

Effect of foliar application of engineered nanomaterials: carbon nanotubes NPK and chitosan nanoparticles NPK fertilizer on the growth of French bean plant

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ABSTRACT

Nanotechnology has the potential to revolutionize the agriculture with new tools to enhancing the ability of plants to absorb nutrients. The uptake of nanomaterials by plants has shown a very recent field of nano agriculture. This work was designed to investigate the beneficial effects of two different types of engineered nanofertilizer namely: carbon nanotubes coated NPK and chitosan nanoparticles NPK on French bean. Here in, we explore the potential influence of different concentration of chitosan nanoparticles loaded with NPK and carbon nanotubes loaded with NPK on the different growth criteria of French bean plants. It was hypothesized that nanomaterials were able to penetrate the plant cell by endocytosis. The results of the combined morphological and anatomical analysis indicate that after about 30 days from the date of planting our experimental conditions, nanomaterials either alone or in combination significantly enhances plant growth and biomass compared to control. TEM images of French bean leaves show the presence of nanomaterials in vascular bundles specifically in sieve tubes of phloem elements in case of chitosan-NPK and in both xylem vessels and sieve tubes in case of carbon nanotubes- NPK. Overall after investigation we conclude that low dose of nanofertilizers have seen to be beneficial, improving water absorption and nutrients uptake, found to enhance the growth of the plants.

Keywords: Carbon nanotubes, chitosan, French bean, nano-fertilizer, transmission electron microscope, uptake.

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INTRODUCTION

Nanotechnology has proved its place in agricultural sciences and related industries (Froggett, 2009). The key focus areas for nanotechnology agricultural research are drug delivery, nano-biofarming, nanopesticides and nanoherbicides and controlled release of nanofertilizers (Agrawal and Rathore, 2014). Nanofertilizers are nutrient carriers of nano-dimensions ranging from 10 to 400 nm and capable of holding nutrient ions due to their high surface area and release it slowly and steadily that commensurate with crop demand. There are slow-release and super sorbent nitrogenous and phosphatic fertilizers (Lal, 2008; Hasaneen et al., 2015).

Chitosan is a linear hydrophilic polysaccharide that is biocompatible, biodegradable and non-toxic nature

biopolymer; react with bioactive molecules (Dai et al., 2009; Cho et al., 2010; Hanafi, 2012). Chitosan nanoparticles (~78 nm) that can be used for controlled release of NPK fertilizer sources such as urea, calcium phosphate and potassium chloride (Corradini et al., 2010).

CNTs acquired an important position due to their unique physical and chemical characteristics, including length, diameter, atomic configuration, impurities, defects and functionality, which allow them to have wide-ranging conductivity, tension strength, flexibility, and chemical reactivity properties (Jackson et al., 2013). Where plants dosed with low concentrations of CNTs can have positive effects on seed germination, root development and water

transport within the plant, or no evidence of phytotoxicity; contrary to the negative effects that can be produced by high dose trials; as they generate harmful reactive oxygen species (ROS) (Mondal et al., 2011).

The uptake of engineered nanomaterials (ENMs) into plant cell can occur via binding to carrier proteins through aquaporin, ion channels, or endocytosis (Nair et al., 2010), ENMs may diffuse apoplectically in the space between the cell wall and plasma membrane; ENMs in apoplastic flow must eventually merge into the symplast so as to penetrate into vascular system (Larue et al., 2012). Foliar uptake was sought to characterize the possibility of phloem-based ENMs transport. There are two possible pathways for foliar uptake of nanoparticles, that is, cuticular and stomatal pathways (Eichert and Goldbach, 2008). The cuticular pathway is usually limited to NPs with sizes below 5 nm due to extremely small sizes of cuticular pores (Eichert et al., 2008). On the other hand, the stomatal pathways allow the penetration of larger NPs since the typical stomatal size is in micrometer size range (Eichert and Goldbach, 2008).

The aim of this work was to investigate the effects of foliar application of chitosan nanoparticles and carbon nanotubes loaded with NPK fertilizer at different concentrations on growth of French bean seedlings, absorption and translocation inside plant tissues.

MATERIALS AND METHODS

Plant materials

Pure strains of *Phaseolus vulgaris* cv. *contender* seeds were kindly supplied by from Horticultural Research Center, Ministry of Agriculture, Giza, Egypt.

