydrogen phosphate [40] Potassium concentration was determined using flame photometer (Atomic spectra AAS vario 6) [41]. Concentrations of calcium and magnesium were measured by using Inductively Coupled Spectrometry Plasma (ICP) Model Ultima 2-JobinYvon. Sulphur was determined by atomic absorption spectrophotometer according to the method [42] and Se was measured in air-dry leaves and seeds of cowpea plants according to the method [43].

Endogenous phytohormones

Phytohormones were analyzed at the Arid Land Agriculture Research (ALAR) and Services Center, Faculty of Agriculture, Ain Shams University. Ten grams fresh young leaves of cowpea developed from different treatments were used for the extraction of phytohormones according to a modified method [44]. The samples were ground in cold 80% ethanol, followed by triple extraction with fresh ethanol for 24 hours at 0°C and the combined extracts were evaporated to the aqueous phase in a rotator flash evaporator. To estimate the amounts of acidic hormones (fraction I), the aqueous phase was adjusted to pH 8.6 and partitioned three times with ethyl acetate. The combined ethyl acetate fraction was evaporated to dryness. The aqueous phase was adjusted to pH 2.8 and portioned three times with ethyl acetate. The combined acidic ethyl acetate was reduced in volume (fraction 1), ready to High-Performance Liquid Chromatography (HPLC) for determination of acidic hormones (IAA, ABA, GA_3). The dried basic ethyl acetate fraction was dissolved in 80% ethanol. The ethanol was evaporated, leaving an aqueous phase which was adjusted to pH 2.8 and partitioned three times with ethyl acetate. The remaining aqueous phase was adjusted to pH 5.5 and portioned three times with water-saturated n-butanol. All butanol phases were combined (fraction 2), reduced in volume and stored at -20°C until HPLC analysis for cytokinins. For identification and determination of hormones, 10 µl of sample was injected into HPLC 510 using data model (Waters 746), detector (U.V Tumable Absorbance), and pump (HPLC 510). The chromatography was fitted (equipped with 3.9 × 300 mµ Bond pack C18 capillary column). The HPLC was operated under temp 25°C. The retention time (RT) and the area of peaks of different authentic standards (IAA, GA_3, ABA, benzyl adenine and kinetin) were used for the identification and characterization of peaks of samples under investigation.

Preparation of sample for light and transmission electron microscopy (LM and TEM)

At fruiting stage, samples of cowpea leaves and seeds were cut

in small pieces (1 × 1 mm) and prepared using standard protocols for electron microscopy [45]. Plant tissues were pre-fixed in 4% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.3 for 4 h, followed by two rinses in the same buffer for a period of 20 min. The tissues were then post-fixed in osmium tetraoxide OsO_4 for 2 h, followed by washing three times for 30 min in sodium cacodylate buffer. The tissue sections were then dehydrated in ascending grades of ethanol from 10 to 100%, 10 minutes in each concentration except the finely one 100% for 30 min for three changes (each one for 10 minutes). The samples were then cleaned in propylene oxide for two changes, each 10 min. The specimens were put in equal volumes of propylene oxide and Epon 812 for one hour at room temperature. Pour off half the (propylene oxide-epon) mixture in a waste bottle and add 2 volume of Epon 812 for 3 h at room temperature. The samples were put in a pure Epon 812 resin overnight and subsequently embedded in Epon 812 resin mixture and polymerized in the oven at 60°C for 24 hours. Semi-thin and Ultra-thin sections were obtained from these blocks by using Reichert-Jung Ultracut ultramicrotome.

For optical microscopy, Semi-thin sections were mounted on glass slides, stained in 1% toluidin blue and observed with OLYMPUS CX31 light microscopy provided with ToupCam HD Camera. Ultra-thin sections from selected blocks were mounted on copper grids, double-stained with urarnyl acetate and lead citrate, and observed with a Jeol-JEM-2100 HR-Japan transmission electron microscope.

Statistical analysis

The data were expressed as the mean of five replicates values for growth criteria and as mean of triplicate values for the photosynthetic pigments, carbohydrates, and proteins. Statistical analysis was performed using one-way analysis of variance ANOVA followed by Duncan's Multiple Comparison Test using IBM Statistical Product and Service Solutions, SPSS Statistics for Windows, Version 21at $p<0.05$ that was denoted as being statistically significant for the means compared, using the least significant difference (LSD at 5% level).

Results

Characterization of the SeNP

UV-vis spectrophotometer: Reduction of selenium ions into selenium nanoparticles by ascorbic acid was observed as a result of the colour change from colourless to light orange (Figure 1b). The UV-vis spectrophotometer in a range of wavelength from 290 to 350 nm was observed for selenium nanoparticles solution (Figure 1a). Strong absorption peak was observed between 290 nm to 310 nm with maxima at 296 nm. This broad peak is corresponding to the selenium nanoparticles. Previous studies have shown that the spherical Se-NPs contribute to the absorption bands at around 250-400 nm in the UV-visible spectra [46] reported λ max at 280 nm, [47] at 380 nm and [48] at 270 nm. In the present study, the broad peak of selenium nanoparticles appeared at approximately 296 nm. This result is in agreement with [49] demonstrating that the reducing agent was strong enough to ensure complete conversion of the precursor molecules into nano-sized selenium particles. SeNPs are known to exhibit a regular maximum absorption in the wavelength region of about 300 cm^{-1} when spherical particles with the size of 30-100 nm are formed, depending on the experimental condition.

TEM analysis and dynamic light scattering (DLS): The TEM micrograph of SeNPs performed at 10 mM sodium selenate and 1.5% ascorbic acid confirms the spherical shape of the particles and their uniform distribution without any significant aggregation at a mean diameter of 33.4 nm (Figure 2a). At the same conditions, an average particle size distribution of approximately 45.9 nm was assessed by using the DLS method (Figure 2b). Particle sizes and distribution were mainly dependent upon spectral analysis [50].

X-ray Diffraction analysis (XRD) of SeNPs: The X-ray Diffraction pattern of nanoselenium in the spectrum of 2θ values ranging from (0) to (90) is shown in Figure 2c. The XRD pattern for the synthesized Se-NPs shows diffraction peaks at 2θ (degrees) of 23.22°, 29.52°, 41.14°, 43.60°, 45.50°, 51.48°, 55.50°, 61.40°, 64.88° and 71.40° which correspond to the (100), (101), (110), (102), (111), (201), (112), (202), (210) and (113) planes of the Se-NPs, respectively. The sharpness of the diffraction peaks revealed that the product is well crystallized. The long sharp peak at (101) plane indicates the approximately uniform size of nano selenium particles formed.

The full-width-at-half-maximum (FWHM) value was measured for 101 planes of reflection and used to calculate the crystallite size of the Se-NPs by using Scherrer's equation (Eq. 1).

$$D=K\lambda/(\beta \cos \theta) \quad (1)$$

Where D is the grain size, K is a constant taken to be 0.94, λ is the wavelength of the X-ray radiation equal 15.405 nm, β is FWHM in radians of the peaks and θ (Bragg angle) is the angle of diffraction.

