Int.J.Curr.Microbiol.App.Sci (2017) *6*(9): 3376-3384

EXCELLENT PUBLISHERS

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 9 (2017) pp. 3376-3384**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.609.416

Biosynthesis and Characterization of ZnO Nanoparticles from Spinach (*Spinacia oleracea*) Leaves and Its Effect on Seed Quality Parameters of Greengram (*Vigna radiata*)

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Keywords

ZnO nanoparticles, *Spinacia oleracea*, *Vigna radiate*, Seed Germination.

Article Info

Accepted:
10 July 2017
Available Online:
10 September 2017

A B S T R A C T

The present work is focused on synthesis and characterization of Zinc oxide nanoparticles from spinach (*Spinacia oleracea*) leaves. The prepared Zinc oxide nanoparticles (Zno NPs) were characterized by using UV-Visible spectrophotometer, particle size analyzer and scanning electron microscope (SEM). The average particle diameter was found to be 40.59 nm. In present study, the different concentration (0, 25, 50, 75, 100, 125, 150, 175 and 200 ppm) of ZnO NPs are used for the treatment in greengram (*Vigna radiata*) seeds to study the effect on seed germination and observed early seedling growth and growth characteristics of *greengram.* The best results were found at 125 ppm of *Zinc oxide nanoparticle for seed quality parameters* over untreated seeds. The experiment was carried out under laboratory conditions. Biological method highlights the necessity for sustainable study on the impacts of nanoparticles on agricultural and environmental sectors.

Introduction

Nanotechnology is the technological innovation in the 21st century. Research and development in this field is growing rapidly throughout the world [1]. A major contribution of this field is the development of new materials in the nano scale [2]. These are usually particulate materials with at least one dimension of less than 100 nanometers (nm) and sometimes even the particles could be zero dimension in the case of quantum dots [1]. Nanoparticles fall in the transition zone between individual molecules and the corresponding bulk materials, which generate both positive and negative biochemical effects in living cell [3]. Metal nanoparticles have been of great interest due to their distinctive features such as catalytic, optical, magnetic and electrical properties [4]. Nanoparticles exhibit completely new or improved properties with larger particles of the bulk materials and these novel properties are derived due to the variation in specific characteristics such as size, distribution and morphology of the particles [5].

The conventional methods of synthesizing nanoparticles using chemical method were found to be more expensive and also found to involve the use of toxic, hazardous chemicals that were responsible for various biological risks [6]. In order to avoid the use of toxic chemicals, scientists have developed better methods which can be done in two ways. First one is the use of microorganisms such as bacteria, fungi and yeast [7]. Using microorganisms for the synthesis of nanoparticles were found to be more tedious and required more steps in maintaining cell culture, intracellular synthesis with more purification steps. Whereas the second one is with the use of plants known as 'Green synthesis' or 'Biogenic synthesis'. This type of biosynthesis shows better advancement over chemical and physical methods as it is lesser toxic, cost effective, environmental friendly [1] and also involves proteins as capping agents [8]. Proteins are biomolecules and are advantageous by giving low toxic degradable end products [9].

Spinach (*Spinacia oleracea*) is a green leafy vegetable belonging to family Amaranthaceae. Normally, it is an annual plant (rarely biennial). Height of the plant is up to 30 cm. The leaves are variable in size about 2-30 cm long and 1-15 cm broad. It is often recognized as one of the functional foods for its wholesome nutritional, antioxidant and anti-cancer composition. The major micronutrients in spinach are vitamins A, C, K and folate, and the minerals, calcium, iron and potassium. Spinach also provides fiber and is low in calories [10].

Zinc nanoparticles are used in various agricultural experiments to understand their effect on growth, germination and various other properties [11]. Application of nanomaterials can help faster plant germination/production and effective plant protection with the reduced environmental impact as compared with traditional methods.