Preparation and characterization of chitosan nanoparticles and carbon nanotubes loaded with NPK fertilizers

The chitosan poly-methacrylic acid (CS-PMAA) nanoparticles were obtained by polymerization of methacrylic acid (MAA) in chitosan (CS) solution in a two-step process (De-Moura et al., 2008; Hasaneen et al., 2014). CNTs easily and inexpensively prepared at room temperatures (Lee and Seo, 2011). Incorporation of NPK into chitosan nanoparticles was carried (Corradini et al., 2010; Hasaneen et al., 2014). Incorporation of NPK into carbon nanotubes was carried out (Yatima et al., 2015).

Determination of electrolyte leakage (EL)

According to Shi et al. (2006), plant leaves were cut into thin discs (10 mm), about twenty leaf discs were placed in a test tube, rinsed three times with 20 cm³ distilled water to remove the electrolytes released during leaf disc excision. Tubes were then filled with 30 ml distilled water and stand in dark for 24 h at room temperature. Electrical conductivity (EC1) of the solution was measured at the end of incubation period using EC meter (HANNA Instrument, HI 8033). The tubes were then heated in a temperature- controlled water bath at 95°C for 20 min and then cooled to room temperature. The final electrical conductivity (EC2) was measured. The electrolyte leakage (EL) was calculated as following:

$$EC = (EC1 / EC2) \times 100$$

Time course experiments

A large scale experiment, carried outdoor, normal day and light conditions, was designed so as to study the effect of four different levels of nanomaterials namely: CS-10%, CS-100%, CNTs-20 µg/L and CNTs-50 µg/L each level being used either alone or in combination with a recommended dose of each of N, P and K attempted fertilizer. Thus, 45 pots, divided into nine groups (each of five pots) were used. One of these groups is left without treatments to serve as water control and the other 8 groups were separately treated with each of the 8 nanomaterial levels either alone or in combination with a recommended dose of each of N, P, and K. Thus, a total of 9 treatments represented all planned possible applications of penta-replicated in a completely randomized design.

Uniformly sized seeds of French bean (*Phaseolus vulgaris* cv. *contender*) seeds were selected, washed thoroughly with tap water and then planted in a mixture of clay-loamy soil (2:1 v/v) in pots (30 × 28 × 26 cm). The soil obtained from the Agriculture Research Station of Mansoura, Dakahlia Governorate, Egypt, was taken from the upper 30 cm arable layer. All pots contained equal amounts of homogenous soil (8 kg) in which 5 to 7 seeds were planted and given 16 days for germination in the soil. That was followed by thinning and only 5 seedling/ pots were left for experimentation. For clarity the following treatments were used:

1. Control (C)
2. Foliar spray of nanochitosan 10% (nanochitosan 10%)
3. Foliar spray of nanochitosan 100% (nanochitosan 100%)
4. Foliar spray of nanochitosan NPK 10% (nanochitosan NPK 10%)
5. Foliar spray of nanochitosan NPK 100% (nanochitosan NPK 100%)
6. Foliar spray of carbon nanotubes-20 µg/L (CNTs-20 µg/L)
7. Foliar spray of carbon nanotubes-50 µg/L (CNTs-50 µg/L)
8. Foliar spray of carbon nanotubes NPK-20 µg/L (CNTs-NPK-20 µg/L)
9. Foliar spray of carbon nanotubes NPK-50 µg/L (CNTs-NPK-50 µg/L)

Treatment of French bean plants with nanofertilizers was carried out after 21 days from the date of germination. The appropriate amount of nanofertilizers and the recommended dose for each of the NPK used were calculated and added foliarily to each pot.

All pots were irrigated with tap water every three days to maintain each of soil at the field capacity throughout the experiment. Samples were taken after 30 days from the date of planting. Sampling was made in a way so as to include all plants allotted for each treatment in the five pots. Samples were used for determination of growth as well as uptake and translocation of nanofertilizer in French bean tissue by transmission electron microscopy (TEM).

The data obtained from triplicate samples were remarkably close, thus only the mean value will be presented. Experimental data were subjected to one-way analysis of variance (ANOVA) with Post Hoc L.S.D. (least significant difference) test in which p value < 0.05 was accepted statistically significant. Statistical analysis was performed with statistical package for social science for windows (SPSS, version 13.0, 2004, Chicago, IL, USA).

Measurement of growth parameters

Root length, shoot length, fresh weight, dry weight, water content and leaf area were determined to evaluate the sequence of growth characters of the different treated French bean plants throughout the entire period of the experiment.