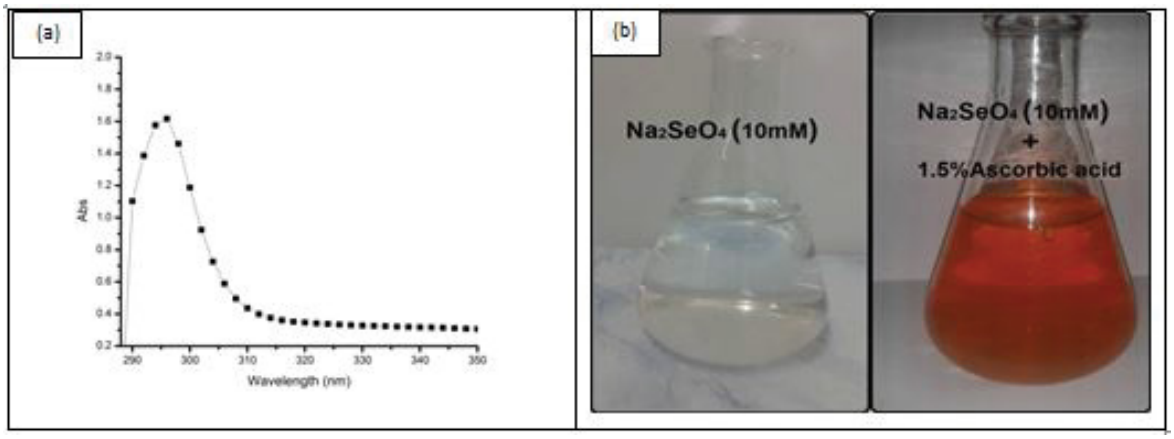

Figure 1: Characterization of the synthesized SeNPs using UV-VIS spectra in an aqueous phase. (a): Effect of 10 mM sodium selenate (Na_2SeO_4) and 1.5% ascorbic acid at pH 2.6; (b) Light orange colour of fabricated SeNPs.

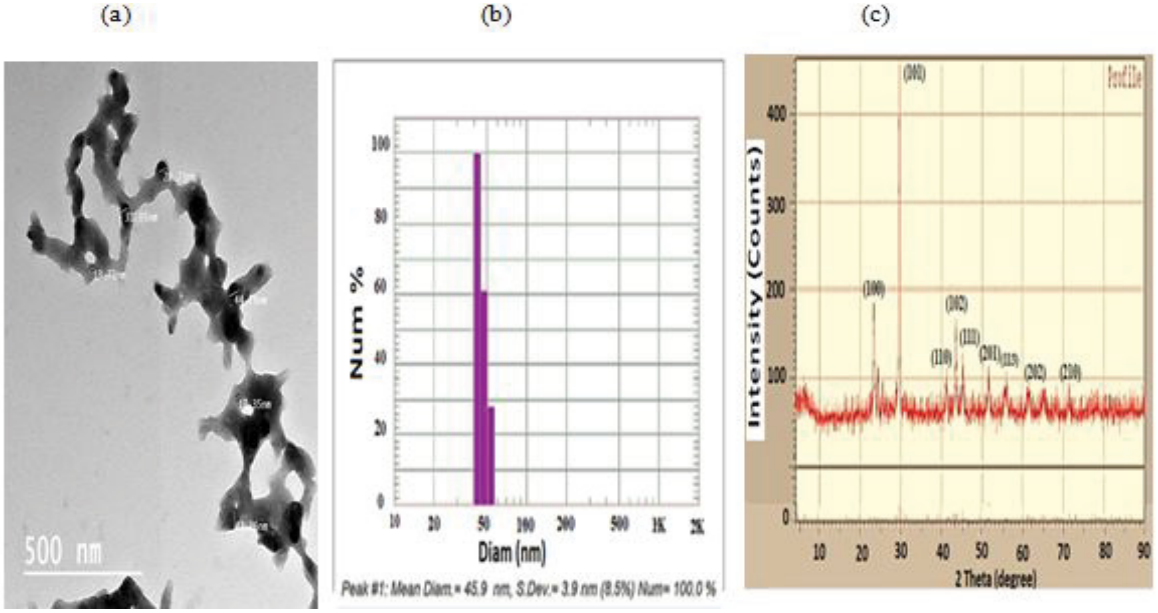

Figure 2: Characterization of the synthesized SeNPs at 10 mM sodium selenate and 1.5% ascorbic acid. (a): TEM micrograph indicates a mean diameter of 33.4 nm; (b): DLS indicates a particle size distribution of approximately 45.9 nm; (c): XRD spectrum of SeNPs.

The calculated average crystallite size of the produced nano selenium was found to be 42.92 nm and is in good agreement with the particle size obtained from the TEM micrograph which confirms the coating of the spherical nanoparticles with a mean diameter of ≈ 33.4 nm and their uniform distribution (Figure 2a).

Fourier transforms infrared spectroscopy: The FTIR spectrum of dry powder L-ascorbic acid, sodium selenate, and the synthesized SeNPs were characterized using FTIR in the range 400 to 4,000 cm^{-1} wavenumber region (Figure 3a, 3b and 3c). The spectrum of SeNPs is almost similar to that of Na_2SeO_4, However, the peak of O-H stretch (3434.6 cm^{-1}) became wider and flatter, indicating that hydrogen bonding was enhanced [51] and the peak of C=O stretching of carbonyl group (scissor bending vibration) at around 1629.55 cm^{-1} became wider and the bending vibration of Se-O band gets broadened at 667 cm^{-1} [52] (Figure 4c). Also, the largest sharp peak of Se-O stretching at 889.0 cm^{-1} in the blank Na_2SeO_4 (Figure 4b) disappeared in FTIR of SeNPs and the one at 424.2 cm^{-1} markedly reduced. On the other hand, the peaks of C-O stretching (1753.94 cm^{-1}), C-OH stretching (1321 cm^{-1}), C-C in plane bending vibration (822.49 cm^{-1}) and C-C out of plane bending (448.38 cm^{-1}) in the blank AA (free of SeNPs) (Figure 4a) disappeared in FTIR of SeNPs (Figure 4c). Apart from the two prominent peaks in FTIR of SeNPs, there are new peaks appeared at 2925.48, 2861, 1184 and 607.487 cm^{-1} representing C-H stretching (acidic asymmetric stretch), and C-O in plane bending (C-H deformations of $-CH_2$ or $-CH_3$ groups), which may be attributed to the carbonyl groups from ascorbic acid can bind strongly to metals, and hence, they can form a coat around NPs to prevent agglomeration (Figure 3c).

Growth parameters

Data presented in Figures 4 and 5 show that foliar application of either Na_2SeO_4 or SeNPs at 6.25, 12.5 and 25 µM significantly promoted almost all measured growth criteria (lengths of stem and root, the stem, root and leaves fresh and dry weights, No. of leaves and total leaves area/plant) of cowpea (*Vigna unguiculata* L) plants compared to the corresponding untreated control plants at 75 DAS.

In all cases, the increment in growth parameters was often highly significant with SeNPs compared to control cowpea plants. The most effective treatments on all growth parameters were SeNPs at 6.25 µM followed by Na_2SeO_4 at the same concentration. Furthermore, Na_2SeO_4 was less effective than SeNPs in increasing vegetative parameters at 6.25-25.0 µM, slight insignificant increase in growth by SeNPs at 50µM was recorded, while Na_2SeO_4 decreased most growth parameters at 50 µM in comparison with untreated controls cowpea plants Figure 5.

Yield (Seed weight): Data presented in Figure 6 shows that foliar application of Na_2SeO_4 or SeNPs up to 25 µM significantly increased 100-seed weight (g) of cowpea plants more than controls at 105 DAS. The highest 100-seed weight was recorded by applying 6.25 µM of either Na_2SeO_4 or SeNPs. Furthermore, SeNPs was more effective than Na_2SeO_4 in increasing 100-seed weight of cowpea plants.