However, the micro elements are required in minute quantity for treating seeds. Recently use of these elements in the form of nanoparticles is gaining importance especially for enhancing seed quality [12]. Germination increases with the decrease in size of the nanomaterials. Reynolds [13] demonstrated that the micronutrients in the form of NPs could be used in crop production to increase the yield. Hence, the present investigation was taken to study the biosynthesis of ZnO NPs from spinach (*Spinacia oleracea*) leaves and its characterization. The biosynthesized ZnO NPs were applied on greengram (*Vigna radiata*) seeds to study the quality parameters.

Materials and methods

The spinach leaves (*Spinacia oleracea*) (Heena variety) were collected from University campus greengram seeds (variety, BGS-9) were collected from seed unit, UAS, Raichur and Zinc nitrate hexahydrate [$Zn(NO_3)_2.6H_2O$] were procured from M/s. High Media, Bangalore.

Biosynthesis and characterization of Zinc oxide nanoparticles

The spinach leaves were washed thoroughly with distilled water and dried using solar tunnel dryer. The dried leaves were ground using a pulverizer to make into a fine powder and passed through a 100 mesh sieve (150 µm). Five grams of dried powder was added to 100 ml of ethanol and kept for 24 h in a 250 ml conical flask and filtered through filter paper (Whatman No.1). The filtrate was stored at 4 ^{0} C for further experiments.

The leaf extract of spinach (50 ml) was boiled at 60 - 80 ^{0} C using magnetic stirrer. Zinc nitrate hexahydrate [$Zn(NO_3)_2.6H_2O$] was used as a precursor. 1 mM Zinc nitrate solution was prepared using distilled water. The solution was added to the leaf extract when temperature reached to 60 ^{0} C and boiled for 30 min or until colour changed. A change in the colour from dark green to pale yellow indicates the formation of ZnO NPs [14]. The sample of biosynthesized ZnO NPs were centrifuged using a centrifuge at 5000 rpm for 15 min. The supernatant was collected and subjected to ultrasonication. The centrifuged sample of biosynthesized ZnO NPs solution was kept in digital ultrasonication bath (Labman Scientific Instruments, LMUC-2.8L, India) for 5 min at 25 ºC for reducing the size of ZnO NPs.

ZnO NPs were confirmed by UV-Visible spectrophotometer (Perkin Elmer, Lamda 35, Germany) in 350 - 410 nm wavelength range. The size and morphology of the synthesized Zinc oxide nanoparticles was characterized by Zetasizer (Malvern, ZETA Sizer, nano383 issue 5.0, England) and scanning electron microscope (Carl Zeiss Microscopy, EVO 10, Germany).

Seed priming

The ZnO NPs solution were dispersed at different concentrations (25, 50, 75, 100, 125, 150, 175 and 200 ppm) in deionized water with continous stirring for 10 min. The priming of greengram seeds for different concentrations was carried out by soaking 100 g of seeds in 35 ml of solution for 3 h. The control was used as without ZnO NPs *i.e.*, seeds treated with deionized water.

Measurement of seed quality parameters

The observations on various seed quality parameters *viz.*, seed germination (%), root length (cm), shoot length (cm), seedling dry weight (mg) [15], seedling vigour index - I and II [16], speed of germination [17], mean germination time [18] and peak value [15] were recorded as per the methods and procedures described by ISTA. The mean data of the laboratory experiments were statistically analyzed by adopting completely randomized design as outlined by [19]. The critical differences were calculated at one per cent level of probability wherever ‘F’ test was found significant for various seed quality parameters under the study.

Results and Discussions

Biosynthesis and characterization of Zinc oxide nanoparticles

The reduction of Zinc ions into ZnO NPs were observed as the colour changed from dark green to pale yellow. The sharp bands of ZnO NPs were observed around 375.4 nm as shown in figure 1 and similar results were obtained by Awwad *et al.*, [20] with 374 nm and Singh *et al.*, [21] with 368 nm. The average particle diameter of ZnO NPs was found to be 40.59 nm as shown in figure 2. Sindhura *et al.*, [22] obtained 53 nm and Supraja *et al.*, [23] obtained 20.3 nm for ZnO NPs. The morphological features of ZnO NPs were characterized by scanning electron microscope and observed to be spindle shaped as shown in figure 3. Similar results were also reported by Noorjahan *et al.*, [24] for ZnO NPs.