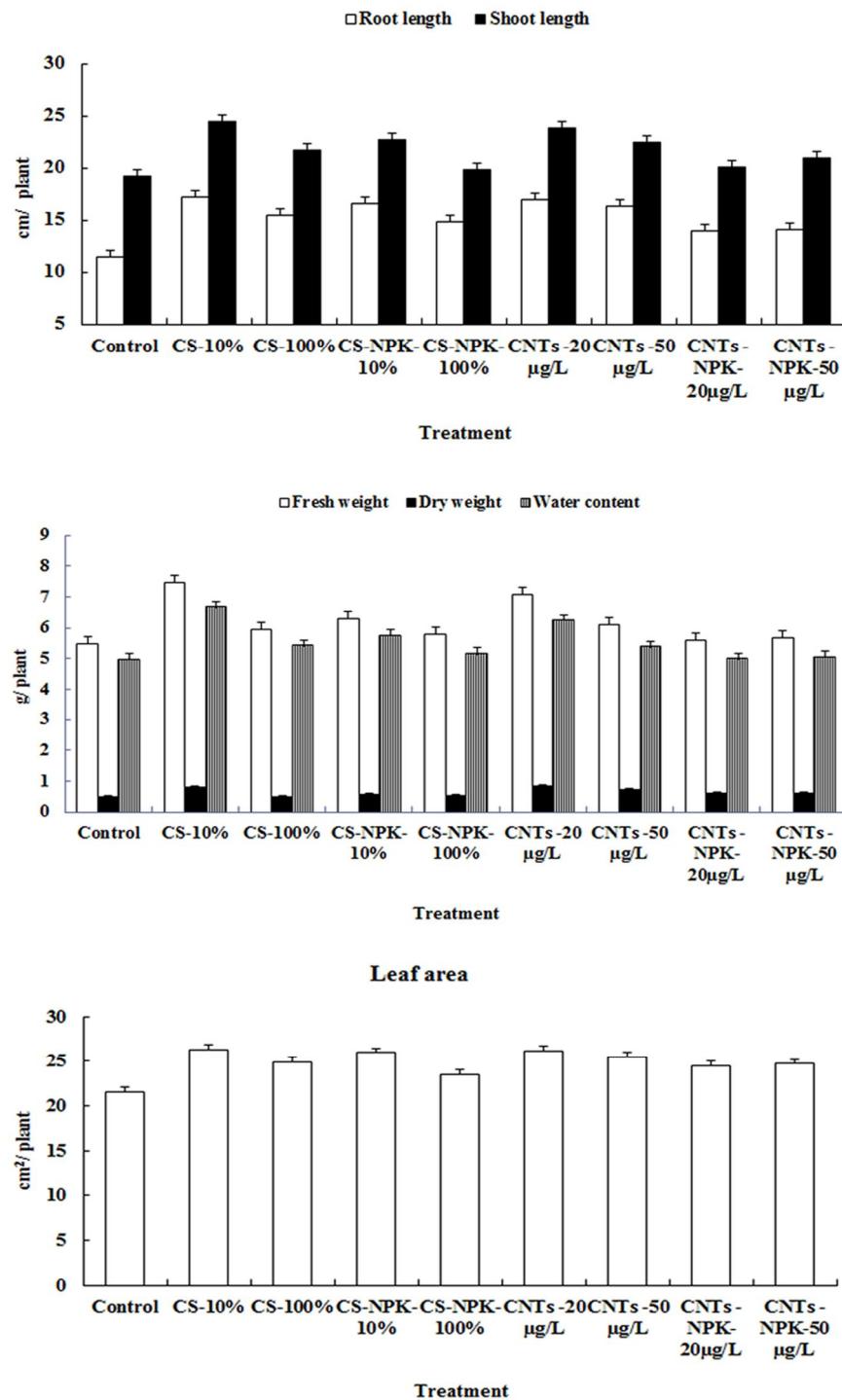


Figure 1. Effect of foliar application of chitosan nanoparticle and carbon nanotubes either alone or loaded with NPK fertilizers on the growth pattern of French bean plants. Vertical bars represent the standard error (\pm S.E.).

The calculated per cent improvement for the various growth parameters; in particular, root length, shoot length, fresh weight, dry weight, water content and leaf area, determined for French bean plants, as a result of a foliar application of engineered nanomaterials; carbon nanotubes NPK and nanochitosan NPK

fertilizer to plants, were as follow 59.3% in dry weight for CS-10%, 66.93% in dry weight for CNTs-20 µg/L. Results revealed synergistic effect of nanomaterials in enhancing dry weight, leaf area and growth rate leading to significant improvement in plant growth (Figure 1).