Photosynthetic pigments: The results presented in Table 1 show that foliar application of either Na_2SeO_4 up to 25 µM or SeNPs up to 50 µM concentration significantly increased the Chl a and b, carotenoids, and consequently the Total Photosynthetic Pigments (TPP) content more than controls at 75 DAS. The most effective concentration was 6.25 µM of either Na_2SeO_4 or SeNPs.

Foliar spray of cowpea plants with Na_2SeO_4 up to 25 µM concentrations significantly increased photosynthetic pigments compared to untreated control plants. On the other hand, a reverse situation was observed at 50 µM of Na_2SeO_4 (Table 1).

Generally, SeNPs was more effective than Na_2SeO_4 in increasing these pigments at all concentrations tested. The highest recorded value of chl a+b, carotenoids, and TPP in the leaves of cowpea plants was obtained with SeNPs at 6.25 µM followed by Na_2SeO_4 at the same concentration.

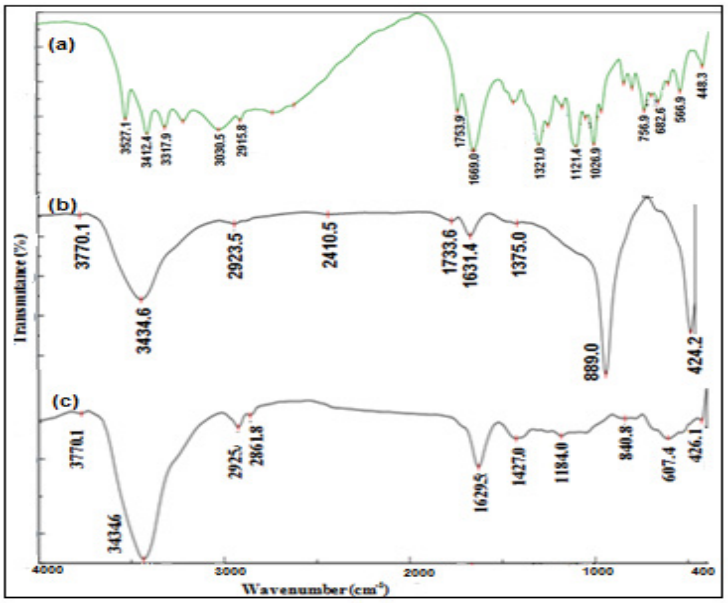

Figure 3: FTIR spectra of (a): ascorbic acid; (b): Na_2SeO_4; (c): SeNPs formed at 10 mM sodium selenate and 1.5% ascorbic acid.

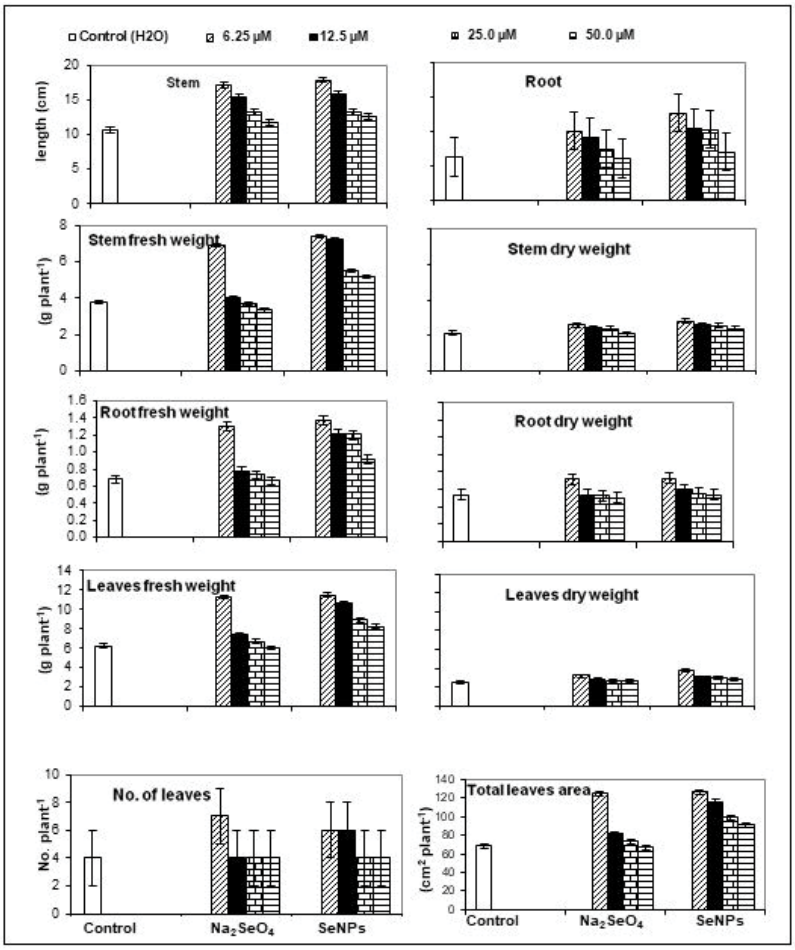

Figure 4: Effect of foliar spray with sodium selenate (Na_2SeO_4) and selenate nanoparticles (SeNPs), each at (0.0, 6.25, 12.50, 25 and 50 µM) on growth criteria of cowpea (*Vigna unguiculata* L.) plants at 75 days from sowing. Each result is a mean of 5 replicates. Statistical analysis was carried out using Duncan test. Vertical bars represent LSD at 5%.

Figure 5: Growth of cowpea (*Vigna unguiculata* L.) plants at 75 days from sowing as affected by foliar spray with sodium selenate (Na_2SeO_4) and selenate nanoparticles (SeNPs), each at (0.0, 6.25, 12.5, 25 and 50 µM). Each group consists of 5 plants.

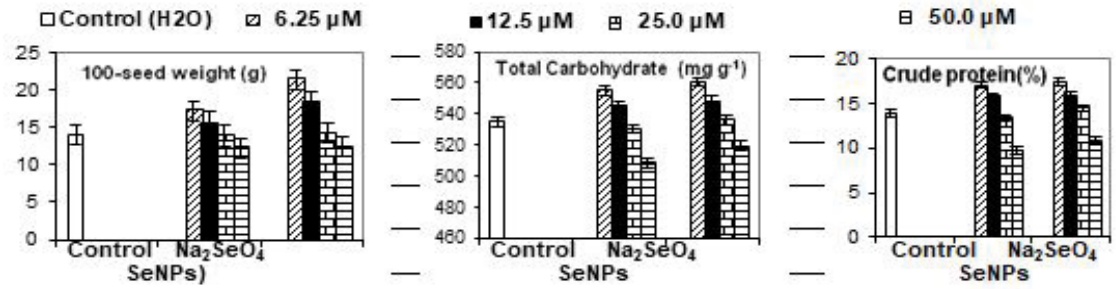

Figure 6: Effect of foliar spray with sodium selenate and selenate nanoparticles, each at 0.0, 6.25, 12.5, 25 and 50 µM on weight and chemical constituents in the dry seeds of cowpea (*Vigna unguiculata* L.) plants at 105 days from sowing. The data are the mean of three replicates. Vertical bars represent LSD at 5%.

Table 1: Effect of foliar spray with sodium selenate (Na_2SeO_4) and selenate nanoparticles (SeNPs), each at 0.0, 6.25, 12.5, 25 and 50 µM on photosynthetic pigments (mg g^{-1} f. wt equivalent) in the leaves of cowpea (*Vigna unguiculata* L.) plants at 75 days from sowing. Statistical analysis was carried out using Duncan. Different letters show significant variation at 0.05 p.

