

## Effect of Priming with Chitosan Nanoparticles on Germination, Seedling Growth and Antioxidant Enzymes of Broad Beans

Heba Mahmoud Mohammad Abdel-Aziz\*

Botany Department, Faculty of Science, Mansoura University, Egypt



### ABSTRACT

This study investigated the effect of two different concentrations (0.05% and 0.1%) of chitosan nanoparticles (CsNPs) as priming solutions (for 6 h) of *Vicia faba* seeds cv. Sakha 1, followed by germination and subsequent growth of seedlings for seven days. Chitosan nanoparticles were prepared using methacrylic acid and showed a mean size of  $20 \pm 2$  nm. Both concentrations of chitosan nanoparticles caused deleterious effects on germination and seedling growth criteria. Germination was greatly reduced in both concentrations as compared to control (distilled water). The magnitude of decrease was much pronounced with the higher concentration of chitosan nanoparticles (0.1%). On the other hand, the lower concentration of CsNPs (0.05%) increased the content of total phenols and the activities of antioxidant enzymes (catalase, ascorbate peroxidase, peroxidase and polyphenol oxidase) as compared with those of the control seedlings. This might indicate that the relatively low concentration of chitosan nanoparticles enhanced the defense system of seeds by increasing total phenols and antioxidant enzyme activities.

**Keywords:** antioxidants, broad beans, chitosan nanoparticles, germination, growth.

### INTRODUCTION

Nanotechnology had proved its vital role in every aspect of day life. Recently, extensive research is being carried out to investigate the possible ways of interaction of nanoparticles in agriculture. Approaches include improvement of soil properties, germination kinetics, uptake of nutrients and fertilization, pest management, drought tolerance and delivery of genetic materials (Choudhary *et al.*, 2016).

Chitosan (Cs) is a polymer produced by fungi, insects and crustaceans, it has positive influences on growth and productivity of many plants and it also has antimicrobial activities (Malerba and Cerana, 2016). Chitosan nanoparticles (CsNPs) are currently being prepared and studied in many aspects of research including industrial applications, medicinal applications, drug delivery, pesticide management and agricultural fertilizers (Malerba and Cerana, 2016). CsNPs are currently being used as nanofertilizers to many crops and their effects as foliar fertilizers were promising giving positive impact on growth and productivity of wheat (Abdel-Aziz *et al.*, 2016), French bean (Hasaneen *et al.*, 2016) and coffee (Van *et al.*, 2013). It is also reported that foliar fertilization using chitosan nanoparticles enhanced tolerance mechanisms against salt stress in beans (Zayed *et al.*, 2017).

Seed priming is a technique in which seeds are soaked in a particular solution for a period of time (no radicle emergence or breaks in seed coat) and then are used for germination (Paparella *et al.*, 2015). The purpose of priming of seeds is to improve germination and seedling growth and to make seeds tolerant to stressful conditions by increasing their resistance (Paparella *et al.*, 2015). Priming with nanoparticles is a new trend in order to obtain highly resistant seeds and to improve germination and seedling growth under different abiotic stress conditions (Maroufi *et al.*, 2011).

Priming of bean seeds with chitosan nanoparticles

(0.1%, 0.2% and 0.3%) for 3 h and germination under 100 mM NaCl enhanced seed germination and radicle length under the influence of salinity (Zayed *et al.*, 2017). The authors reported also that proline, chlorophyll a and antioxidant enzyme activities of bean seedlings treated with 0.1% chitosan nanoparticles under salt stress showed significant improvement as compared with salt-stressed untreated ones (Zayed *et al.*, 2017). Priming with chitosan nanoparticles could have some toxic effects on seeds when applied at higher concentrations; thus, the applied concentrations of chitosan nanoparticles should be monitored to provide good results with less toxic effects (Zayed *et al.*, 2017).

The aim of the present study was to investigate the effect of priming of broad bean seeds (Sakha 1) with two different concentrations of chitosan nanoparticles: 0.05% and 0.1% on germination and the possible changes maintained in total phenols and some antioxidant enzyme activities.