Effect of Zinc oxide nanoaparticles treatment on *V. radiata*

Seed germination (%)

The germination percentage was significantly higher (84.75 %) in 125 ppm compared to all other treatments and control (77.25 %) as shown in figure 4. The concentration at 125 ppm recorded highest germination 8.84 % over the control. The enhanced seed germination was due to the penetration of ZnO NPs into seed coat and this stimulated the growth hormones especially indole acetic acid (IAA) [25]. The decreased germination at higher concentrations could be the increase in absorption and accumulation of these nanoparticles, both in extracellular space and within the cells resulting in reduction in cell division, cell elongation and inhibition of the hydrolytic enzymes involved in food mobilization during the process of seed germination [12]. Similar findings were also reported earlier by Prasad *et al.,* [26] for ZnO NPs at 1000 ppm in groundnut, Jayarambabu *et al.,* [27] for ZnO NPs at 20 mg in mungbean and Shyla and Natarajan [28] for Zinc oxide, silver and titanium dioxide nanoparticles at 1000 mg/kg in groundnut.

Root length and shoot length

The concentration of 125 ppm of ZnO NPs recorded highest root length of 29.2 % over control. The concentration of 200 ppm of ZnO NPs, showed that 3.58 % increase in root length over control.

The shoot length increased to 9.36 % in 125 ppm concentration over the control. Higher concentration showed decreased shoot length. In 200 ppm concentration of ZnO NPs, there is 0.77 % increase in shoot length over the control and 9.48 % decrease over the treatment of 125 ppm (figure 5).

This might be due to the enhancement of the growth hormones in seed by the treatment of different concentrations of Zinc oxide nanoparticles [29]. However, at higher concentration, the growth of root and shoot was found to decline. Narendhran *et al.,* [30] also reported on *Sesamum indicum* which showed maximum growth attributes at 0.5 g/l concentration with ZnO NPs.

Seedling length and seedling dry weight

The best seedling length response was recorded as 2.51 % in 125 ppm over the control. Pearson's correlation coefficients showed positive linear relationship (r = 0.90) between seedling length and seedling dry weight. The increase in seedling dry weight was 15.03 % in 125 ppm over the control. In case of higher concentration at 200 ppm of ZnO NPs, it was 0.72 % increase over control and 16.84 % decrease over 125 ppm (figure 6). This increase in seedling length could be ascribed to activity of hydrolytic enzymes during the early phase of germination. The effective mobilization of the available food reserves in the seeds resulted in the early emergence and growth of the seedlings. In proportion to increase in seedling growth, dry matter production was also increased.

The reduction in seedling length growth at higher doses might be due to the penetration of nanoparticles into cell wall and plasma membrane of epidermal layers in shoot and root and accumulation in vascular tissues [12]. Hence, the overall seedling growth of greengram reduced at higher concentration.

These results are in good agreement with previous findings of Mahajan *et al.,* [31] for ZnO NPs in mung at 20 ppm and gram at 1 ppm, Jayarambabu and Kumari [32] for ZnO NPs at 50 and 100 mg in mungbean and Reddy *et al.,* [25] for ZnO NPs at 150 mg in mungbean.

Seedling vigour index - I & II

The data on seedling vigour index revealed significant difference due to the application of ZnO NPs to the seeds. The maximum seedling vigour index - I & II were recorded in 125 ppm concentration of ZnO NPs, which were 25.43 % and 22.48 % highest over the control as shown in figure 7.

Fig.1 Average particle diameter (nm) of Zinc oxide nanoparticles

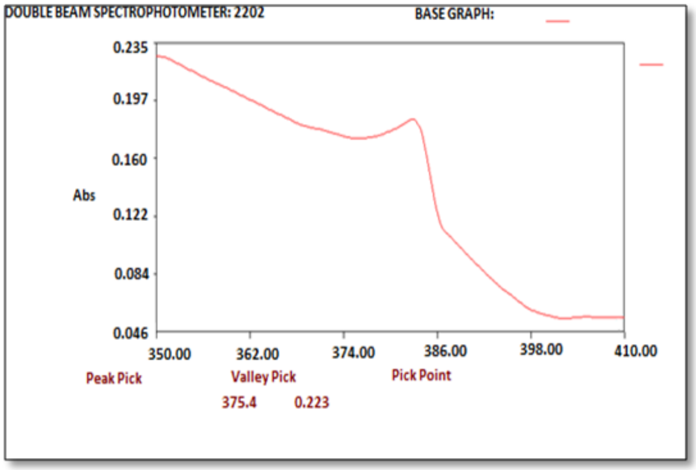


Fig.2 UV-Visible spectrum analysis of Zinc oxide nanoparticles from the Leaves extract of spinach