Treatments (µM)	Photosynthetic pigments (mg g^{-1} f. wt equivalent)					
	Ch.a	Ch.b	Cha/b	Ch.a+b	Carotenoids	Total pigments
Control (H_2O)	1.51^{e}	1.10^{e}	1.37^{d}	2.61^{g}	4.05^{h}	6.66^{h}
Na_2SeO_4 6.25	2.67^{b}	1.86^{a}	1.44^{bc}	4.53^{b}	6.89^{b}	11.42^{b}
Na_2SeO_4 12.50	2.38^{bc}	1.55^{bc}	1.54^{a}	3.93^{c}	5.71^{d}	9.64^{d}
Na_2SeO_4 25.00	1.89^{d}	1.29^{d}	1.47^{b}	3.18^{e}	5.32^{e}	8.50^{f}
Na_2SeO_4 50.00	1.48^{e}	1.05^{e}	1.41^{c}	2.53^{g}	3.98^{i}	6.49^{j}
SeNPs 6.25	2.87^{a}	1.87^{a}	1.53^{a}	4.74^{a}	6.93^{a}	11.67^{a}
SeNPs 12.50	2.43^{bc}	1.58^{b}	1.54^{a}	4.01^{c}	5.92^{c}	9.93^{c}
SeNPs 25.00	2.09^{cd}	1.49^{c}	1.40^{c}	3.58^{d}	5.18^{f}	8.76^{e}
SeNPs 50.00	1.75^{de}	1.25^{d}	1.40^{c}	3.00^{f}	5.05^{g}	8.05^{g}
L.S.D at 0.05	0.49	0.15	0.06	0.18	0.07	0.17

T**otal carbohydrates, total soluble sugars, total soluble proteins and proline:** Data presented in Table 2 shows that foliar application of either Na_2SeO_4 or SeNPs at 6.25, 12.5 and 25 µM concentration significantly increased the Total Carbohydrates (TC) and Total Soluble Protein (TSP) content accompanied by decrease in the Total Soluble Sugar (TSS) in the dry leaves of cowpea plants as compared with control plants. Moreover, the result showed that SeNPs at 6.25 µM showed higher enhancement of TC and TSP contents than Na_2SeO_4 and vice versa for TSS content in cowpea plants.

Furthermore, the results obtained indicate that Na_2SeO_4 and SeNPs at all tested concentrations insignificantly decreased proline content in cowpea leaves.

Chemical constituents in the seeds (Seed quality): Data presented in Figure 6 shows that foliar application of Na_2SeO_4 or SeNPs at 6.25 and 12.5 µM increased the Total Carbohydrate (TC) and Crude Protein (CP) in the dry seeds of cowpea more than controls at 105 DAS. On the other hand, a reverse situation was observed at 25 and 50 µM of Na_2SeO_4 and at 50 µM SeNPs. The highest values of TC and CP were recorded by applying 6.25 µM of either Na_2SeO_4 or SeNPs. Moreover, SeNPs at the four used concentrations was more effective than Na_2SeO_4 in increasing TC and CP in cowpea seeds.

Mineral contents: Data presented in Figure 7 show that foliar application of sodium selenate or selenate nanoparticles at 6.25 and 50 µM increased different minerals (N, P, K, Ca, S and Mg mg l^{-1}),

Table 2: Effect of foliar spray with sodium selenate (Na_2SeO_4) and Selenate Nanoparticles (SeNPs), each at 0.0, 6.25, 12.5, 25 and 50 µM on total carbohydrate (TC), Total Soluble Sugars (TSS), Total Soluble Protein (TSP) and proline (mg g^{-1} d. wt equivalent) in the leaves of cowpea (*Vigna unguiculata* L.) plants at 75 days from sowing. Statistical analysis was carried out using Duncan. Different letters show significant variation at 0.05 p.

Treatments (µM)	**Total carbohydrates**	**Total Soluble sugars**	**Total Soluble protein**	**Free proline** (mg g^{-1} d. wt equivalent)
Control (H_2O)	164.46^{i}	59.09^{a}	81.65^{i}	8.92^{a}
Na_2SeO_4 6.25	287.37^{b}	49.45^{e}	128.20^{b}	8.84^{ab}
Na_2SeO_4 12.50	218.78^{d}	51.66^{d}	116.56^{d}	8.85^{ab}
Na_2SeO_4 25.00	195.32^{f}	56.37^{b}	103.49^{f}	8.87^{ab}
Na_2SeO_4 50.00	168.86^{h}	57.35^{b}	91.70^{h}	8.88^{ab}
SeNPs 6.25	289.65^{a}	44.79^{f}	133.63^{a}	8.83^{b}
SeNPs 12.50	248.85^{c}	50.40^{d}	118.50^{c}	8.84^{ab}
SeNPs 25.00	197.17^{e}	54.65^{c}	106.50^{e}	8.85^{ab}
SeNPs 50.00	180.00^{g}	57.14^{b}	100.29^{g}	8.87^{ab}
L.S.D at 0.05	1.85	1.74	2.12	0.08

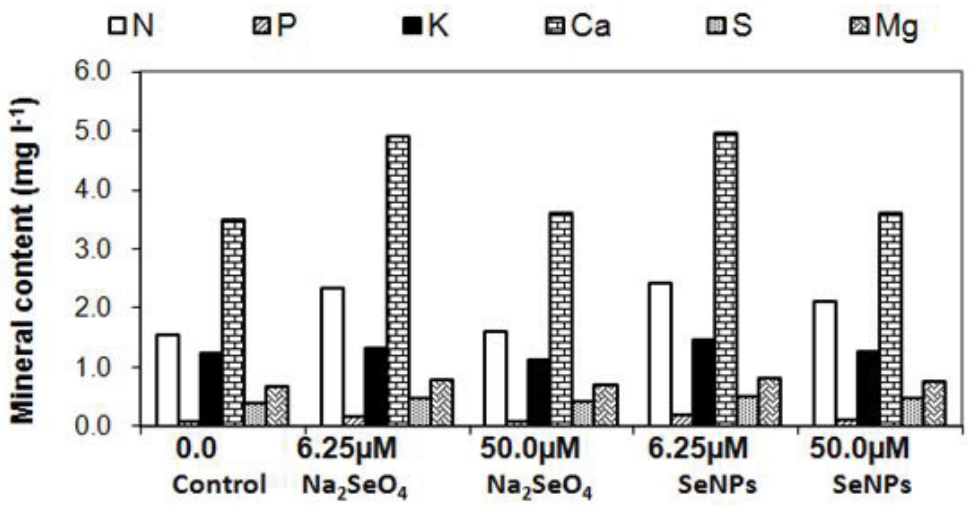

Figure 7: Effect of foliar spray with sodium selenate (Na_2SeO_4) and selenate nanoparticles (SeNPs), each at (0.0, 6.25 and 50 µM) on the mineral content (mg l^{-1}) of air dry leaf tissue of cowpea (*Vigna unguiculata* L.) plants at 75 days after sowing.

content in the dry leaves of cowpea plants more than controls, except K decreased at 50 µM Na_2SeO_4. The highest values of N, P, K, Ca, S and Mg were recorded by applying SeNPs at 6.25 µM, followed by Na_2SeO_4 at the same concentration.

