

Effect of Using Two Different Types of Engineered Nanomaterials on The Growth and Antioxidant Enzymes of French Bean Plants

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ABSTRACT

The application of nanomaterials in agriculture is one of the most greatly studied areas in nanotechnology as it includes the using of nanofertilizers to transport nutrient elements to the plant at the needed time and required amount, thereby improving the use efficiency. This work was designed to investigate the effects of soil incorporation of two different types of engineered nanofertilizer namely: carbon nanotubes and chitosan nanoparticles alone or loaded with nitrogen, phosphorus and potassium (NPK) on the different growth criteria of French bean plants. The results indicate that after about 30 days from the date of planting, nanomaterials either alone or in combination with NPK significantly decrease plant growth and increase the activity of antioxidant enzymes implying that French bean plants were exposed to an oxidative stress.

Keywords: carbon nanotubes, chitosan, French bean, nanofertilizer, antioxidant enzymes

INTRODUCTION

As nanotechnology is an interdisciplinary technology which solves problems and enters in all stages of production, processing, storing, packaging and transport of agricultural products, it is proved its place in agriculture and correlated industries (Froggett, 2009). Nanofertilizers are known as nutrient carriers with size ranging from 30 to 40 nm and due to their high surface area they are able to holding plentiful nutrient ions and release it slowly and gradually in a timely manner to a rhizospheric target that proportionate with crop demand and have high use efficiency. Nanofertilizers are ultrahigh absorption able to increase production, rise photosynthesis, and expansion of leaves surface area (INIC, 2014). Nanomaterials might be toxic than its bulk compound due to their superior contact surface area, especially in an agricultural ecosystem (Lang *et al.*, 2011; Rizzello and Pompa, 2014).

Chitosan nanoparticles (nanochitosan) are one of the engineered nanomaterials generating from chitosan which is a linear hydrophilic polysaccharide that is biodegradable, biocompatible, non-toxic nature biopolymer and react with bioactive molecules due to the presence of $-\text{NH}_2$ and $-\text{OH}$ functional groups so, it has been used as a functional biopolymer in food and pharmaceuticals (Dai *et al.*, 2009; Hanafi, 2012; Cho *et al.*, 2010). 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). While, carbon nanotubes (CNTs) is six times lighter and hundred times stronger than stainless steel, as hard as diamond and its thermal capacity is twice that of pure diamond, their current carrying capacity is 1000 times higher than that of copper and thermally stable up to 4000 Kelven (Kumar, 2011). These physical and chemical properties enable carbon nanomaterials to be used in agriculture to increase the germination process, root growth, photosynthesis and crop yield (Flores *et al.*, 2014). CNTs are used for the controlled release of nutrient, agrochemical, drug and biological delivery to specific cells of these reagents (Irin *et al.*, 2012).

The aim of this work was to investigate the effects of soil incorporation of chitosan nanoparticles and carbon nanotubes loaded with NPK fertilizer at

different concentrations on growth of French bean plants and their antioxidant enzymes.

MATERIALS AND METHODS

1. Plant materials

Pure strain of *Phaseolus vulgaris* cv. Contender seeds were kindly supplied by Horticultural Research Center, Ministry of Agriculture, Mansoura, Egypt.

2. 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 according to De Moura *et al.* (2008) and Hasaneen *et al.* (2014). Meanwhile, CNTs easily and inexpensively prepared at room temperatures according to Lee and Seo (2011).

Incorporation of NPK into chitosan nanoparticles and carbon nanotubes was carried out according to Corradini *et al.* (2010) and Hasaneen *et al.* (2014).

3. 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 $\mu\text{g/l}$ and CNTs- 50 $\mu\text{g/l}$ 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 (30x 28x 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 (8kg). The

appropriate amount of nanofertilizers and the recommended dose for each of the NPK used were calculated and mixed with soil nanofertilizers before sowing. About 5 to 7 seeds were planted in each pot and given 16- days for germination in the soil. That was followed by thinning and only 5 seedling/ pots were left for experimentation.

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

Experimental data were statistically analysis using one-way analysis of variance (ANOVA) with Post Hoc L.S.D. (least significant difference) test. * P value < 0.05 was accepted statistically significant performed with statistical package for social science for windows (SPSS, version 13.0, 2004, Chicago, IL, USA).