### MATERIALS AND METHODS

#### Plant material and growth conditions

Seeds of *Vicia faba* (cv. Sakha 1) were supplied by Sakha Agricultural Research Station, Kafr El-Sheikh, Egypt. A homogenous lot of seeds were selected and surface sterilized by soaking in 1% sodium hypochlorite solution for 3 min, then washed with sterilized distilled water three times. Seeds were soaked in sterilized distilled water with aeration for 12 h.

Two different concentrations of chitosan nanoparticles (CsNPs) (0.05% and 0.1%) were used as priming solutions. Hundred seeds were soaked in each concentration for 6 h and then allowed to germinate in plastic boxes (28×16×12 cm) (in four replicates). Each plastic box contained 25 seeds germinated on a Whatmann no.1 filter paper soaked with 20 ml distilled water. Control group was soaked in distilled water and germinated using sterilized distilled water. Seeds were incubated for

\* Corresponding author e-mail: hebamabdelaiz@mans.edu.eg

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7 d under dark conditions in an incubator at  $25 \pm 2^\circ\text{C}$ . The experiment was repeated three times.

Germination percentage was calculated from the first day of germination until the seventh day. Emergence of the radicle for 2 mm was an indicator of successful germination as indicated by ISTA (2009). Data of germination percentage presented here are those of the 7th day. At the end of the experiment seedlings were collected for estimation of different growth variables and calculation of germination rate, seedling vigours, mean daily germination (MDG), pick value (PV = maximum germinated seed number at one day/day number), germination value ( $\text{GV} = \text{PV} \times \text{MDG}$ ) according to the equations provided in Azimi *et al.* (2013). Samples were taken for the estimation of total phenols and some antioxidant enzymes.

### Preparation and characterization of chitosan nanoparticles

Chitosan nanoparticles were prepared using methacrylic acid according to the method developed by DeMoura *et al.* (2008), Corradini *et al.* (2010) and Hasaneen *et al.* (2014). About 0.2 g chitosan (degree of deacetylation, 98%) were dissolved in 0.5 (v/v) of methacrylic acid aqueous solution for 12 hours under magnetic stirring. Then, about 0.005 g potassium persulfate was added to the previous solution with continuous stirring till the solution became clear. After that, the solution was heated up at  $70^\circ\text{C}$  for 1 hour under magnetic stirring to ensure the formation of chitosan nanoparticles. Finally, the solution was cooled in an ice bath to stop the reaction.

The average size and zeta potential of the obtained chitosan nanoparticles were determined by measuring zeta size using Zetasizer NanoZS (Malvern Instruments, UK). On a carbon coated grid, one drop of the prepared chitosan nanoparticles was spread and then the grid was dried at room temperature and examined using a JEOL 1010 transmission electron microscope at 80 kV (JEOL, EM unit, Mansoura University).

### Estimation of total phenols

Total phenols were estimated in sample tissues according to the method developed by Ainsworth and Gillespie (2007). Fresh seedlings (0.2 g) were homogenized with 5 ml 95% methanol and kept in the dark at room temperature for 48 h. Samples were centrifuged at 13000 g for 5 min and supernatant was collected. The reaction mixture contained 0.5 ml of sample, 1 ml of 10% Folin-Ciocalteu reagent and 4 ml of 700 mM sodium carbonate. Methanol was used as blank. The reaction mixture was kept at room temperature for 2 h and the absorbance of the resulting colour was read at 765 nm using a spectrophotometer. Gallic acid solutions were used to make standard curve and total phenols were estimated from that curve.

### Enzyme extraction

Broad bean seedlings (0.2 g) were homogenized in a mortar with 5 ml of chilled phosphate buffer. For APX, the extraction medium was 0.1 M phosphate buffer at

pH 7.8 and for CAT, POX and PPO, 0.1 M phosphate buffer at pH 6.8 was used. The homogenate was filtered through cheesecloth and the filtrate was centrifuged in a refrigerated centrifuge at 10,000 g for 20 min. The supernatant served as enzyme extract. All operations were carried out at  $4^\circ\text{C}$  (Agarwal and Shaheen, 2007).