Uptake of selenium in nano and bulk forms: Atomic absorption spectroscopic studies show low selenium concentration in soil (0.11 mg Kg^{-1}) and negligible accumulation (<0.3 mg Kg^{-1}) of both forms of selenium (either sodium selenate or nano selenium) in the leaves and seeds of cowpea plants at 50 µM of either Na_2SeO_4 or SeNPs as compared with undetected amount (0.0 mg Kg^{-1} dry weight) at 6.25 µM of both forms of selenium and untreated control plants**.**

Endogenous phytohormones concentrations: The results presented in Table 3 show that foliar application of either sodium selenate or selenate nanoparticles at 6.25 µM concentration increased the content of IAA (Indole Acetic Acid), GA_3 (gibberellin), Kin (kinetin) and BA (benzyl adenine) as well as GA_3/ABA ratio, as compared with control (untreated) cowpea plants. On the other hand, a reverse situation was observed with the content of ABA (abscisic acid) and ABA/Cks ratio (Table 3).

Light and transmission electron microscopy (LM and TEM): The effects of treatment with SeNPs at 6.25 and 50 µM on cowpea leaves and seeds at fruiting stage were examined using light microscopy and TEM. Light microscopy observations were unable to show the differences between the leaves and seeds of cowpea plants sprayed with SeNPs and cells appeared almost similar to their respective controls (Figures 8a, 8d, 8g and 9a). Differences among the treatments were observed using TEM analysis of cell ultrastructure. In leaves and seeds of cowpea plants sprayed with SeNPs at 6.25 and 50µM, the main effect observed by TEM was in evidence small, dark deposits in cells exposed to SeNPs. These deposits were never observed in control leaves and seeds cells (Figures 8f-8h, 8j-8l and 9f-9h, 9j-9l).

Discussion

The present study indicates that the foliar application of Se as

Table 3: HPLC analysis for endogenous hormone concentration (µg/100 g fwt equivalent) for young leaves of cowpea (*Vigna unguiculata* L.) plants as affected by foliar spray with sodium selenate (Na_2SeO_4) and selenate nanoparticles (SeNPs), each at (0.0 and 6.25 µM) after 75 days from sowing.

Treatments (µM)	Endogenous hormone concentration (µg 100 g^{-1} f. wt. equivalent)							
	IAA	GA_3	Kin	BA	ABA	CKs (Kin+BA)	ABA/Cks	GA_3/ABA
Control (H_2O)	342.00	410.00	151.90	92.35	8.24	244.25	0.034	49.76
Na_2SeO_4 6.25	529.00	1125.00	364.00	241.58	4.49	605.58	0.007	250.6
SeNPs 6.25	788.00	3322.00	709.00	272.87	3.99	981.87	0.004	832.6
IAA: Indole-3-Acetic Acid; GA_3: Gibberellic Acid; ABA: Abscisic Acid; Kin: Kinetin; BA: Benzyl Adenine; CKs: (Kin+BA)								

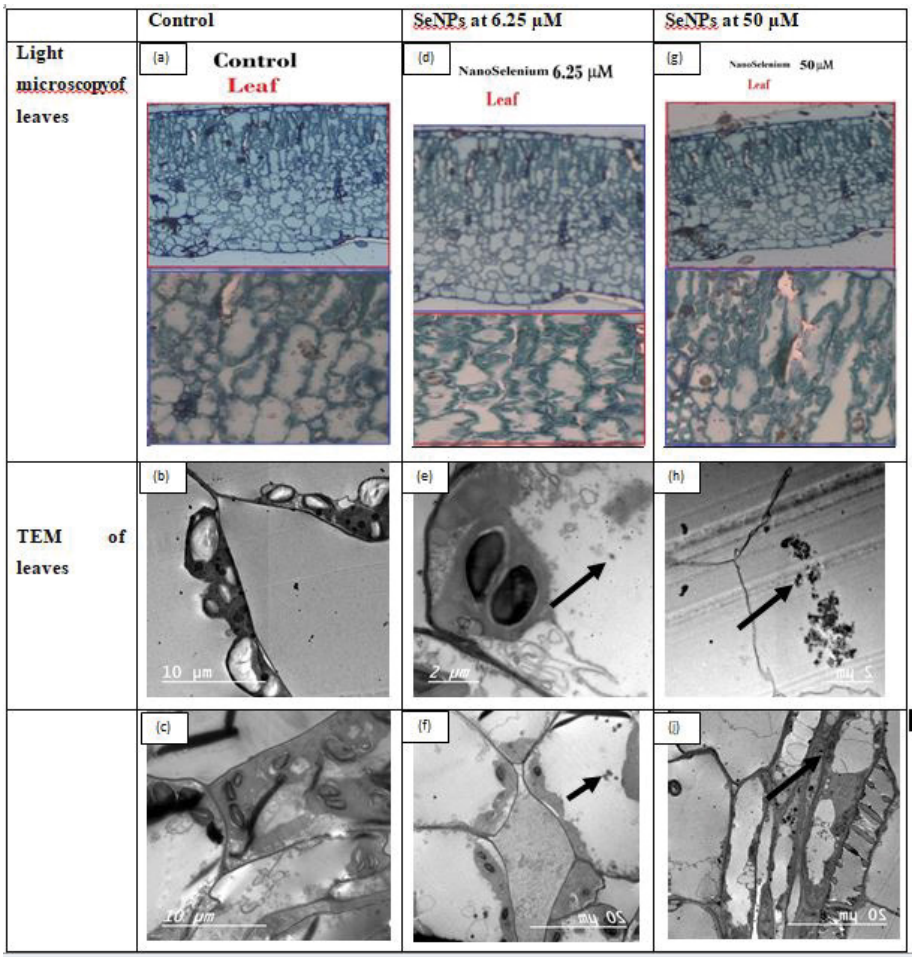

Figure 8: Light (LM) and Transmission Electron Microscopy (TEM) of cowpea (Vigna *unguiculata*) leaves at fruiting stage. (a, d and g)-Light microscopy of cowpea leaves under control (a): treatment with SeNPs at 6.25 µM (d): and 50 µM (g): at a magnification of 10x and 60x. (b, c), (e, f), (h, i)-TEM of palisade and spongy cells from cowpea leaves at control (b, c), treatment with SeNPs at 6.25 µM (e, f) and 50 µM (h, i), respectively. In e, f, h and i, the arrows indicate the location of the small dark deposits which probably originated from Se nanoparticles absorbed by the leaves at magnification of 2 µm-20 µm.

Na_2SeO_4 or SeNPs at low concentrations (up to 25 µM), acts as an antioxidant and greatly promoted the vegetative growth of cowpea plants, whereas a reverse situation was observed at 50 µM of Na_2SeO_4 (Figure 4). The stimulation of cowpea growth possibly through participation in the synthesis of auxin and/or cytokinin, enhancement of cell division, nutrients uptake and Chl accumulation. Similarly, the dual effect of Se on plant growth (positive or toxic) was found in other plant species depending on concentrations of Se. In canola, foliar spray with sodium selenate (2.5 and 5.0 mg L^{-1}) significantly increased plant growth (shoot length, number of leaves $plant^{-1}$, leaves area $plant^{-1}$ as well as fresh and dry weights of shoots) and yield, while higher concentration of Se (10 mg L^{-1}) significantly decreased growth parameters under normal and salt stress conditions [21]. In two wheat genotypes seedlings, Se foliar spray at 7.06 µM was effective in maintaining higher growth, fresh and dry matter drought tolerance indexes [22]. In *Brassica juncea* plants, low level of Na_2SeO_4 (10 µM) improved growth and photosynthesis by increasing the efficacy of 24-epibrassinolide (as a phytohormone) and acting as quasi essential micronutrient, whereas higher concentrations of Se (80 µM) induced deleterious effect [53]. In *Nicotiana tabacum* L leaves, Na_2SeO_3 at 6 mg kg^{-1} improved plant growth by enhancing photosynthesis, stomatal conductance, carboxylation efficiency and Rubisco content [25]. In our present study foliar application of Na_2SeO_4 at 50 µM reduced the vegetative growth and dry-matter production of cowpea plants,