4. Estimation of antioxidant enzymes

Enzyme extraction

Enzyme extracts were prepared by grinding 0.2 g of 30- d old fresh French bean leaves with 5 cm³ of chilled phosphate buffer, for determination of SOD, CAT, POX and GR activities. For SOD the extraction medium buffer was 0.1M phosphate buffer at pH 7.8, while for CAT, POX and GR the extraction medium buffer was 0.1M phosphate buffer was at pH 6.8. The mixture then was filtered through cheesecloth and the filtrate was centrifuged at 10000 rpm for 20 minutes at 4°C. The supernatant served as enzyme extract (Agarwal and Shaheen, 2007).

Estimation of Superoxide Dismutase activity (SOD, EC1.15.1.1)

The SOD activity was determined according to the method of Giannopolitis and Ries (1977). The reaction mixture was consisted of 0.1 cm³ of enzyme extract, 1cm³ of working buffer (10 cm³ of phosphate buffer, 1cm³ of nitro-blue-tetrazolium (NBT) and 1cm³ of NADH) and mixed well. To initiate the reaction, 0.1 cm³ of phenazine methosulphate (PMS) was added. The absorbance was measured at 560 nm.

Estimation of catalase activity (CAT, EC1.11.1.6)

A reaction mixture containing 1cm³ of potassium phosphate buffer and 0.2 cm³ of H₂O₂ was add to 1.5 cm³ of plant extract to initiate the reaction and the activity was measured at 240 nm over 1 min by monitoring the removal of H₂O₂ (Gomes-Junior *et al.*, 2007). CAT activity is expressed as $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein.

Estimation of Glutathione reductase activity (GR, EC1.8.1.7)

The method proposed by Beutler (1984) was adopted for assaying the activity of glutathione reductase. 1.5 cm³ of buffer was added to 0.5 cm³ of ethylene diamine tetra acetic acid (EDTA), 0.2 cm³ of oxidized glutathione (GSSG) and 0.1cm³ of NADPH. To initiate the reaction, 0.2 cm³ of enzyme extract was added to the mixture. The enzyme activity is calculated intermediate of micromoles of NADPH oxidized /min/mg protein.

Estimation of Peroxidase activity (POX, EC1.11.1.7)

Peroxidase activity was assayed by the method of (Devi, 2002). 3cm³ of pyrogallol buffer in 0.1 M phosphate buffer was added to 0.1 cm³ of enzyme extract, and 0.5 cm³ of 1% H₂O₂. The absorbance at 420 nm was read to determine the amount of formed purpurogallin. The enzyme activity was expressed in units. One unit is defined as unit per g fresh weight per min.

Estimation of polyphenoloxidase (PPO, EC. 1.10.3.1)

1cm³ of enzyme extract was added to 2 cm³ of phosphate buffer and 1 cm³ of Pyrogallol. The reaction mixture was incubated at 25⁰ C for 1 minute, after that the reaction was stopped by the addition of 1cm³ of 2.5 N H₂SO₄. The absorbance of the formed purpurogallin was recorded at 420 nm. The enzyme activity was expressed in units. One unit is defined as unit per g fresh weight per minute. About 0.1 cm³ of the supernatant was added to 0.5 cm³ of phosphate buffer and 1 cm³ of 2,6-, dichlorophenyl indophenole (DCPIP). The absorbance was measured at 520 nm within 1 h (Devi, 2002).

RESULTS AND DISCUSSION

1. 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. Treatment of French bean seeds or plants with either nanocomposite (nanochitosan NPK) or nanoengineered (carbon nanotubes NPK) fertilizers through soil incorporation technique led to significant progressive decrease in all growth parameters below the control values. The following sequence of treatments: control > CNTs-20 $\mu\text{g/L}$ > CNTs-NPK-20 $\mu\text{g/L}$ > CNTs-50 $\mu\text{g/L}$ > CNTs-NPK-50 $\mu\text{g/L}$ > CS-10%> CS-NPK-10%> CS-100%> CS-NPK-100% was displayed with respect to French bean plants grown on clay-sandy soil and treated with soil incorporation technique throughout the entire period of the experiment (Table 1 and figure 1).

