



Research Article

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## ***Copper Sulfate Nanoparticales In vitro Applications on Verbena bipinnatifida Nutt. Stimulating Growth and Total Phenolic Content Increasments.***

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### ABSTRACT

Nanotechnology opens a large scope of novel applications in the fields of biotechnology, medicine, nutrition and energy. Since nanoparticles (NPs) have unique physicochemical properties, i.e. high surface area, high reactivity, tunable pore size and particle morphology. Nanoparticles can serve as "magic bullets" containing herbicides, nanopesticide fertilizers or genes, which target specific cellular organelles in plant and release its contents. Here, we report novel successful in vitro effects of CuSO<sub>4</sub>-NPs on seeds of *Verbena bipinnatifida* Nutt. to enhance phenolic contents which are important compounds in pharmaceutical industries especially anti oxidant medicaments.

**Keywords:** Copper Sulfate Nanoparticales (CuSO<sub>4</sub>-NPs), In vitro, Germination, Total Phenolic content, *Verbena bipinnatifida* Nutt.

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### INTRODUCTION

Nanotechnology, a new emerging and fascinating field of science, permits advanced research in many areas and open widely novel applications in the field of biotechnology, agriculture, electronics, energy, medicine and life science(1). Nanotechnology offers an expanding research for reproductive science technology, conversion of agricultural and food wastes to energy, useful byproducts production through enzymatic nanobioprocessing, chemical sensors production, cleaning water, diseases prevention and plant treatment applications using various nanocides(2,3). The use of modern techniques in the field of nanotechnology is one of the important recent trends for improving active constituents production in the field of tissue culture(4). Researchers from their findings suggested positive effects on plant growth and development. The impact of engineered nanoparticales (ENPs) on plants depends on the composition, concentration, size and physical and chemical properties of ENPs as well as plant species(5). Many researches used nanoparticles like nano silicon(6-8), nano iron (9-11) and nanocopper on plant species(12-14). *Verbena bipinnatifida* Nutt. Family Verbenaceae (VbV) is cultivated in Egypt as an ornamental plant(15) and it is widely distributed as small herbaceous plant with small purple erect flowers (16). The main medicinal uses of verbena species are antioxidant(17), sleep promoting (18) and hepatoprotective activity(19). Several investigations showed that *Verbena bipinnatifida* Nutt. Family Verbenaceae contains phenolic compounds and iridoids (20-22). Here we report on the effect of different concentrations of both CuSO<sub>4</sub> and CuSO<sub>4</sub>-NPs and their effect on growth parameters (seed germination, shoot length, root length and fresh weight) and total phenolic content of *Verbena bipinnatifida* Nutt.

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## MATERIALS AND METHODS

### 2.1. Preparation of CuSO<sub>4</sub>-NP suspension

CuSO<sub>4</sub>-NPs (1g) was dissolved in 50 ml distilled water to prepare different concentrations of CuSO<sub>4</sub>-NPs (5, 10 and 15 µg L<sup>-1</sup>). Prior being added to culture media, nanoparticle suspensions were centrifuged and filtered to avoid particles aggregation (23).

### 2.2. Surface Sterilization:

Seeds of *Verbena bipinnatifida* Nutt. were surface sterilized under aseptic condition in laminar flow hood (NUAIRE™, USA) with 0.1% fungicide for 10 min followed by soaking for 2 min in 70 % (v/v) ethanol, then washed once with sterile D. D. H<sub>2</sub>O, then seeds were rinsed with 20 % (v/v) commercial Clorox® (5.25% Sodium hypochlorite) supplemented with few drops of Tween 20 for 20 min, followed by soaking for 2 min in sterile D. D. H<sub>2</sub>O, then rinsed five times in sterile D.D. H<sub>2</sub>O.

### 2.3. Culturing of explants

Seeds were cultured onto germination medium (GM) basically contains Murashige and Skoog salts (Murashige and Skoog 1962)(24) supplemented with 30 g sucrose, 8 g agar as a solidifying agent. Seven treatments were conducted as following:

Control(T1); 5 µM L<sup>-1</sup> CuSO<sub>4</sub> (T2); 10 µM L<sup>-1</sup> CuSO<sub>4</sub> (T3); 15 µM L<sup>-1</sup> CuSO<sub>4</sub> (T4); 5 µM L<sup>-1</sup> CuSO<sub>4</sub>-NPs (T5); 10 µM L<sup>-1</sup> CuSO<sub>4</sub>-NPs (T6); 15 µM L<sup>-1</sup> CuSO<sub>4</sub>-NPs (T7) were added to germination medium.