### Assay of catalase activity

Catalase (CAT) activity was assayed by the method of Sinha (1972). The enzyme extract (0.5 ml) was added to the reaction mixture containing 1 ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M  $\text{H}_2\text{O}_2$ , 0.4 ml  $\text{H}_2\text{O}$  and incubated for one minute. The reaction was terminated by the addition of 2 ml of acid reagent (5% potassium dichromate/ glacial acetic acid mixture, 1:3 by volume). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10 minutes and the absorbance was read at 610 nm. Catalase activity was expressed in  $\mu\text{moles}$  of  $\text{H}_2\text{O}_2$  consumed/min/g fresh tissue.

### Assay of ascorbate peroxidase activity (APX)

Ascorbate peroxidase activity was assayed by measuring the decrease in absorbance at 290 nm due to ascorbate oxidation; as adopted and described by Nakano and Asada (1981). The reaction mixture contained 0.83 ml of 0.5 mM AsA in phosphate buffer (pH 7), 0.13 ml of 2 mM  $\text{H}_2\text{O}_2$  and 0.04 ml of enzyme extract in a final volume of 1 ml at  $25^\circ\text{C}$ . Ascorbate peroxidase activity was expressed in terms of  $\mu\text{moles}$  of AsA oxidized/min/g fresh tissue.

### Assay of peroxidase activity (POX).

POX activity was assayed as the increase in absorbance at 420 nm due to the formation of purpurogallin (Devi, 2002). The reaction mixture contained 3 ml of pyrogallol phosphate buffer (0.05 M pyrogallol in 0.1 M phosphate buffer, pH 6), 0.1 ml of enzyme extract and 0.5 ml of 1%  $\text{H}_2\text{O}_2$ . One enzyme unit is defined as unit per min per g fresh weight.

### Assay of polyphenol oxidase activity (PPO)

PPO activity was assayed as the increase in absorbance at 420 nm due to the formation of purpurogallin (Devi, 2002). The reaction mixture contained 2  $\text{cm}^3$  of 0.02 M phosphate buffer (pH 7), 1  $\text{cm}^3$  of 0.1 M pyrogallol and 1  $\text{cm}^3$  of enzyme extract. The reaction mixture was incubated for 1 min at  $25^\circ\text{C}$  and the reaction was stopped by adding 1  $\text{cm}^3$  of 2.5 N  $\text{H}_2\text{SO}_4$ . One enzyme unit is defined as unit per g fresh weight per min.

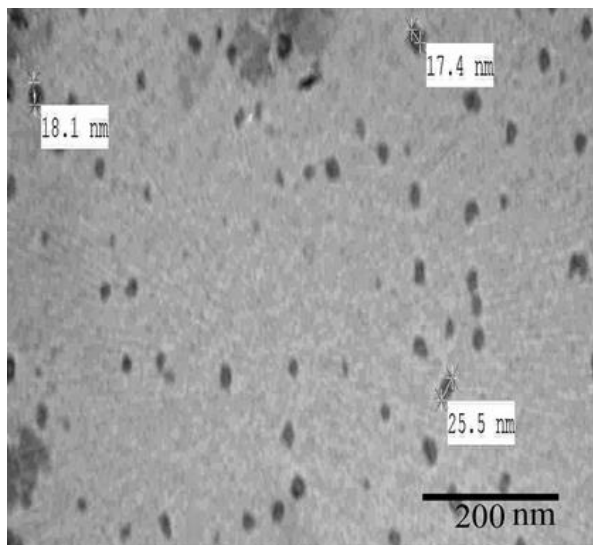
### Statistical analysis

Mean values of all three replicates are represented here. Data were subjected to one-way analysis of variance (ANOVA) with Post Hoc L.S.D. (least significant difference) test. \**P* value  $\leq 0.05$  was accepted statistically significant. Statistical analysis was performed with statistical package for social science for windows (SPSS, version 20.0, 2011, IBM Corp., Armonk, NY, USA).

## RESULTS

**Characterization of chitosan nanoparticles**

The obtained chitosan nanoparticles showed a mean size of  $20 \pm 2$  nm. The average zeta potential obtained for nanochitosan was 81.3 mV. Transmission electron microscopy revealed that chitosan nanoparticles appeared round in shape (Fig. 1).