In particular, the use of nanomaterials for improving plant growth, generally related to the concentrations of nanoparticle applied to various plant organs or tissues (Khodakovskaya *et al.*, 2009). Generally, nanoparticles may be applied to seeds or roots. But the interactions between nanoparticles, soil and plants may be complicated and cause ecological safety problems (Wang *et al.*, 2013). Furthermore, soil application of nanomaterials at low concentration can enhance growth of plants while nanomaterials applied through nutrient solution increase the bioavailability and accumulation in plants thereby inhibiting growth (Razzaq *et al.*, 2016). Nanoparticles toxicity depends on the differences in root anatomy because xylem structures determine the speed of water transport and different xylem structures may demonstrate different uptake kinetics of nanoparticles (Lee *et al.*, 2010).

Table 1: The effects of soil incorporation technique of nanocomposite (chitosan nano NPK) and nanoengineered (carbon nanotubes NPK) fertilizers on the growth of 30- d old French bean plants (vegetative stage). *Mean values listed are significant different from control at $p \leq 0.05$

Parameters	Root length (cm plant ⁻¹)	% change	Shoot length (cm plant ⁻¹)	% change	Fresh weight (g plant ⁻¹)	% change	Dry weight (g plant ⁻¹)	% change	Water content (g plant ⁻¹)	% change	Leaf area (cm ² plant ⁻¹)	% change
Control	11.20	—	19.20	—	—	—	0.499	—	4.98	—	24.33	—
CS-10%	9.50*	-15.17 %	15.90*	-17.18 %	2.35*	-57.11 %	0.332*	-33.46 %	2.02*	-59.43 %	20.78*	-14.59 %
CS-100%	—	—	—	—	—	—	—	—	—	—	—	—
CS-NPK-10%	9.30*	-16.96 %	15.50*	-19.27 %	2.18*	-60.21 %	0.326*	-34.66 %	1.85*	-62.85 %	18.22*	-25.11 %
CS-NPK-100%	8.90*	-20.53 %	14.60*	-23.95 %	2.06*	-62.40 %	0.280*	-43.88 %	1.78*	-64.25 %	17.36*	-28.64 %
CNTs -20 µg/L	10.40*	-7.14 %	18.40*	-4.16 %	3.95*	-27.91 %	0.443	-11.22 %	3.51*	-29.51 %	23.74*	-2.42 %
CNTs -50 µg/L	9.90*	-11.60 %	17.50*	-8.85 %	2.90*	-47.08 %	0.388*	-22.24 %	2.51*	-49.59 %	22.08*	-9.24 %
CNTs -NPK 20µg/L	10.00*	-10.71 %	18.10*	-5.72 %	3.49*	-36.31 %	0.380*	-23.84 %	3.11*	-37.55 %	22.39*	-7.97 %
CNTs -NPK-50 µg/L	9.60*	-14.28 %	17.20*	-10.41 %	2.74*	-50.00 %	0.372*	-25.45 %	2.37*	-52.40 %	21.53*	-11.50 %

