Accumulation of nutrient contents and biochemical changes in shallot, *Allium ascalonicum* grown in soil amended with CuO nanoparticle

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Abstract

Aim: The aim of the current study was to investigate plants growth, physiological, biochemical and nutrients changes in shallot grown in soil loaded with CuO nanoparticle (CuONPs).

Methodology: The shallot seedlings were treated with increasing doses (25, 50, 100, 200 mg kg⁻¹) of CuONPs; the rate of seedling growth, plant biomass, photosynthetic pigment content level, antioxidant enzyme activities and nutrient elements were estimated and compared with the control.

Results: CuONPs treated plants exhibited increased shoot and root growth (123.31% and 184.47%) and biomass compared to the control. Also, the level of photosynthetic pigments namely, chlorophyll a (277.24%), chlorophyll b (301.42%) and carotenoids (104.81%) increased in the CuONPs exposed plants. The activity of antioxidative enzymes viz. superoxide dismutase (SOD, 382.77-330.29%), peroxidase (POX, 197.51-166.86%) and catalase (CAT, 234-317.35%) were found to be significantly high in 100 mg NPs treated plants compared to others. Hence, the nutrient elements in 100mg NPs treated plants were estimated and found to be higher than control.

Interpretation: Results indicate that the engineered phycomolecule loaded CuONPs may possibly be used as nanofertilizer to increase crop productivity and it can be used for enhancing growth of agricultural crops in the future.

Key words: *Allium ascalonicum*, Antioxidative enzyme, Copper oxide nanoparticle, Phycomolecule, Seaweeds

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Introduction

In the global market, nanoparticles (NPs) are tiny materials that are being used in various consumer products, and by 2020 it can rise upto 3 trillion USD by 2020. Currently, more than 1000 viable products that contain NPs are available in the market (Marslin *et al.*, 2017). Bio-synthesis routes offer added benefit, they are easily available, nontoxic, easy handling, rich in nutrient, and cost-friendly (Santhoshkumar *et al.*, 2019). Nanofertilizers are engineered nanomaterials or nano complex (ENPs) responsible for providing essential nutrients to the cultivated crop plants for improving their growth and crop productivity (Liu and Lal, 2015). Based on the type of nutrient requirements, bionanofertilizer can be divided into different categories: macro- and micro- nanofertilizers. Macro-nutrients such as nitrogen (N), magnesium, phosphorus, potassium and calcium, etc., are essential macro elements required for plant growth. The frequently used micronutrients are zinc, copper and silver, etc., for standard plant growth and development. Nanofertilizers play significant role in enhancing the growth of plants as well as crop yield (Ha *et al.*, 2019).

The function of copper nanoparticles as bionanofertilizers and bio-solids agricultural fields can present a feasible way of exposure to the plants (Rawat *et al.*, 2018). Copper improves the production of bioactive compounds, reactive oxygen species, chlorosis, necrosis, altered root system and inhibition of plant growth. Previous studies have reported that CuO NPs released from various products accumulate in food crops and agricultural soils (Shaw *et al.*, 2014; Nair and Chung, 2014). Copper is an essential crop micronutrient, participating directly in redox enzymes and is involved in chlorophyll synthesis and protein and carbohydrate metabolism (Wang *et al.*, 2019). It is a crucial element when mutual with specific enzymes and proteins that have essential functions in the nutrition and growth of plants; it is concerned with photosynthetic pigment transport, oxidative stress responses, mitochondrial respiration, cell wall metabolism and hormone signalling (DaCosta and Sharma, 2016). Shallot (*A. ascalonicum*) products are increasing every year in line with the population growth and food industries, in particular instant food using red onions as flavouring agents, but an increase in crop production has not accompanied the rise in demand. This is due to the extensive cultivation of onion and productivity is still low. Alliums are the most crucial horticulture crop worldwide, after tomatoes, with current annual production around 66 million tonnes. Production of onions has increased by more than 25% in the last ten years (Lemes *et al.*, 2019).

Recently, it has been reported that the application of seaweed extracts on agriculture crops has led to increased percent seed germination, enhanced plant growth and development, resistance against pathogens, pests and also protection against nutrient deficiency. Seaweed based fertilizers have been applied in agricultural land to improve the soil quality management, water use efficiency, nutritional, strategies and drought tolerance etc. (Arioli *et al.*, 2015; Ghaderiardakani *et al.*,

2019). Different marine environments cover around 70% of the earth's surface and have remained unexplored. Earlier studies indicate that most algae can perform as biofactories for the production of nanoparticles (Fawcett *et al.*, 2017).