Cultured seeds were then incubated at dark for two days in controlled growth chamber (Shel-Lab, USA) at 25°C and then transferred to a 16-h photoperiod. After 15 d the seedlings were collected and the shoot length, root length and fresh weight were recorded.

### 2.4. Statistical Analysis

Data obtained were exposed to the proper statistical analysis of complete randomized design as described by Snedecor and Cochran (25) in three replicates. Means obtained were differentiated using Duncan's new multiple range test as described by Duncan (26).

### 2.5. Determination of total phenolic content:

#### 2.5.1. Extraction of phenolic content.

The method described by the International Organization for Standardization (ISO) 14502-1 was used. Briefly, (0.001 g of each sample was weighed in an extraction tube, and 5 ml of 70% methanol at 70 °C was added. The extract was mixed and heated at 70 °C on a vortex for 10 min. After cooling at room temperature, the extract was centrifuged at 200g for 10 min. The supernatant was decanted in a graduated tube. The extraction step was repeated twice. Both extracts were pooled and evaporated.

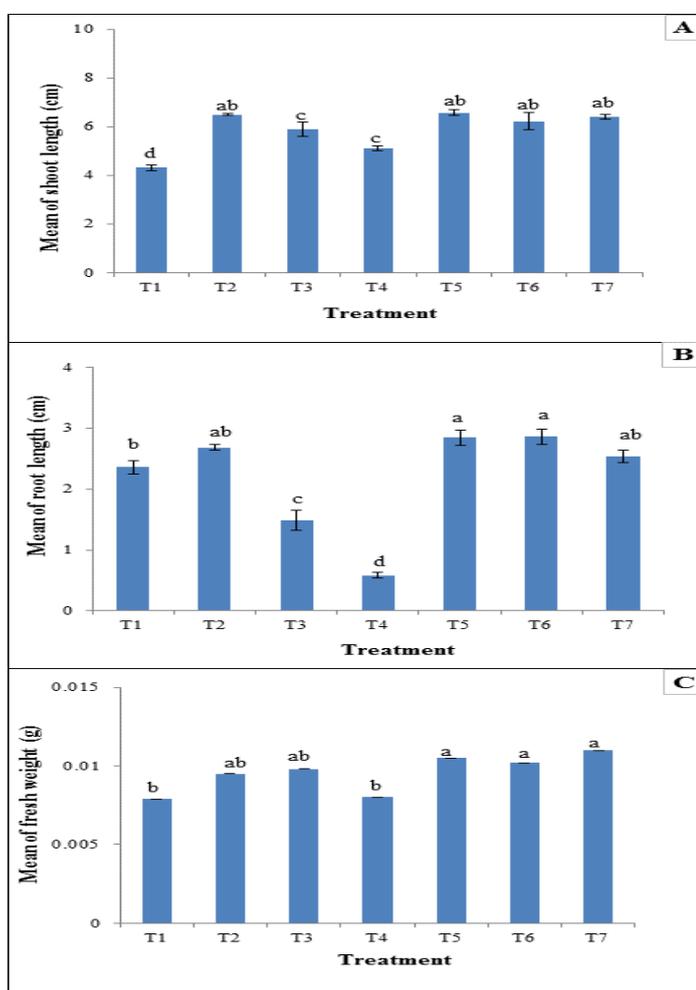
#### 2.5.2. Determination of total phenolic content.

The total phenolic content (TPC) was determined by spectrophotometry, using gallic acid as standard, The concentrated extracts were dissolved in the least volume of methanol then completed to 10 ml, 100 µl of this extract was diluted with 8 ml distilled water. To the sample 0.5 ml of 50 % Folin-Ciocalteu reagent was added and left 8 min, and then 1.5 ml of 5% sodium carbonate was added, mixed and allowed to stand for 60 min., protected from light. The absorbance was measured at 725 nm using 95% methanol as a blank. The total phenolic content was measured in terms of gallic acid equivalent.