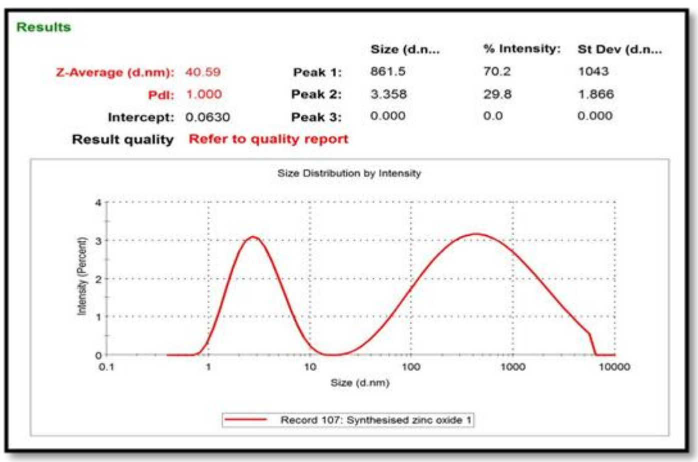


Fig.3 Scanning electron microscope image of Zinc oxide nanoparticles

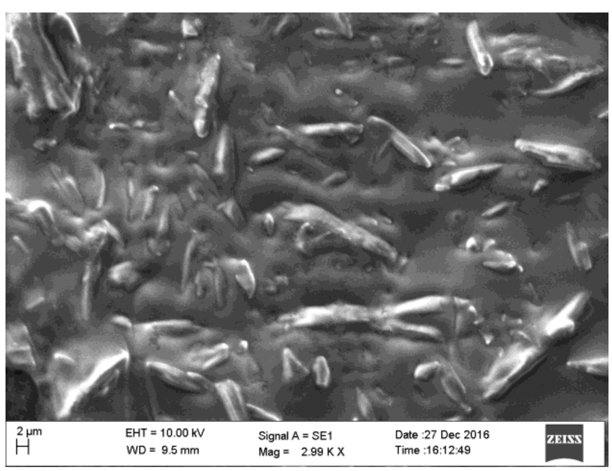


Fig.4 Effect of ZnO NPs seed germination

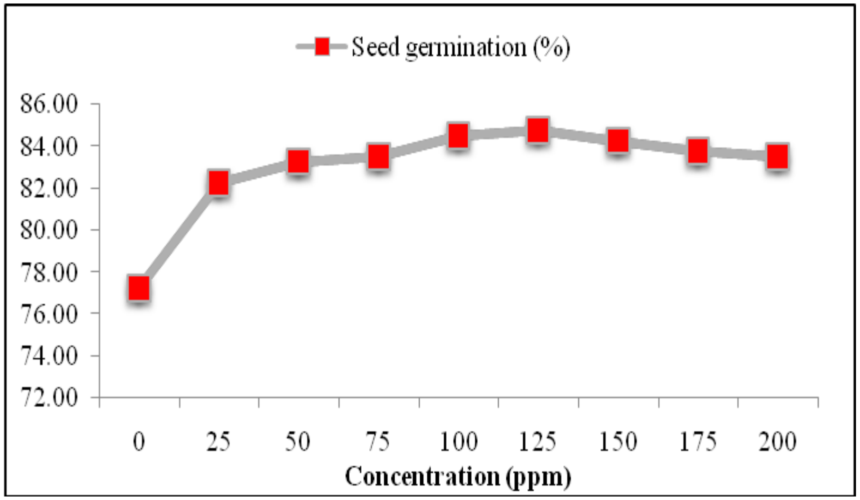


Fig.5 Effect of ZnO NPs on root and shoot length

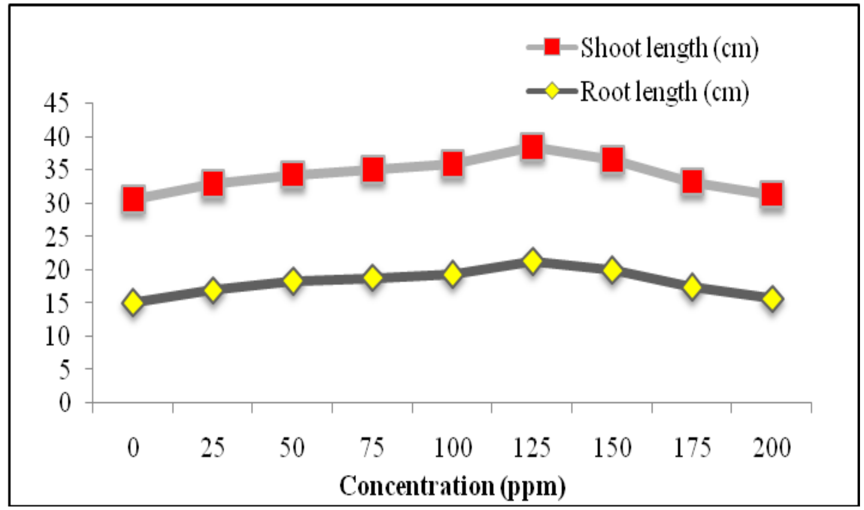


Fig.6 Effect of ZnO NPs on seedling length and seedling dry weight

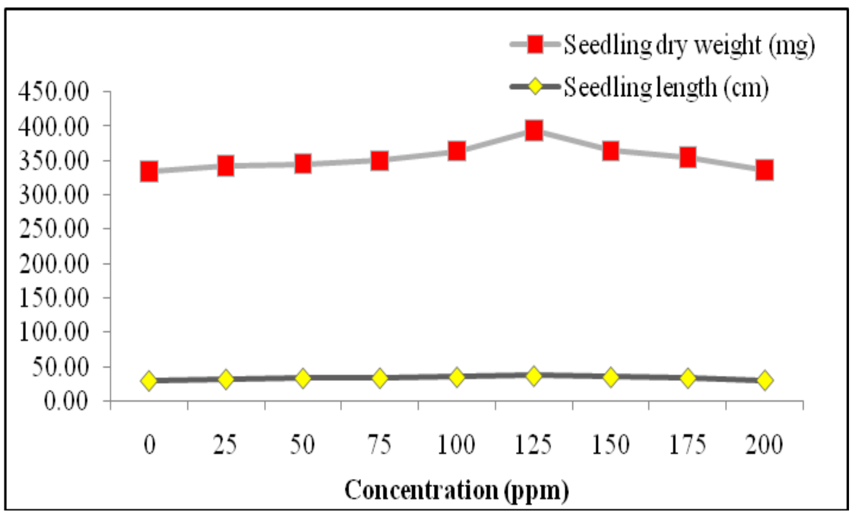


Fig.7 Effect of ZnO NPs on seedling vigour index - I & II