**Figure (1):** Transmission electron micrograph of chitosan nanoparticles.

**Changes in germination features and growth variables**

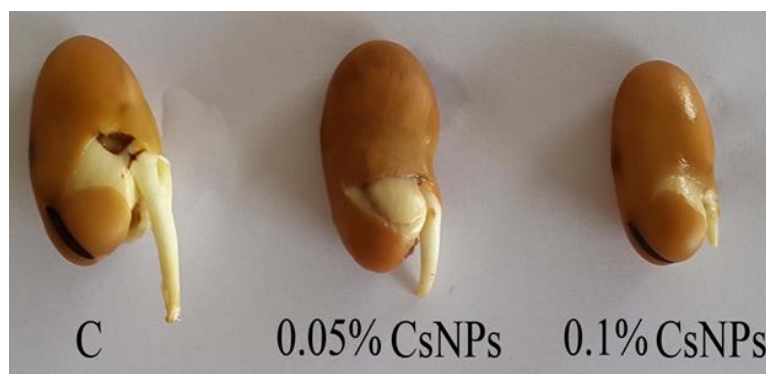
Table (1) revealed that treatment with chitosan nanoparticles resulted in significant decreases in all the determined germination features as compared with control. Germination percentage was greatly decreased due to treatment with 0.1% CsNPs. It should be mentioned that treatment with higher concentrations more than 0.1% of CsNPs ceased germination. The following sequence of treatments was shown: C > 0.05% CsNPs > 0.1% CsNPs with respect to the determined germination percentages. Priming of broad bean seeds with chitosan nanoparticles (0.05% and 0.1%) also caused significant decreases in the mean day for germination, germination rate, pick value (PV), germination value (GV) and the two vigor indices (I, II), as compared with control untreated seeds. The pattern of germination of broad bean seeds in each treatment is shown in Figure (2).

Priming of broad bean seeds with two different concentrations (0.05% and 0.1%) of chitosan nanoparticles resulted in decrease of radicle length, fresh weight and water content of seedlings (7 d old). The magnitude of decrease was much observed in treatment with 0.1% CsNPs. In general the following sequence was found: Control > 0.05% CsNPs > 0.1% CsNPs. Dry weight of seedlings was not changed due to treatment with 0.05% CsNPs but was decreased due to treatment with 0.1% CsNPs as compared with control seedlings (Table 2).

**Table (1):** Effect of chitosan nanoparticles on germination features of 7 d old broad bean seedlings. Data are the means of three replicates  $\pm$  standard error.

Treatment	Germination (%)	MDG (day)	Germination rate	PV	GV	Vigor index I	Vigor index II
C	90.91 $\pm$ 0.50	12.99 $\pm$ 0.05	11.03 $\pm$ 0.50	5.71 $\pm$ 0.06	74.17 $\pm$ 0.10	154.55	239.09
0.05% CsNPs	76.67 $\pm$ 0.60*	9.52 $\pm$ 0.12*	10.09 $\pm$ 0.30*	4.29 $\pm$ 0.11*	40.84 $\pm$ 0.21*	90.00*	166.00*
0.1% CsNPs	37.78 $\pm$ 0.20*	5.40 $\pm$ 0.10*	4.76 $\pm$ 0.20*	2.43 $\pm$ 0.30*	13.12 $\pm$ 0.30*	30.98*	78.96*

\* Means are significantly different from control at  $p \leq 0.05$



**Figure (2):** Pattern of germination of broad bean seeds of the control (C), and those primed with chitosan nanoparticles (CsNPs) at the end of the experiment.

**Table (2):** Effect of chitosan nanoparticles on growth parameters of 7 d old broad bean seedlings. Data are the means of three replicates  $\pm$  standard error.

Treatments	Radicle length (cm/seedling)	Fresh weight of seedling (g/ seedling)	Dry weight of seedling (g/ seedling)	Water content (g/ seedling)
C	1.70 $\pm$ 0.04	2.63 $\pm$ 0.05	0.77 $\pm$ 0.03	1.86 $\pm$ 0.06
0.05% CsNPs	1.35 $\pm$ 0.17*	2.49 $\pm$ 0.17	0.77 $\pm$ 0.05	1.72 $\pm$ 0.12
0.1% CsNPs	0.82 $\pm$ 0.02*	2.09 $\pm$ 0.12*	0.68 $\pm$ 0.03	1.41 $\pm$ 0.10*