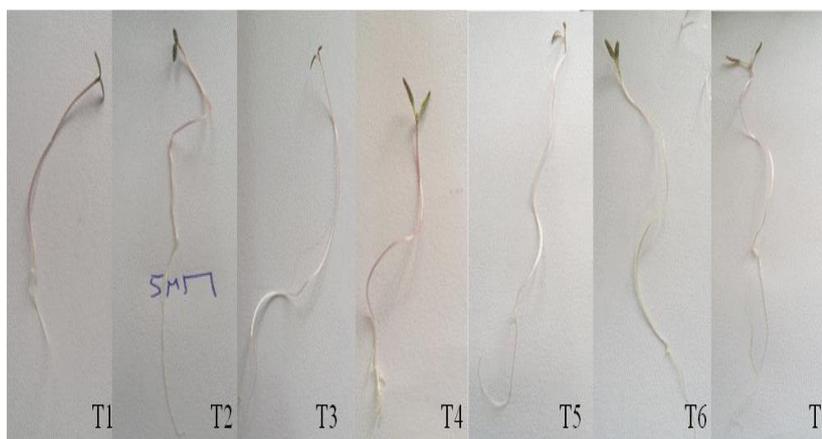
## RESULTS

Response of *Verbena bipinnatifida* Nutt. seedlings grown in MS medium supplemented varied significantly across different concentrations of  $\text{CuSO}_4$  and  $\text{CuSO}_4$ -NPs (Figure 1 and 2). The verbena seedling treated with the  $\text{CuSO}_4$ -NPs showed the significantly high values in most of the growth parameters tested. Results showed that the  $\text{CuSO}_4$  and  $\text{CuSO}_4$ -NPs concentrations had significant effect on increment of shoot length over control. At  $5 \mu\text{M}$  of  $\text{CuSO}_4$ -NPs the highest shoot length was recorded which reached about 52 % more than control. A marked increase in root length was observed at different levels of  $\text{CuSO}_4$ -NPs which reached to 21 % and 20 % at  $5 \mu\text{M}$  and  $10 \mu\text{M}$ , respectively. On the other hand, increasing  $\text{CuSO}_4$  concentrations above  $5 \mu\text{M}$  caused significant reduction in root length, whereas, highest reduction (75%) in root length was recorded at  $15 \mu\text{M}$ . Moreover, increasing the level of  $\text{CuSO}_4$  and  $\text{CuSO}_4$ -NPs was accompanied by a significant increment in fresh weight, whereas  $15 \mu\text{M}$   $\text{CuSO}_4$ -NPs reached to 39% increment as compared with control.

Data recorded in (Table 1) represent the effect of  $\text{CuSO}_4$ -NPs at different concentration on total phenolic content of *Verbena bipinnatifida* Nutt. germinated seeds. It was clearly shown from data that the treatment with  $\text{CuSO}_4$ -NPs at concentrations,  $5 \mu\text{M}$ ,  $10 \mu\text{M}$  and  $15 \mu\text{M}$  led to increments in total phenolic content compared with control and  $\text{CuSO}_4$  treatments, while the minimum values were recorded at control and  $15 \mu\text{M}$   $\text{CuSO}_4$ .



**Figure (1):** Effect of different concentrations of  $\text{CuSO}_4$  and  $\text{CuSO}_4$ -NPs addition to control on growth parameters of (VbV): (A) shoot length; (B) root length and (C) fresh weight; Control: T1;  $5 \mu\text{M}$   $\text{L}^{-1}$   $\text{CuSO}_4$ : T2;  $10 \mu\text{M}$   $\text{L}^{-1}$   $\text{CuSO}_4$ : T3;  $15 \mu\text{M}$   $\text{L}^{-1}$   $\text{CuSO}_4$ : T4;  $5 \mu\text{M}$   $\text{L}^{-1}$   $\text{CuSO}_4$ -NPs: T5;  $10 \mu\text{M}$   $\text{L}^{-1}$   $\text{CuSO}_4$ -NPs: T6;  $15 \mu\text{M}$   $\text{L}^{-1}$   $\text{CuSO}_4$ -NPs: T7.