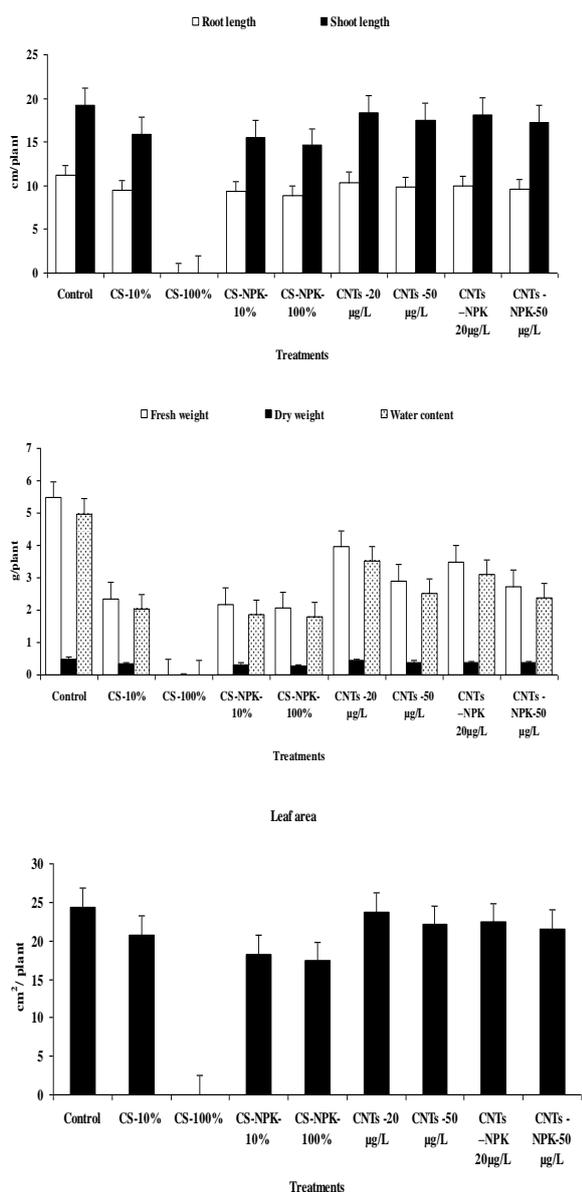


Fig. 1. Effect of nanocomposite (chitosan nano NPK) and nanoengineered (carbon nanotubes NPK) fertilizers on the growth parameters of 30- d old French bean plants treated by soil incorporation technique. Vertical bars represent the standard error (\pm S.E.).

2. Changes in enzymatic antioxidants

Tables 2 and figures 2 show the activity of SOD, POX, PPO, GR and CAT enzymes of the control as well as the differently treated French bean plants with soil incorporation technique. In the variously treated plants, there appeared a progressive increase in all the activities of the determined enzymes throughout the duration of the experimental 30- day- period. The following sequence of treatments in variously treated plants: Control < CNTs-20 µg/L < CNTs -NPK- 20µg/L < CNTs -50 µg/L < CNTs -NPK-50 µg/L < CS-10 % < CS-NPK-10 % < CS-100 % < CS-NPK-100 % was displayed with respect to SOD, POX, PPO, GR and CAT activities of French bean plants treated with soil incorporation technique.

The percentage of improvement in SOD, POX, PPO, GR and CAT activities of French bean plants treated with soil incorporation technique were shown in table 2. For example in French bean plants treated with CNTs- 20 µg/L were found to be: 13.58 %, 4.33 %, 15.00%, 1.61 % and 11.91 % and in French bean plants treated with CS-NPK-100 % were: 25.54 %, 64.00 %, 16.00 %, 15.90 % and 46.18 % respectively.

After entering the cell, nanoparticles may induce intracellular oxidative stress which represents a common mechanism for cell damage induced by nanoparticles (Pulskamp *et al.*, 2007) by disturbing the balance between both oxidant and antioxidant processes and generating harmful ROS due to their large surface areas. Excessive oxidative stress may also alter membranes, lipids, proteins and nucleic acids, which stimulates the antioxidant defense system resulting in cellular injury or leads to cell death (Moore, 2006; Laware and Raskar, 2014). The response of plant to nanoparticles stress has been evaluated in various crops at physiological and biochemical levels (Hossain *et al.*, 2015).

Our results are in agreement with Tan *et al.* (2009) observation since antioxidant enzymes in rice cells were significantly induced when treated with 20 mg/L of MWCNTs. This enzyme contents increment indicates that the cells are under oxidative stress and it may lead to cell proliferation decrease or cell death through the apoptotic pathway or necrosis. Tea leaves treated chitosan nanoparticles showed significant increase in accumulation of POX, PPO, SOD, CAT and

phenol content. In chitosan nanoparticles treated leaves, both enzyme activity and mRNA expression levels of CAT and SOD were found to be significantly higher than control plants. Hence, the increased expression of SOD and CAT might protect the plants from the oxidative stress (Chandra *et al.*, 2015).

Due to mechanical stress and injury, plants change their morphology or growth rate to adapt to stress. This phenomenon has known as thigmomorphogenesis. Also plant hormones play an

important role in this process (Chehab *et al.*, 2009) as the activity of jasmonic acid, the plants stress hormone, increases, while, the activity of auxin, morphogenesis and plant growth hormone decreases. This alteration in plant hormone levels may be associated with the increase of peroxidase activity (Woodward and Bartel, 2005) and this increase is accompanied with the oxidative stress caused by nanomaterials and initiates the mechanism of response of the plant to stress.