Transmission electron microscope determination (TEM)

Freshly harvested leaves were cut into small pieces (1 mm) with a sharp razor blade under 2.5% (v/v) glutaraldehyde. Leaf tissues were transferred to vials of 2.5% (v/v) glutaraldehyde in 1M phosphate buffer at pH 7.5 at 4°C for 24 h.

Following fixation, the specimens were embedded in gelatin capsules and left in an oven at 60°C for 60 h. The gelatin capsules were dissolved in boiling water for 1 to 2 h. Ultra-thin sections were cut on a Reichert ultra-microtome using glass knife. Silver or pale gold interference sections were picked up on the dull surface of form-coated 100 or 200 mesh copper grids (Juniper et al., 1970). The grids with sections were left on a clean filter paper to dry. Ultra-thin sections were stained by 2% aqueous uranyl acetate (Juniper et al., 1970). A drop of stain was put in a clean plastic Petri dish and the grids were gently floated, with the sections facing down, on a drop of the stain. The grids were washed by a stream of distilled water and then transferred to drops of lead citrate (Reynolds, 1963) which were placed on a wax plate in a Petri dish. Pellets of sodium hydroxide were placed in the Petri dish to remove carbon dioxide. The grids were left in lead citrate for 10 to 20 min and then rinsed by distilled water, dried under a bench lamp and stored in a grid box. The stained sections were examined and photographed with a JEOL 1010 transmission electron microscope at 80 kV.

RESULTS AND DISCUSSION

Changes in growth parameters

Root length, shoot length, fresh weight, dry weight, water content and leaf area were determined to evaluate the sequence of growth characters of the different treated plants. It was observed that with decrease in nanofertilizer concentrations, all growth criteria increase as shown in CS-10%, CS-NPK 10% and CNT-20 µg/L compared to control and decrease at higher concentration of nanofertilizers (Figure 1). Nanofertilizers harmonized the release of fertilizer-N and -P with their uptake by crops, so preventing undesirable nutrient losses to soil, water and air via direct entry by crops, and avoiding the interaction of nutrients with soil, microorganisms, water and air (De-Rosa et al., 2010).

Based on studies on nanoparticles effects on seed germination and growth mechanism, it could conclude that nanoparticles might help the water absorption by the seeds, increase nitrate reductase enzyme concentrations and promote seed antioxidant system (Lu et al., 2002). It was found that nanoparticles reduced antioxidant stress by reducing H₂O₂, superoxide radicals, and malonyldialdehyde content, and increased some enzymes such as superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, and catalase activities (Lei et al., 2008) which resulted in enhancement of seed germination in some plant species (Feizi et al., 2012).

Carbon nanotubes can regulate cell division and plant growth by a unique molecular mechanism that is related to the activation of water channels (aquaporins) and major gene regulators of cell division and extension (Khodakovskaya et al., 2012).

The present results concerning the increased growth

parameters of French bean plants as influenced by foliar application of either nanocomposite NPK nanoparticles or nanoengineered CNTs-NPK, in particular, at low concentration 10% for chitosan nanoparticles and CNTs-20 µg/L can be explained on the basis the sprayed nanoparticles may get absorbed through the stomata of french bean leaves and translocated in plants. The selective uptake, bioabsorption and transport of various nanoparticles by a model plant have been high reactivity because of more specific surface area, more density of reactive areas or increased reactivity of these areas on the particles surface. This features in nanoscalesimplify their absorption in plants (Dhoke et al., 2013). Mahmoodzadeh et al. (2013) and Hasaneen et al. (2015) reported that direct exposure of wheat plants to specific type of nanoparticles caused significant increase in all growth parameters determined at optimum concentrations of nanosolution. Furthermore, based on studies on nanoparticles effects on seed germination and plant growth mechanism, it could conclude that nanoparticles might help the water absorption by the seed, increase nitrate reductase enzyme concentrations, promote seed antioxidant system (Lu et al., 2002), reduce antioxidant stress by reducing H₂O₂, superoxide radicals, and malonyldialdehyde content, and increased some enzymes such as superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, and catalase activities (Lei et al., 2008) which resulted in enhancement of seed germination in some plant species (Feizi et al., 2012).

Changes in electrolyte leakage

Treatment of French bean plants grown in clay-sand soil with increasing concentration of either nanocomposite NPK (CS-NPK) fertilizers or nanoengineered carbon nanotubes (CNTs-NPK) fertilizers throughout the entire period of experiment appeared, in general, to significantly decrease the leakage of ions from the variously treated plants below those control levels; the response being more operative with the nanofertilizers (Figure 2).