**Figure (2):** Effect of different concentrations of CuSO<sub>4</sub> and CuSO<sub>4</sub>-NPs addition to control on growth parameters of (VbV): Control: T1; 5  $\mu\text{M L}^{-1}$  CuSO<sub>4</sub>: T2; 10  $\mu\text{M L}^{-1}$  CuSO<sub>4</sub>: T3; 15  $\mu\text{M L}^{-1}$  CuSO<sub>4</sub>: T4; 5  $\mu\text{M L}^{-1}$  CuSO<sub>4</sub>-NPs: T5; 10  $\mu\text{M L}^{-1}$  CuSO<sub>4</sub>-NPs: T6; 15  $\mu\text{M L}^{-1}$  CuSO<sub>4</sub>-NPs: T7.

**Table (1):** Total phenolic content in *Verbena bipinnatifida* Nutt. with control and different CuSO<sub>4</sub> and CuSO<sub>4</sub> NPs tested treatments

Sample	Total phenolic content (mg GAE/g DW)
Control	18.2
5 $\mu\text{M L}^{-1}$ CuSO <sub>4</sub>	28.1
10 $\mu\text{M L}^{-1}$ CuSO <sub>4</sub>	26.4
15 $\mu\text{M L}^{-1}$ CuSO <sub>4</sub>	21.2
5 $\mu\text{M L}^{-1}$ CuSO <sub>4</sub> NPs	42.7
10 $\mu\text{M L}^{-1}$ CuSO <sub>4</sub> NPs	34.3
15 $\mu\text{M L}^{-1}$ CuSO <sub>4</sub> NPs	31.4

## DISCUSSION

The elements (Co, Cu, Fe, Mn, Mo, Ni and Zn) called micronutrients where they play vital functions in plant cells. Micronutrients are essential for biosynthesis and function of nucleic acids, growth substances, chlorophyll and secondary metabolites, carbohydrates as well as for growth and stress resistance, on the other hand, they are required in a small traces, whereas, turns toxic at higher concentrations. The nanoparticles can be reported with minimal toxicity on test plants if it has no negative effect on root growth at such a high concentration according to the USEPA guidelines.

Properties of nanoparticles, the chemical composition, chemical structure, particle size and surface area play an important role in the mechanism of toxicity of nanoparticles. Toxicity of nanoparticles may be attributed to two different actions (1) a chemical toxicity based on the chemical composition e.g., release of toxic ions and (2) stress or stimuli caused by the surface, size and shape of the particles (27).

Several studies concentrate on the adverse effect of the nanoparticle treatment at higher concentrations. However, here in this research the lower concentration of nanoparticles shows significantly positive response. Our results showed

that the lower concentrations of CuSO<sub>4</sub> and CuSO<sub>4</sub>-NPs induced increasing in shoot length, root length and fresh weight of vby. Verma *et al.*,(2011)(28)mentioned that copper promoted the growth of *Vigna radiata* seedlings when presented at low level, while at high levels, copper retarded growth by interfering with normal cellular metabolic events. Hafeez *et al.*,(2015)(29) stated that MS medium blended with low concentration of Cu-NPs significantly increased fresh weight of wheat as compared with control plants. Nanoparticles may not have a direct effect on plant growth but may be responsible about the effect via indirect mechanisms. This decrease reported here in root length may be due to reduction in cell division. Similar results has been reported by Verma *et al.*, (2011)(28) who found that the growth of root system of mung bean was significantly decreased with increasing level of copper sulfate. Hong *et al.*,(2015)(30)reported that high level of Cu-NPs reduced root length in hydroponically grown alfalfa by 47.65% compared with control. Copper nanoparticles had shown to be toxic with two crop species i.e. mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*) as demonstrated by the reduced seedling growth rate (31).

In brief, the main significant results obtained from determination of TPC by the Folin-Ciocalteu method(32,33) showed that the total phenolic content was increased in *Verbena bipinnatifida* Nutt. by more than two folds surpassing control by using the treatment of 5 μM L<sup>-1</sup> CuSO<sub>4</sub> NPs. Moreover, the total phenolic content was also decreased when the concentration of CuSO<sub>4</sub> and CuSO<sub>4</sub> NPs increased than 5 μM L<sup>-1</sup>.

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