\* Means are significantly different from control at  $p \leq 0.05$

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### Changes in total phenols and antioxidant enzymes

Data presented in table (3) represent the effects of using chitosan nanoparticles as priming solutions for broad bean seeds on total phenols and different antioxidant enzymes of broad bean seedlings (7 d old). Priming of broad bean seeds with 0.05% CsNPs resulted in a significant increase in total phenols, CAT, APX,

POX and PPO activities, as compared with untreated control seeds. On the other hand, treatment with 0.1% CsNPs resulted in a significant decrease in all the determined enzyme activities as well as total phenol contents, as compared to control seedlings. In this respect, the following sequence was displayed: 0.05% CsNPs > Control > 0.1% CsNPs.

**Table (3):** Effect of chitosan nanoparticles on total phenols and antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX) and polyphenol oxidase (PPO) of 7 d old broad bean seedlings. Data are the mean of three replicates  $\pm$  standard error.

Treatments	Total phenols mg gallic acid/ g fwt	CAT mmol H <sub>2</sub> O <sub>2</sub> / min/ g f.wt	APX $\mu$ mol AsA / min/ g f.wt	POX U/ min/ g f.wt	PPO U/ min/ g f.wt
C	2.90 $\pm$ 0.05	24.30 $\pm$ 0.95	2.68 $\pm$ 0.06	83.06 $\pm$ 3.22	67.06 $\pm$ 1.05
0.05% CsNPs	3.03 $\pm$ 0.13*	26.16 $\pm$ 0.43*	4.92 $\pm$ 0.25*	86.34 $\pm$ 5.06*	69.07 $\pm$ 2.15*
0.1% CsNPs	2.14 $\pm$ 0.11*	9.40 $\pm$ 0.35*	1.49 $\pm$ 0.07*	60.13 $\pm$ 3.17*	54.00 $\pm$ 2.33*

\* Means are significantly different from control at  $p \leq 0.05$

## DISCUSSION

Nanotechnology proved its importance in agriculture nowadays. Research is being developed to provide nutrients to crop plants using nanofertilizers. Nano fertilization could be done by three methods: seed priming, foliar application or soil incorporation (Divya and Jisha, 2018). Foliar application of nanofertilizers showed different results with several crops with respect to enhancement of growth and increased productivity. Foliar application of chitosan nanoparticles improved growth and yield of wheat plants (Abdel-Aziz *et al.*, 2016), French bean plants (Hasaneen *et al.*, 2016) and coffee seedlings (Van *et al.*, 2013).

In the present work, characterization of chitosan nanoparticles showed stable round particles with mean diameter of  $20 \pm 2$  nm and zeta potential of + 81.3 mV, hence showing the cationic nature of the obtained nanoparticles.

Nanopriming of seeds is a new technique developed to improve seed germination and seedling growth especially under stressful conditions such as drought and salinity (Maroufi *et al.*, 2011). In the present study, priming of broad bean seeds with 0.05% and 0.1% chitosan nanoparticles resulted in reduction of germination percentage and decrease in radicle length and fresh weight of seedlings, as compared with those of the control. Similar results were also obtained for different germination features: germination rate, seedling vigor, mean daily germination (MDG), pick value (PV) and germination value (GV). The magnitude of decrease was most pronounced at 0.1% chitosan nanoparticles. These results are in accordance with the findings of Omar (2017) who showed that priming of French bean seeds with 100% chitosan nanoparticles resulted in failure of germination of seeds. Meanwhile treatment with 10% CsNPs resulted in a decrease of germination percentage and all growth variables including length of radicle, length of plumule, as well as fresh and dry weights of seedlings. In the same context, Behboudi *et al.* (2017) showed that priming of barley and wheat grains with chitosan nanoparticles showed no significant changes in germination rate, MDG, PV and GV. Meanwhile, lower concentration (30 ppm) of chitosan

nanoparticles showed increased seedling vigor index, whereas increased concentrations (60 and 90 ppm) of CsNPs showed lower values of seedling vigor index for both wheat and barley seedlings. Few reports showed that chitosan nanoparticles had stimulating effects on seed germination and seedling growth as illustrated in tomato and maize (Saharan *et al.*, 2015 and 2016).