Normal and nanofertilizers appeared to reduce the amount of malonyldialdehyde (MDA) and ion leakage in the treated wheat plants grown on clay, clay-sand or sand soils throughout the entire period of experiment, as reported by Oancea et al. (2009) who hypothesized that controlled release of active plant growth stimulators and other chemicals encapsulated in nanocomposites made of layered double hydroxides (anionic clay) could be another feasible option for organic agriculture.

Of interest, these results might indicate that nanocomposite NPK fertilizers mitigated the increase in the plasma membrane permeability and cell mortality under nanoparticle effects in wheat plants (Du et al., 2011). Similar results were observed in watermelon plant after foliary uptake of nanocomposite (Wang et al., 2013; Hasaneen et al., 2015).

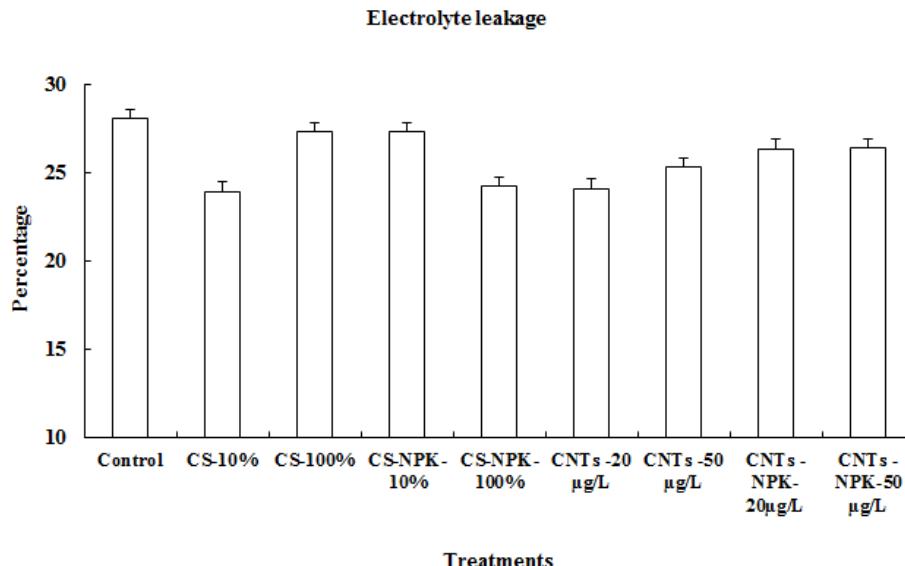


Figure 2. Effect of foliar application of nanocomposite (chitosan nano NPK) and nanoengineered (carbon nanotubes NPK) fertilizers on the electrolyte leakage of adult French bean plants. Vertical bars represent the standard error (\pm S.E.).

Nair et al. (2010) observed that the uptake efficiency and the effect of various nanoparticles on the growth and metabolic functions vary differently among plants. Foliar uptake (uptake through the leaves) of nanoparticles by plants represents another possible way for this purpose (Hasaneen et al., 2015). Leaves are important plant organs primarily for photosynthesis, transpiration and gas exchange (Nadakavukaren and McCracken, 1985).

Uptake and translocation of nanocomposite-NPK and carbon nanotubes NPK fertilizers

In all chitosan nanofertilizer treated plants, nanoparticles were observed inside phloem tissue; especially in sieve tubes. No nanoparticles, however, can be observed inside the xylem tissue. On the other hand, in all CNTs treated plants, nanotubes were observed in both xylem and phloem; especially in vessels and sieve tubes, respectively, confirm the penetration of plant leaves and lead to a strong support to the observed changes in growth of French bean plants affected by nanofertilizer as shown in Figures 3 and 4.

Thus, it can be conducted that adequate amounts of nano-NPK fertilizer, after being sprayed to the foliage of French bean plants, were taken up as N, P and K and assimilated within French bean plants. The water content, fresh and dry matter accumulation, stem and root length and leaf area in French bean plants at fully vegetative growth stages of adult plants (Figures 1 and 5).

These increases in various growth parameters may be directly related to the increased flux of nanocomposite NPK to the leaf (Hasaneen et al., 2015).

Examination of Figures 3 and 4 revealed that nanocomposite chitosan NPK appeared in leaf phloem tissue, in particular in sieve tubes of French bean plants treated with chitosan nano-NPK fertilizer at fully vegetative stage.