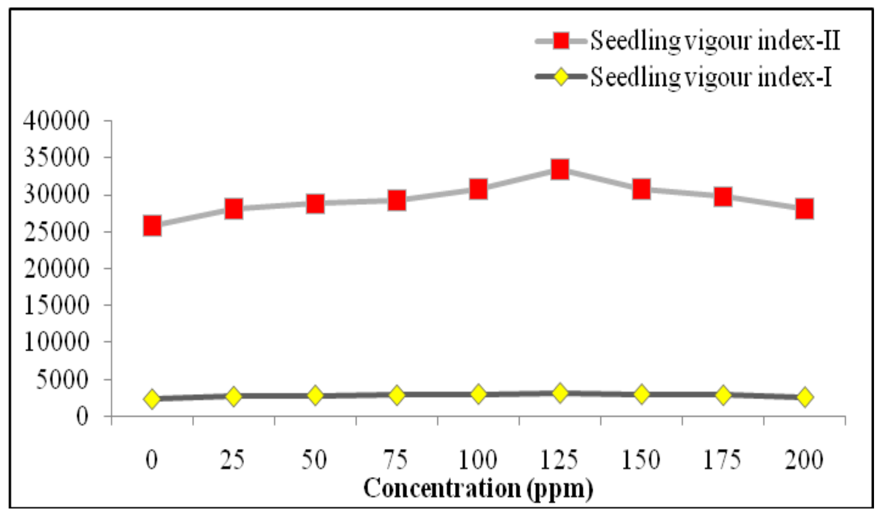


Fig.8 Effect of ZnO NPs on speed of germination and mean germination time

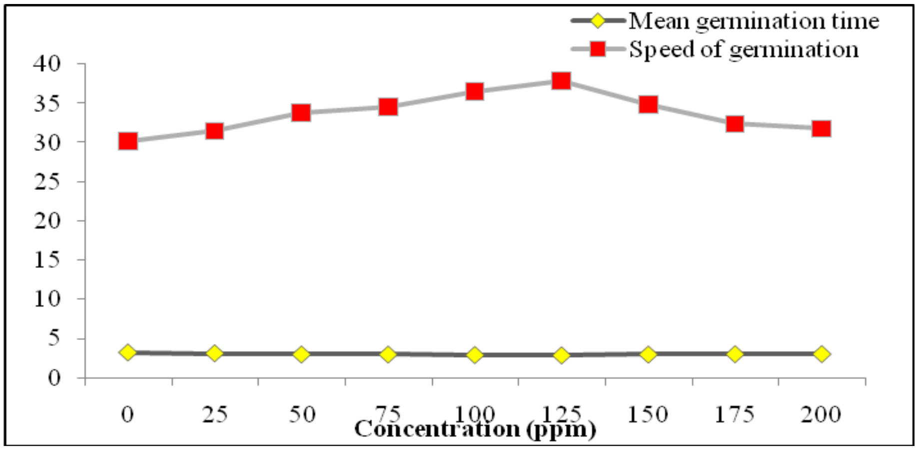
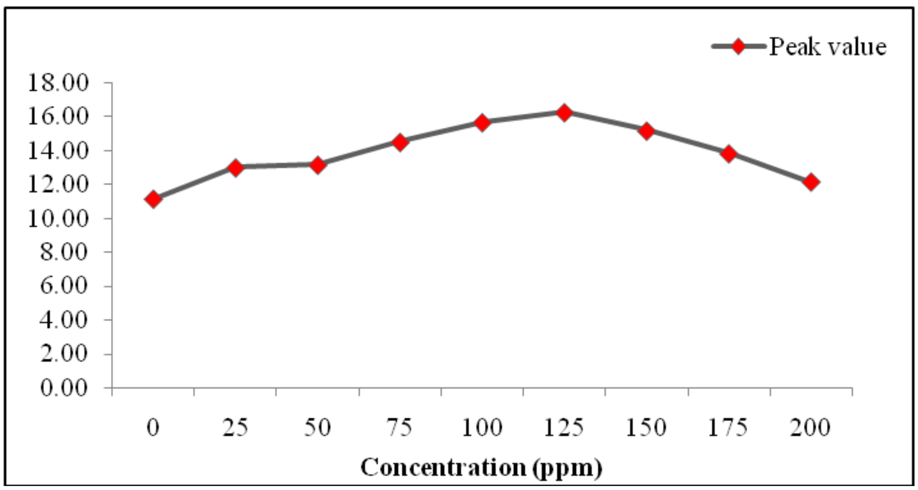


Fig.9 Effect of ZnO NPs on peak value



The probable reason might be due to the effect of ZnO NPs in improving the seed quality during germination, resulting in the quenching of free radicals in the germinating seeds. In turn, oxygen produced in such process could also be used for respiration, which would further promote germination resulting in improving seedling vigour [28]. The Pearson's correlation coefficient showed positive linear relationship between seed germination and seedling vigour index - I & II. The present findings corroborate with Prasad *et al.*, [26] for ZnO NPs at 1000 ppm in groundnut, Gokak and Taranath [33] for bulk Zinc at 100 ppm and nano Zinc at 10 and 50 ppm in horse gram seeds and Vinoth and Udayasoorian [34] (2014) for Zinc oxide, aluminium oxide and titanium dioxide nanoparticles at 500 ppm in maize.