Table 2: Effect of soil incorporation of nanocomposite (chitosan nano NPK) and nanoengineered (carbon nanotubes NPK) fertilizers on the activities of antioxidant enzymes (SOD; U/g fresh weight, POX; U/g fresh weight/ min, PPO; U/g fresh weight/ min, GR; U/g fresh weight, CAT; U/g fresh weight) of 30- d old French bean plants. *Mean values listed are significant different from control at $p \leq 0.05$

Parameters	SOD	% changes	POX	% changes	PPO	% changes	GR	% changes	CAT	% changes
Control	14.13	—	3.00	—	1.00	—	14.84	—	5.37	—
CS-10%	17.27*	22.22 %	4.11*	37.00 %	1.16*	16.00 %	16.89*	13.81 %	7.33*	36.49 %
CS-100%	—	—	—	—	—	—	—	—	—	—
CS-NPK-10%	17.52*	23.99 %	4.45*	48.33 %	1.16*	16.00 %	17.05*	14.89 %	7.42*	38.17 %
CS-NPK-100%	17.74*	25.54 %	4.92*	64.00 %	1.16*	16.00 %	17.20*	15.90 %	7.85*	46.18 %
CNT -20 µg/L	16.05*	13.58 %	3.13*	4.33 %	1.15*	15.00 %	15.08*	1.61 %	6.01*	11.91 %
CNT -50 µg/L	16.37*	15.85 %	3.80*	26.66 %	1.10*	10.00 %	16.14*	8.76 %	6.95*	29.42 %
CNT -NPK- 20µg/L	16.18*	14.50 %	3.25*	8.33 %	1.15*	15.00 %	15.23*	2.62 %	6.63*	23.46 %
CNT -NPK-50 µg/L	16.80*	18.89 %	3.87*	29.00 %	1.14*	14.00 %	16.37*	10.30 %	7.28*	35.56 %

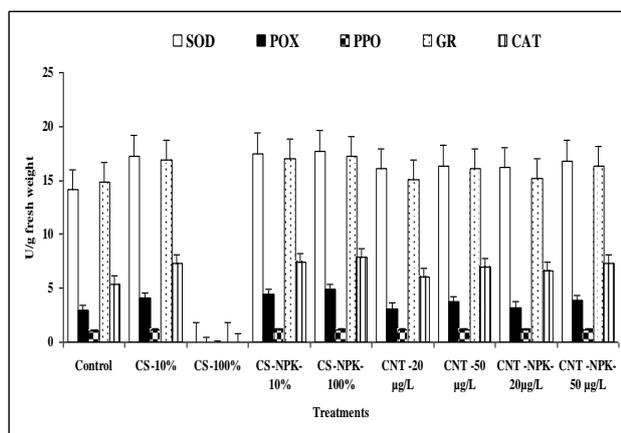


Fig. 2. Effect of nanocomposite (chitosan nano NPK) and nanoengineered (carbon nanotubes NPK) fertilizers on antioxidant enzymes of 30- d old French bean plants treated by soil incorporation. Vertical bars represent the standard error (\pm S.E.).

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**تأثير استخدام نوعين من المواد النانومترية على النمو والإنزيمات المضادة للأوكسدة في نبات الفاصوليا الحمراء
محمد نجيب عبد الغنى حسنين ، هبة محمود محمد عبد العزيز و آية محب عمر بدير
قسم النبات – كلية العلوم – جامعة المنصورة**

يعتبر استخدام المواد النانومترية في الزراعة أحد أهم تطبيقات تكنولوجيا النانو الحيوية حيث تستخدم كمخصبات تعمل على نقل العناصر الغذائية إلى النبات في الوقت المحدد وبالكمية المطلوبة لحاجة النبات. في هذا البحث تم إجراء تجربة لدراسة تأثير استخدام نوعين من تلك المواد وهما جزيئات الكيتوزان النانومترية وأنابيب الكربون النانومترية سواء منفردتين أو محلين بسماد النيتروجين والفسفور والبولتاسيوم على نمو نبات الفاصوليا الحمراء. وأوضحت الدراسة أن خلط تلك المحاليل بالتربة قبل الزراعة أثر سلبي على نمو نبات الفاصوليا الحمراء (عمر ٣٠ يوم من الزراعة). كما أدى إلى زيادة ملحوظة في نشاط الإنزيمات المضادة للأوكسدة مما يعنى أن النبات تعرض للإجهاد.