Thus, CNTs possess excellent tensile strength and are possibly the strongest, smallest fiber known. Most studies are increasingly carried out in order to obtain the uptake and transport mechanism of carbon-based nanomaterials into intact plant cells. There is proof that CNTs could translocate to systemic sites, such as fruits, leaves and roots, which could involve a strong interaction with the cells of the tomato seedling (Aslani et al., 2014).

Esmaeili et al. (2015) stated that since the first characteristic of the influence of stress on plants is growth reduction, the nanotubes and activated carbon at these concentrations cannot act as tension materials because they have a positive effect on callus growth. It showed that MWCNT-COOH can act as elicitors enhancing rosmarinic acid. These materials may increase primary metabolites such as amino acids tyrosine and phenylalanine which in turn increase the synthesis of rosmarinic acid.

The epidermis foliar of French bean plants is made up of cells with the exposed walls waterproofed by hydroxylated fatty acids (cutin and waxes), which form a cuticle membrane. Epidermis is provided with stomata ranging from 100 to 1300 per mm^2 , consisting of two guard cells, which, through expansion (turgor), form a pore between them ranging from 3 to 12 μm in width and 10 to 30 μm in length, for gas exchange (Eichert et al., 2008), according to the polar pore model (Schreiber, 2005), estimated 2 to 2.4 nm as the exclusion limit of the