Speed of germination, mean germination time and peak value

The control showed an average speed of germination in (30.17), while the concentration of 125 ppm recorded highest speed of germination 37.90, but higher concentrations showed decline in speed of germination. The reason for rapid germination could be that the nanoparticles might have penetrated into the seed coat facilitating the influx of water inside the seed or nanoparticles might have entered into the seed through the cracks present over the surface of the seeds and activated the enzymes in early phase, thereby enhancing the speed of germination [35]. These results are in conformity with the findings of Korishettar *et al.*, [12] for Zinc nanoparticles at 750 ppm and iron nanoparticles at 500 ppm in pigeonpea. As a general rule, lower mean germination time (MGT) represents a faster germination speed. The results revealed that at 125 ppm, recorded the lowest mean germination time of 2.83 days, which was reduced by 13.78 % in comparison to the control (3.22 days), but higher concentrations increased mean germination time (figure 8). It is proposed that activation of respiration and rapid ATP production appears to be most important metabolic events induced by faster seed germination [36]. This might be the reason for decrease in mean germination time. Maximum peak value was recorded in 125 ppm, which was 31.32 % higher than the control as shown in figure 9. Similar results were also obtained by Gokak and Taranath [33] (2015) for bulk Zinc at 100 ppm and nano Zinc at 10 and 50 ppm in horse gram seeds and Almutairi [37] (2016) for nano silicon in tomato.

The results of present investigation showed potential application of biosynthesized ZnO NPs to encourage speed of germination, breaking seed dormancy and improve plant production of greengram. To our knowledge, this effort is the first information related to biosynthesis of ZnO NPs from spinach leaves. The effect of biosynthesized ZnO NPs on seed quality parameters of greengram was analyzed. The average particle diameter was found to be 40.59 nm and the sharp bands were observed at a wavelength of 375.4 nm. The morphological features of ZnO NPs were found to be spindle in shape. Significantly highest seed quality parameters *viz.*, seed germination percentage (84.75 %), root length (21.30 cm), shoot length (17.20 cm), seedling length (37.57 cm), seedling dry weight (356.90), seedling vigour index - I (3181), seedling vigour index - II (30218), speed of germination (37.90), mean germination time (2.83 days) and peak value (16.25) was recorded in 125 ppm concentrations. Increase in concentrations from 150 to 200 ppm showed a slight decrease in seed quality parameters over the superior treatment but positive effect over the control. Regarding the toxic levels, future studies should focus on levels of uptake and retention, the mechanism of phytotoxicity and interactions within cells. Intensive, research at molecular level can be undertaken to understand the mechanism of entry of ZnO NPs and their mode of action in invigorating the seeds during priming with nanoparticles that would help to increase the yield by improving seed quality.

Acknowledgement

The authors thank the University of Agricultural Sciences, Centre for nanotechnology Department of Processing and Food Engineering and Department of Seed Science and Technology, Raichur, Karnataka, India to provide the facility to carry out the experimental work and also thanks to Indian Council of Agriculture Research (ICAR) for the financial support under National Talent Scholarship during research work.

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How to cite this article:

Sagili Jhansi Lakshmi, R.S. Roopa Bai, H. Sharanagouda, C.T. Ramachandra, Sushila Nadagouda and Shivanagouda R. Doddagoudar. 2017. Biosynthesis and Characterization of ZnO Nanoparticles from Spinach (*Spinacia oleracea*) Leaves and Its Effect on Seed Quality Parameters of Greengram (*Vigna radiata*). *Int.J.Curr.Microbiol.App.Sci.* 6(9): 3376-3384.
doi: https://doi.org/10.20546/ijcmas.2017.609.416