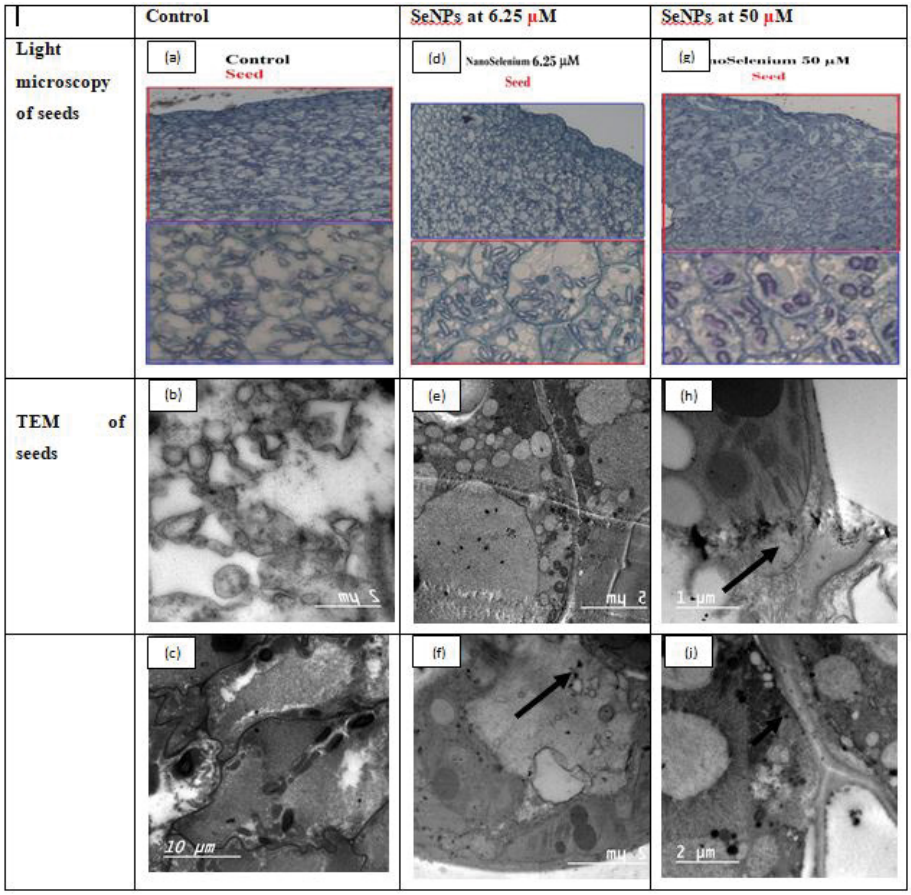

Figure 9: Light (LM) and Transmission Electron Microscopy (TEM) of cowpea (Vigna *unguiculata*) seeds at fruiting stage. (a, d and g)-Light microscopy of cowpea seeds under control (a) treatment with SeNPs at 6.25 µM; (d): and 50 µM; (g): at a magnification of 10x and 40x. (b, c), (e, f), (h, i)-TEM of subepidermal layer from cowpea seeds at control (b, c), treatment with SeNPs at 6.25 µM (e, f) and 50 µM (h, i), respectively. In e, f, h and i, the arrows indicate the location of the small dark deposits which probably originated from Se nanoparticles absorbed by the leaves and accumulated in the seeds at magnification of 1 µm-10 µm.

possibly due to the replacement of S atoms by Se in S-containing amino acids, resulting in changes in the structure and activity of proteins and consequently decrease cowpea growth. Similarly, in sugar cane plants, symptoms of metal toxicity as stunted growth, reduced plant height, vigor, root, shoot weight and leaf chlorosis were observed at 50 and 100 ppm Na_2SeO_3 [54]. The toxicity of a high Se concentration is supposed to be due to its pro-oxidant ability to catalyze the oxidation of thiols and simultaneous generation of superoxide that can damage cellular components [27] and the replacement of S atoms by Se in S-containing amino acids such as cysteine and methionine; result in changes in the structure and activity of Se-substituted proteins and consequently resulting in a decrease in plant growth [26]. The inhibitory effects of high level of selenate were reported in arabidopsis plants [55] and cucumber by application of either selenate at 80 or selenite at 20 µM concentrations [29]. Comparing the values of the toxicity threshold, it is obvious that cowpea plants are not as sensitive to Se in the selenate form as lettuce [56] but less than cucumber plants [29], where 50 µM of selenate significantly reduced cowpea growth.

On the other hand, SeNPs up to 50 µM showed, in this study, a much better ability than Na_2SeO_4 to promote growth and yield of cowpea plants. In this respect, nano-Se showed less pro-oxidative effects than selenite as measured by cell growth [57]. Nano-Se has a higher efficiency in up-regulating selenoenzymes and exhibits less toxicity than selenite [27]. In tobacco callus cultures, SeNPs (265-530 µM) significantly stimulated the organogenesis and the growth of root system (40%) while, they completely inhibited by selenate, since the selenate ion can get into plant tissue and in excess as a pro-oxidant can damage directly and/or indirectly the regenerating calli growth and regeneration of explants [30]. In tomato, a low concentration of Se (2.5 µM) and N-Se (1 µM) can improve plant growth parameters more effectively than a higher concentration of Se/N-Se under high and low-temperature stress [31]. In Cluster bean, fertilization with selenium nanoparticles at 400 mg improved the growth and biochemical characteristics [32].

Cowpea yield (100-seed weight (g)) was significantly enhanced by foliar application of Na_2SeO_4 and SeNPs, especially at 6.25 µM possibly through enhancing growth-promoting hormones, photosynthetic activity, accumulating dry matter, and consequently increasing translocation and accumulation of certain metabolites in plant organs, which affected their yield (Figure 6). In accordance, foliar application of sodium selenate (up to 5 mg L^{-1}) significantly increased number of pods $plant^{-1}$, number of seeds pod^{-1} and weight of seeds $plant^{-1}$ in canola plants under normal and salt stress conditions [21], improved grain yield and quality in wheat plants under normal and water-deficit conditions through influences nutrients uptake, maintenance of turgor and gas exchange characteristics and enhancement in antioxidant system activity [22] and significantly increased lentil grain yield compared to soil application [23]. In our present study

foliar application of Na_2SeO_4 at 50 µM, reduced cowpea yield. In accordance, higher concentration of Na_2SeO_4 (10 mg L^{-1}) significantly decreased canola yield under normal and salt stress conditions [21]. Se at high concentration has a pro-oxidant role, resulting in metabolic disturbances and a reduction in yield [28]. Also, Se at 3 µg L^{-1}, slightly reduced seed weight (g $plant^{-1}$) and weight of 1000 seeds (g) in two wheat genotypes [29].

Furthermore, SeNPs at 6.25-25 µM increased cowpea yield (100-seed weight) more effectively than Na_2SeO_4 by increasing the content and activity levels of endogenous growth promoters and hormones (IAA, GA_3, Kin and BA, as well as GA_3/ABA ratios) and antagonized the growth inhibitory effect of abscisic acid, especially at 6.25 µM SeNPs. In accordance, Se-NPs fertilization improved yield performance in the cluster [32]. And significantly increased the seed set percentage and seed yield in sorghum under high-temperature stress compared to control plants [58].