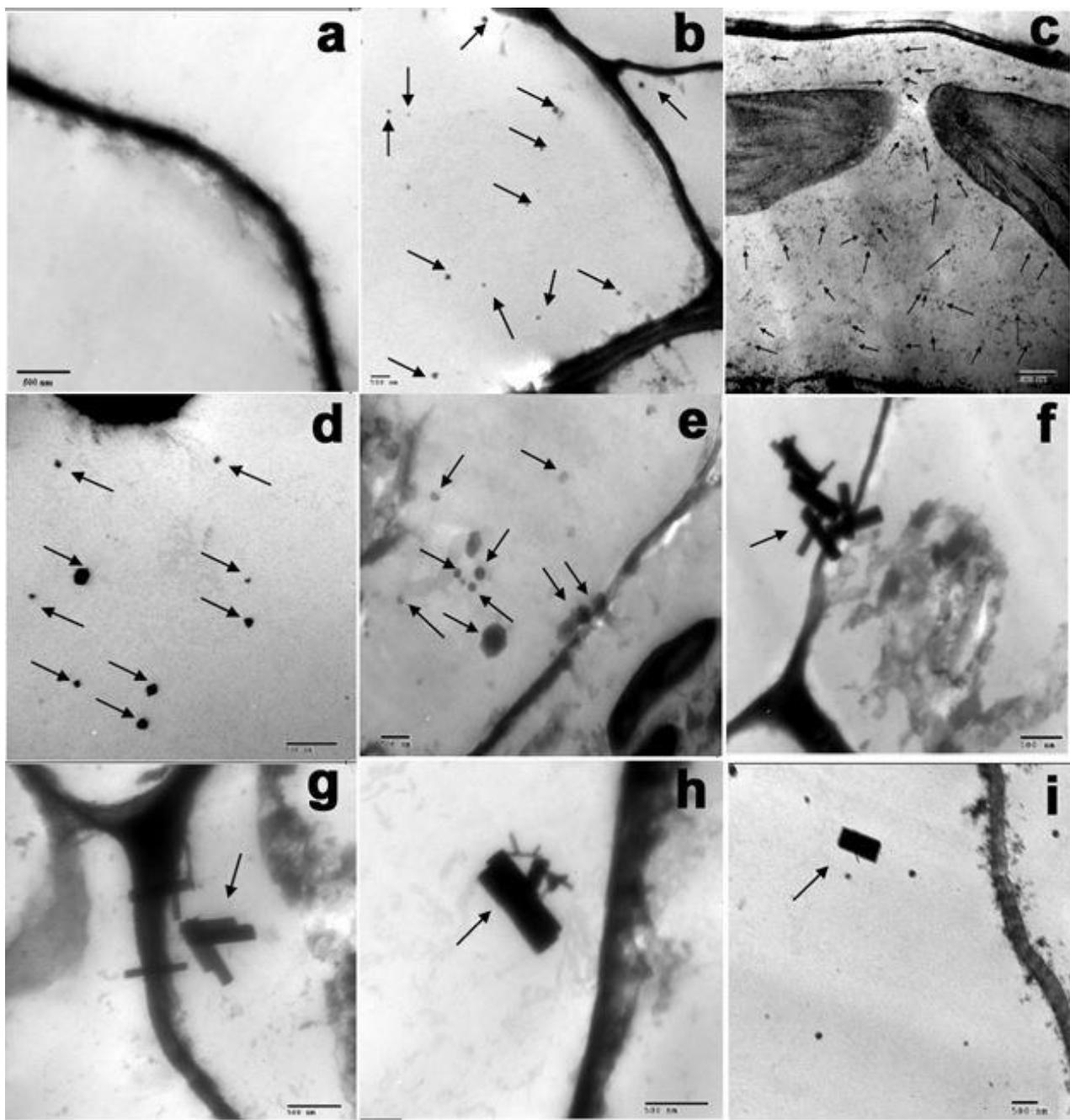


Figure 3. Transmission electron microscope images of chitosan nanoparticles and carbon nanotubes alone or loaded with NPK in sieve tubes of phloem elements of French bean leaves. a: Control, b: CS - 10%, c: CS - 100%, d: CS - NPK 10%, e: CS - NPK 100%, f: CNTs - 20 µg/L, g: CNTs - 50 µg/L, h: CNTs-NPK - 20 µg/L, i: CNTs-NPK - 50 µg/L.

pore radius for polar and ionic solutes to penetrate the cuticle, while for diffusion via the stomatal surface the pore radius was quite variable and always exceeded 20 nm (Asli and Neumann, 2009) and more often are around 5 nm. The progression to protoplasm (symplastic way) is made possible by cell membrane-embedded protein carriers and ionic channels, or by membrane invagination (endocytosis), which forms vesicles around the passenger. The transport cell to cell is made dynamic by cytoplasmic channels, the plasmodesmata, which are 20 to 50 nm in diameter at the midpoint and usually let inside small particles, around 3 nm (Dietz and Herth, 2011).

The pathway for penetrating MWCNTs the cell is through the openings of the primary cell wall, consisting of a polysaccharidic-proteic structure which, depending on the pectic component, is more or less porous (Fleischer et al., 1999); the pore sizes range from 3.5 to

20 nm (Asli and Neumann, 2009) and more often are around 5 nm. The progression to protoplasm (symplastic way) is made possible by cell membrane-embedded protein carriers and ionic channels, or by membrane invagination (endocytosis), which forms vesicles around the passenger. The transport cell to cell is made dynamic by cytoplasmic channels, the plasmodesmata, which are 20 to 50 nm in diameter at the midpoint and usually let inside small particles, around 3 nm (Dietz and Herth, 2011),