The present obtained results of reduction in germination percentage and different germination features due to priming of broad bean seeds in 0.05% and 0.1% chitosan nanoparticles would verify that nanoparticles could penetrate pores of seed coat. Then chitosan nanoparticles are predicted to reach the embryo cells causing cytotoxic effects as indicated by Khalifa and Hasaneen (2018) who stated that higher concentrations of chitosan nanoparticles (1%, 0.5%, 0.25%) that reduced root elongation of pea seedlings resulted in genotoxic effects on root cells of seedlings (Khalifa and Hasaneen, 2018).

In the present study, priming of broad beans with 0.05% chitosan nanoparticles resulted in significant increase in total phenols and antioxidant enzymes, as compared with those of the control seedlings. On the other hand, the higher concentration of chitosan nanoparticles (0.1%) caused serious drop in the antioxidant enzyme system of broad bean seedlings. This could be explained on the bases of comparison with the effect of lower concentrations of chitosan nanoparticles that were found to act as stimulating factor for the antioxidant system of treated seeds with respect to the control. A similar trend was also observed in French bean (Hasaneen *et al.*, 2016) and coffee seedlings (Van *et al.*, 2013). Priming with chitosan nanoparticles also increased the activity of catalase enzyme and proline content in salt-stressed *Phaseolus vulgaris* seedlings (Zayed *et al.*, 2017). On the other hand, high concentrations of chitosan nanoparticles decreased antioxidant enzyme activities of maize and tomato (Saharan *et al.*, 2015 and 2016). These findings might be interpreted on the bases that lower concentrations of chitosan nanoparticles acted as signals which stimulated the expression of genes responsible for synthesis of different antioxidant enzymes and total phenols. On the other hand, high concentrations of chitosan nanopar-

ticles are assumed to induce oxidative stress in embryo cells that would then induce serious harmful effects on seed germination and reduce production of antioxidant enzymes and phenols accumulation (Divya and Jisha, 2018).

In conclusion, priming of broad beans with chitosan nanoparticles could be used at lower concentrations (0.5% or lower) to enhance antioxidant enzymes and total phenols accumulation which could help make seeds to further tolerate oxidative stress conditions. The most important thing is that the cytotoxic effects of priming with chitosan nanoparticles should be monitored and controlled to alleviate harmful effects on human health and environment.

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تأثير معاملة البذور باستخدام جسيمات الكيتوزان النانوية على الإنبات و نمو البادرات و أنشطة الإنزيمات المضادة للأكسدة في نبات الفول

هبة محمود محمد عبد العزيز

قسم النبات، كلية العلوم، جامعة المنصورة، مصر

الملخص العربي

في هذا البحث تمت دراسة تأثير تركيزين من الجسيمات النانوية للكيتوزان (0.05 %، 0.1 %) كمحاليل تهيئة لبذور الفول البلدى سخا 1 ثم الانبات لمدة سبعة أيام. و قد تسبب كل من تركيزى الكيتوزان النانوية فى آثار سلبية على الإنبات و دالات الانبات المختلفة. وأدت المعاملات إلى انخفاض ملموس فى عملية الإنبات إثر المعاملة بكل من التركيزين بالمقارنة مع المعاملة الضابطة (البذور المنقوعة فى الماء المقطر). وكان حجم الانخفاض واضحاً بشكل كبير مع التركيز الأعلى من جسيمات الكيتوزان النانوية (0.1%). و من ناحية أخرى، فقد أحدث التركيز المنخفض من الكيتوزان (0.05 %) زيادة فى نظام المقاومة فى البادرات عن طريق زيادة محتوى الفينولات الكلية وأنشطة الإنزيمات المضادة للأكسدة (الكاتلايز، الاسكوربات البيروكسيديز، البيروكسيديز والبوليفينول أوكسيديز) مقارنة مع البادرات فى المعاملة الضابطة. وقد خلصت النتائج إلى أن التركيزات المنخفضة نسبياً من جسيمات الكيتوزان النانوية تؤدي لتعزيز نظام الدفاع فى البذور وبالتالي تصبح ملائمة لنمو النبات تحت ظروف الاجهاد.