In the present study, foliar application of either Na_2SeO_4 or SeNPs, especially at 6.25 µM increased the total carbohydrate and crude protein in the dry seeds of cowpea, possibly due to the bioregulatory effect on enzymatic activity and translocation processes from leaves to seeds, linking or converting to other plant metabolites. In two wheat genotypes, Se treatment increased soluble carbohydrates and soluble protein concentrations in the leaves and roots under well-watered and drought conditions [29]. In the present study a reverse situation was observed for the total carbohydrate and crude protein in the dry seeds of cowpea at 25 and 50 µM of Na_2SeO_4 and at 50 µM SeNPs. In this respect, the minimum carbohydrate content was detected in canola leaves received the highest Se dosage (10 mg L^{-1}) [21]. With respect to nitrogen, N concentration was not significantly affected by selenate in *Brassica oleracea* [59] and in alfalfa (*Medicago sativa* L) [60]. Also, the level of N remained at the control level at low selenite (2, 6 µM), but significantly decreased under a highly phytotoxic selenite concentrations (30 and 60 µM) in the aboveground organs in cucumber [19].

In our study, SeNPs at 6.25-50 µM concentration was more effective than Na_2SeO_4 in increasing the TC and CP in the dry seeds of cowpea, with the superiority of 6.25 µM concentration (Figure 6). In cluster bean, the protein content was higher by fertilization with SeNPs at 400 mg than 500 mg [32].

Photosynthetic pigments of cowpea leaves were significantly enhanced by the application of Na_2SeO_4 and SeNPs up to 25 µM, but inhibited at high concentrations (50 µM) of Na_2SeO_4 (Table 1). At low concentrations, the Na_2SeO_4 and SeNPs might concomitantly increase cell metabolic rate and retard senescence by protecting and preventing chloroplasts from senescing and retarding Chl destruction and/or increase Chl biosynthesis. In our study, Na_2SeO_4 and SeNPs increased N and Mg levels (structural component of Chl), enhanced Chl accumulation, which led to a greater rate of photosynthesis and may protect the photosynthetic apparatus when a plant is subjected to stress, by scavenging the excessively free radicals. In canola, Na_2SeO_4 counteracted the adverse effect of salt stress on chlorophyll by decreasing ROS levels, reactivation of antioxidants, restore the structure of the damaged chloroplasts and protect photosynthetic apparatus from oxidative stress [21]. In lettuce plant, sodium selenate at 50 mg m^{-2} increased chlorophyll content at early stages of plant development, while delayed the decrease in total chlorophyll content (senescence prevention) and promote carotenoids accumulation at 100 and 200 mg m^{-2} [61]. In *Nicotiana tabacum* leaves, Na_2SeO_3 at 6 mg kg^{-1} enhanced photosynthesis, carboxylation efficiency and Rubisco content [25]. In our present study foliar application of Na_2SeO_4 at 50 µM, reduced the photosynthetic pigments of cowpea leaves. In this respect, [62] did not find any influence of selenate on barley chlorophyll fluorescence parameters which commonly used to characterize the primary PSII photochemistry. They suggest that the high selenate dosage had a harmful effect on photosynthesis via changes in activity and/or biosynthesis of enzymes, rather than via alteration of PSII, which is interrelated with the photosynthetic capacity**.** In spinach plants, chlorophyll b was more sensitive to the Se stress than chlorophyll a [63].

In cucumber, the decrease in the chlorophyll levels at the low Se concentration was the primary bioindicator of trace elements phytotoxicity [19].

Furthermore, SeNPs was more effective than Na_2SeO_4 in increasing total photosynthetic pigments in the leaves of cowpea, especially at 6.25 µM concentrations. This positive effect of SeNPs could be attributed to the increase in CO_2 assimilation, photosynthetic rate, and mineral uptake. In tomato leaves, application of N-Se at 1 µM improved the chlorophyll content by 27.5% while Na_2SeO_4 at 2.5 µM increased by 19.2% under low-temperature stress [31].

In this study, foliar application of either Na_2SeO_4 or SeNPs up to 25 µM concentration significantly increased the levels of Total Carbohydrates (TC) and Total Soluble Protein (TSP) content in the dry leaves of cowpea plants accompanied by decrease in the contents of Total Soluble Sugars (TSS) that are required for the plant osmotic adjustment under stress (Table 2). The increase in TC content might be explained on the bases of enhancement of photosynthetic capacity, predicted to result from increased carotenoids and total photosynthetic pigments, while the TSP increased due to increase in protein biosynthesis. In two wheat genotypes, Se treatment increased soluble carbohydrates and soluble protein concentrations in the leaves under well-watered and drought conditions associated with lower free α-amino acids in the leaves that imply depletion of amino acids pool following elevated protein synthesis by Se in the leaves [29]. In the present study a reverse situation was observed for the TC, TSP and CP in the leaves of cowpea at 25 and 50 µM of Na_2SeO_4 and at 50 µM SeNPs. In this respect, the minimum carbohydrate contents, soluble sugars and polysaccharides in canola were detected in leaves received the highest Se dosage (10 mg L^{-1}) [21]. In cucumber, the level of N in the aboveground organs significantly decreased under a highly phytotoxic selenite concentrations (30 and 60 µM) [19].

In our study, SeNPs at 6.25 to 50 µM concentration was more effective than Na_2SeO_4 in increasing the TC and TSP accompanied by a decrease in TSS in the dry leaves of cowpea plants, with the superiority of 6.25 µM concentration. In cluster bean, fertilization with SeNPs at 400 mg increased the protein content more than 500 mg [32].

On the other hand, there was no significant change in proline content in cowpea leaves by application of Na_2SeO_4 and SeNPs at any concentration. In this respect, Se application increased leaf proline concentration in drought-stressed wheat [64] and canola plants [21]. In two wheat genotypes, application of Na_2SeO_4 at 15 µg L^{-1} decreased the leaf proline concentration in 'Homa' of both well-watered and drought-stressed plants whereas increased it in 'Sara', but both genotypes had slightly lower proline concentration in the roots [29].

From the obtained results foliar application of Na_2SeO_4 and SeNPs

at 6.25 and 50 µM enhanced the content of N, P, K, Ca, S and Mg (mg l^{-1}) in the leaves of cowpea plants, but decreased K at 50 µM Na_2SeO_4 (Figure 7). The increase might be due to Se-mediated changes in the root length and proliferation that improves the absorption of nutrients from soil. In *Arabidopsis thaliana*, exogenous selenate induced sulphate bioaccumulation in aboveground plant organs, by preventing a reduction in the abundance or/and activity of sulphate transporters by sulphate and its derivatives [65]. Selenium addition increased S accumulation in shoots of *B. oleracea* [66] as well as shoots of rape and wheat [67]. Se treatment at 5 mg L^{-1} had a positive effect on P^{3+} and Mg^{2+} content in canola plants under normal and salt stress condition [21]. Na_2SeO_4 at 3 µg L^{-1} level, increased P, K, and Ca contents in the leaves of two wheat genotype [29]. In our study, application of Na_2SeO_4 at 50 µM slightly increased the content of N, P, Ca, S and Mg, but decreased K in the leaves of cowpea plants. Similarly in cucumber plants, application of selenate at of 2-60 µM increased P content in a dose-independent manner and resulted in a slightly higher concentration of Ca, but significantly decreased K levels if selenate concentration in the growth media passed 6 µM. Selenate at concentrations higher than 6 µM, caused elevated S-SO_4 accumulation in cucumber shoots, and the impact of SeO^{-4} ions, as SO^{-4} ions analog, was more evident [19].