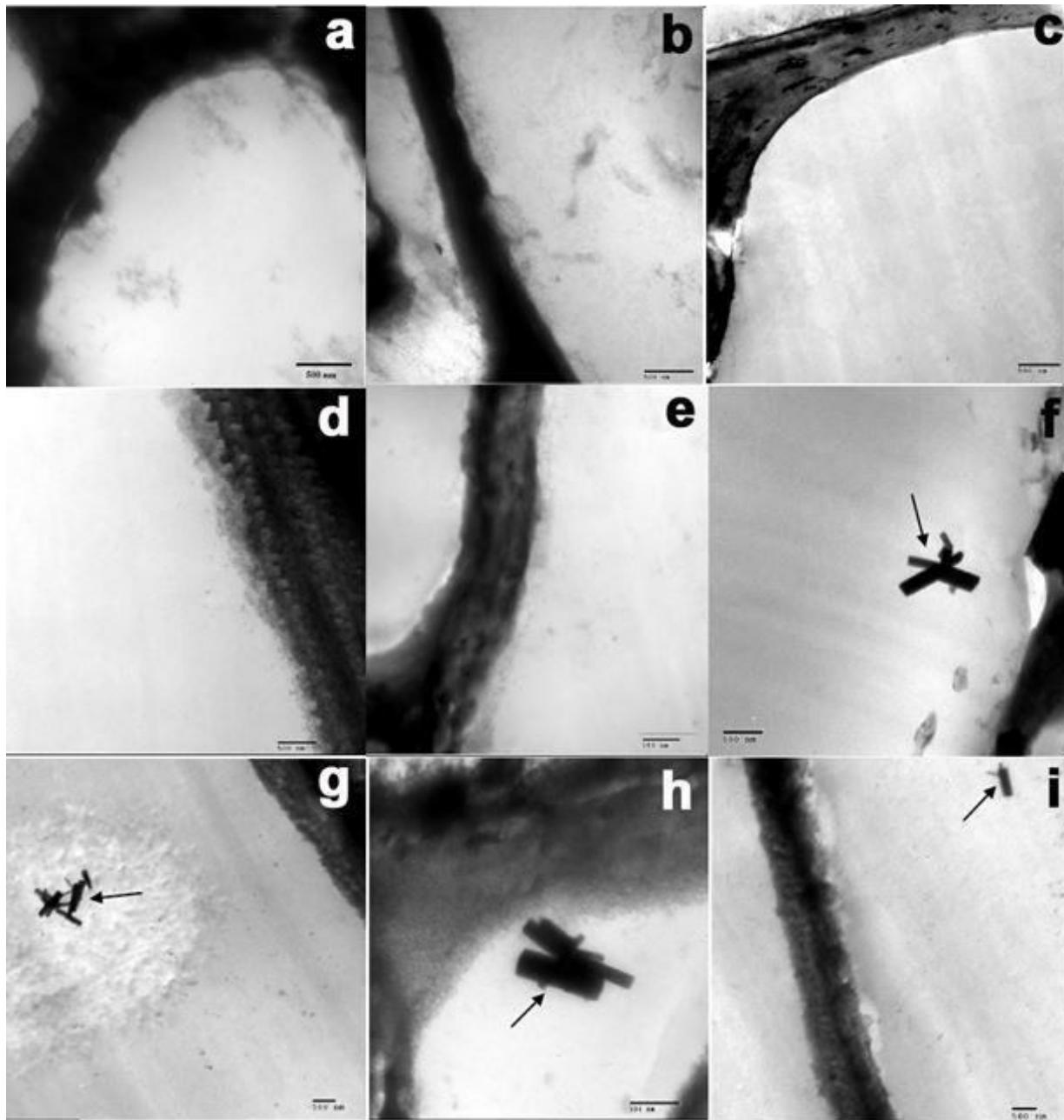


Figure 4. Transmission electron microscope images of chitosan nanoparticles and carbon nanotubes alone or loaded with NPK in xylem vessels of French bean leaves. a: Control, b: CS - 10%, c: CS - 100%, d: CS - NPK 10%, e: CS-NPK 100%, f: CNTs - 20 $\mu\text{g/L}$, g: CNTs - 50 $\mu\text{g/L}$, h: CNTs-NPK – 20 $\mu\text{g/L}$, i: CNTs-NPK – 50 $\mu\text{g/L}$.

but the exclusion limits are subject to variations as endogenous proteins mediate crossing. Below the foliar epidermis, the photosynthetic palisade (Chichiriccò and Poma, 2015) tissue provides small intercellular spaces which, together with the cell walls, form the apoplastic pathway. Through the symplastic and apoplastic pathways, small particles can reach the photosynthate conducting system (phloem vessels), made up of living sieve-cells devoid of a nucleus and most organelles, while being connected to one another and to the

surrounding tissues by wall sieve pores of 0.2 to 0.4 μm (Chichiriccò and Poma, 2015).

For successful foliar uptake, in addition to particle size other various factors should also be considered such as working environment (light, water and gas), plant species and nanoparticle application methods. In the present work, after chitosan nanoparticles and carbon nanotubes entering the stomata; the nanoparticles are translocated by the phloem system (Figures 3 and 4). The nanoparticles are carried in this sugar flow through the



Figure 5. Photograph showing the effect of foliar application of chitosan nanoparticle and carbon nanotubes either alone or loaded with NPK fertilizers on the growth pattern of French bean plants. a: Control, b: CS - 10%, c: CS - 100%, d: CS-NPK 10%, e: CS-NPK 100%, f: CNTs -20 µg/L, g: CNTs - 50 µg/L, h: CNTs-NPK - 20 µg/L, i: CNTs-NPK - 50 µg/L.

phloem sieve tubes to shoots and roots as a result of pressure differentials between source (leaves) and sink (e.g., growing shoot apex) based on mass flow or pressure flow hypothesis which indicate the presence of chitosan nanoparticles inside phloem tissue of French bean plants and not present in xylem tissue. On the other hand, carbon nanotubes were observed in both xylem vessels and phloem sieve tubes. There are evidences that radial transport from cell to cell occurs (Corredor et al., 2009), which may involve the trafficking pathway to plasmodesmata. Once the nanoparticles are inside the cells, they can be transported via endosomes toward other areas.

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