Moreover, SeNPs was more effective in enhancing the absorption of minerals than the Na_2SeO_4, especially at 6.25 µM, which might be attributed to the better proliferation and absorption of root reflected as better growth of cowpea by SeNPs than the Na_2SeO_4 application as recorded in our present work [68] demonstrated that nano-Se has comparable efficacy to selenite in up-regulating selenoenzymes and Se levels in tissue, but is less toxic. Comparing with selenomethionine, nano-Se has lower toxicity and possesses equal efficacy in increasing the activities of seleno-enzymes [27]. These results indicated that nano-Se can serve as an antioxidant with reduced risk of Se toxicity [69].

Our study show negligible accumulation of both forms of selenium (either sodium selenate or nano selenium) in leaves and seeds of cowpea plants at 50 µM of Na_2SeO_4 and SeNPs, as compared with an undetected amount at 6.25 µM and untreated control plants. In this respect, application of sodium selenate at 10 and 20 g Se ha^{-1}, increased Se contents of barley grain and straw and red clover forage [70]. Se-NPs at 50 and 100 mg L^{-1}, increased Se concentrations to 1.7 and 3.4 µg g^{-1}, respectively compared with <0.05 µg g^{-1} Se for the control sorghum leaf [58]. The intracellular CuO nanoparticle concentration increased with increasing CuO nanoparticles' exposure in wheat roots [71]. Increasing the applied concentration of AuNPs up to 100 ppm increased the uptake of gold nanoparticles (AuNPs) to 21.36l µg/g fresh weight in the leaf tissues of *Brassica juncea* [72] and increased the uptake of nanosilver and silver nitrate; recording uptakes of 0.35 and 0.49 µg/g dry weight for the leaves of Bronco and Nebraska varieties, respectively compared with 0.25 and 0.29 µg/g dry weight for their respective controls plants at 60 ppm gum arabic-coated silver nanoparticles (GA-AgNPs) [73].

In the present study, foliar application of Na_2SeO_4 and SeNPs at 6.25 µM enhanced the growth-promoting hormones, expressed as IAA, GA_3, Kin, BA, as well as GA_3/ABA ratios in the leaves of cowpea plants, which might represent the primary hormonal signal correlated with the increased metabolic activity and consequently growth of these plants, whereas a reverse situation was observed with ABA and ABA/Cks ratio and thus preventing the growth inhibitory effects of ABA (Table 3). In this connection, abscisic acid (ABA) induces the abscission process through stimulation of the ethylene (ET) biosynthesis, while auxin is effective in delaying abscission by reducing the sensitivity of cells to ethylene [74]. Silver ions can displace copper ions from the receptor proteins, consequently blocking ethylene perception, since copper ions play a critical role in ethylene binding upon receptors [75]. In borage, inhibition of ET action reduces the event of abscission which is one of the important reasons for increase in leaf number [76]. In tomato, foliar pretreatment with 1 mgL^{-1} sodium selenate effectively delayed fruit ripening and maintained fruit quality. Gene expression revealed that the repression of ethylene biosynthetic genes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase decreased ethylene production and respiration rate [77].

Moreover, application of SeNPs at 6.25 µM was much better than Na_2SeO_4 in improving cowpea growth by increasing the growth-promoting hormones and GA_3/ABA ratios more than in those with Na_2SeO_4. This might be attributed to the effect of SeNPs on initiating growth promoter's biosynthesis and/or preventing its destruction, or it might be due to the synergistic effect on the stimulatory action of promoters through the transformation of inactive forms into active forms leading to changes and stimulation in the endogenous growth-promoting hormones. Also, SeNPs may affect the phytohormone ethylene (ET) signaling and biosynthesis by antagonizing ACC and reducing the expression of ACC oxidase 2 (ACO_2) and ACC synthase 7 (ACS7) the enzymes that catalyze essential steps in the biosynthesis of ethylene expecting that NPs may act as inhibitors of ET perception and may interfere with ET biosynthesis [78] using AgNPs on arabidopsis seedlings. This agrees with [79] who mentioned that $AgNO_3$ and AgNPs may have a bioregulatory effect on phytohormones balance involved in gene expression regulating the signaling activities or levels of growth-regulating substances leading to an increase in metabolic compounds and consequently growth and yield of common bean plants. In our present study, selenium (as Na_2SeO_4 and SeNPs) at low concentration may also have a similar effect to Ag, resulting in the inhibitors of ET and enhancement of growth promoters levels that have highly positive impacts on the growth and yield of cowpea plants.

In the present study, light microscopy observations show no differences between the leaves and seeds of cowpea plants sprayed with SeNPs and cells appeared almost similar to their respective controls (Figures 8a, 8d, 8g and 9a, 9d and 9g). These results are consistent with those reported [67] for rape seedlings treated with 2 µM Na_2SeO_4, Se led to an increase of chloroplast size reduced by Cd treatment, rebuilt the chloroplast ultrastructure and caused a partial reversal of the detected changes of chloroplast envelopes i.e. fatty acid saturation and fluidity. In Sorghum, Se-NPs protected the thylakoid membrane by restoration of the chloroplast ultrastructure through distribution of thylakoid membranes and granal stacking under heat stress [58].

Differences among the leaves and seeds of cowpea plants sprayed with SeNPs at 6.25 and 50 µM were observed using TEM analysis of cell ultrastructure, the main effect observed by TEM was in evidence small, dark deposits in cells exposed to SeNPs, which probably originated from the nanoparticles absorbed onto the leaf (Figure 8e, 8f, 8h and 8i) and transferred to seeds which suggests that they accumulate in the seeds. (Figure 9f-9h, 9j-9l). These small deposits were less obvious in samples treated with SeNPs at 6.25 µM (Figures 8f-8h and 9f-9h). However, these deposits were never observed in control leaves and seeds cells (Figures 8b-8d and 9b-9d). In this connection, Selenium concentrations in leaves of plants decline before, or upon, flowering, when Se is translocated from leaves to reproductive organs [79]. Selenium is readily redistributed in the

phloem as both selenate and the organoselenium compounds SeMet and SeMSeCys [80]. In non-accumulator plants, much of selenate fertilizer is translocated to the shoot and sequestered in the vacuoles of cells within the vasculature and leaf mesophyll [81]. However, there is no evidence about biotransformation of SeNPs in plant systems [58].

Conclusion

The present study describes the synthesis of Se nanoparticles with a mean diameter of about 33.4 nm by the superficial one-step method using a biocompatible substance ascorbic acid as a reductant and coating at 1.5% and Na_2SeO_4 at 10 mM concentration. SeNPs were characterized by different physicochemical techniques such as UV-Vis spectroscopy, DLS, TEM, FT-IR spectroscopy and XRD. SeNps have a narrow distribution, small size a uniform spherical and crystalline shape. A comparison between the physiological effect of Se in form of Na_2SeO_4 and NanoSelenium particles was performed. The result showed that foliar application of either Na_2SeO_4 or SeNPs can influence the physiological processes and mineral balance of plants under pot-grown experiment. Application of Na_2SeO_4 and SeNPs at 6.25 μM, resulted in a significant increase in every morphological and biochemical attribute, particularly seed weight and quality. However, the application of SeNPs was more effective than Na_2SeO_4 at all used concentrations. This study justifies further work on cowpea plants under a broader range of field conditions to further evaluate the possibility of using Na_2SeO_4 and SeNPs for increasing cowpea yield, improving quality and nutritional value on a larger scale.

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