Among the green algae, *Caulerpa racemosa* and is predominantly well known abundant in the Indo-Pacific region. *C. racemosa* is largely consumed as salads. They are rich in fiber, proteins, minerals (calcium and magnesium), folic acid, ascorbic acid, vitamin A and vitamin B1. *Caulerpa* possess vitamins, polyunsaturated fatty acids, fibre and bioactive antioxidants (deGaillande *et al.*, 2017; Nagappan and Vairappan, 2014). It is well known that *C. racemosa* produces various secondary metabolites which are involved in defence mechanisms against pathogen infection. In addition, when crude extracts of *C. racemosa* are directly applied on the leaves, the compounds triggered changes in photosynthetic activity. In this context, *C. racemosa* has been selected for the synthesis of nanoparticles and studied for its role on shallot seedlings. In view of the above, the present study mainly focused on the synthesis and characterization of bioengineered nanoparticles loaded with phycomolecules, and to study its growth promoting role on plant growth, physio-chemical changes as well as accumulation of nutrients in shallot plants.

Materials and Methods

Phycosynthesis and characterization of copper oxide nanoparticle (CuONPs): *Caulerpa racemosa*, was procured from the coast of Rameswaram, Tamil Nadu, India. It was thoroughly washed with deionized water and powdered after shade drying. The algae powder was boiled under microwave irradiation and the filtrate was mixed with copper sulphate (CuSO₄) solution, incubated at room temperature to prepare CuO nanoparticle. The nanoparticle pellets were centrifugated and thoroughly washed. Dried nanoparticles were further characterized by Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction (XRD) analysis, Scanning Electron Microscopy (SEM), Energy Dispersive X-ray (EDX) spectra analysis and Dynamic Light Scattering (DLs).

Effect of CuONPs on plant growth and biomass: Mature (30 days) shallot bulbs (*A. ascalonicum*) were placed in a beaker containing distilled water and mixed for 3hr until hydrated. Organic potting (500g) mix was placed in each pot, creating three replicates per treatment. The seeds were placed about 1.5 cm deep in the soil. Pots were placed in a greenhouse (25/25°C day/night temperature). For germination, seeds of same size (3.5 cm) were selected for nanoparticle exposure. For nanoparticle treatment, various doses of Cu (25 mg), CuONPs (25 mg, 50 mg, 100 mg and 200 mg) along with control (water) and algal extract (25 mg) were used in the experiment and treatment was carried out for 30days. After 30 days of treatment, seedlings were collected for estimating the growth tolerance index, biomass, photosynthetic pigments level and antioxidative enzyme activity. The remaining plants from each pot were maintained for

maturation. Matured plants were collected and assessed for various biochemical parameters.

Estimation of MDA content: The amount of lipid peroxidase or malondialdehyde (MDA) content in the shoot and root tissues of treated plants were determined following the method of Michael and Krishnaswamy (2011). MDA content was calculated by the following formula: $\text{MDA } (\mu\text{mol g}^{-1}) = [6.45 \times (A_{532} - A_{600}) - (0.56 \times A_{450}) \times V_t/W]$, where $V_t = 0.0021$; $W = 0.2\text{g}$.

Estimation of photosynthetic pigments: The content of chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (Car) was extracted from leaves with 80% acetone. The amount of chlorophyll a, b and carotenoid content in treated plants was quantified spectrophotometrically at 665, 649 and 470 nm according to Lichtenthaler (1987) and expressed in $\text{mg g}^{-1}\text{f.wt}$.

Estimation of total soluble protein: Total soluble protein content of shoot and root was determined by Bradford method (1976). Approximately, 100 mg of leaf tissue was homogenized with 1ml of 0.1 M Tris- HCl buffer using pre-chill mortar and pestle. The extract was centrifuged (10,000rpm) for 10 min at 4°C. The supernatant was transferred to a sterile Oakridge tube and used for the estimation of total soluble protein content. Bovine serum albumin served as standard and protein content was expressed in $\text{mg g}^{-1}\text{f.wt}$.

Estimation of antioxidative enzyme activity: Fresh leaf and root tissues (100 mg) from treated plants were collected separately and homogenized in a mortar and pestle under ice-cold conditions with 1.0 ml of extraction buffer, containing 50 mM phosphate buffer (pH-7.5) and 1 mM EDTA. The homogenate was centrifuged (10,000 rpm) for 10 min, and the supernatant was collected and then used to estimate the activity of antioxidative enzymes. Superoxide dismutase (SOD) activity was estimated by measuring its capacity to inhibit the photochemical reduction of nitro-blue tetrazolium (Beauchamp and Firdovich, 1971). The reaction mixture (3ml) contained 100mM potassium phosphate buffer (pH 7.8), 3mM EDTA, 200mM methionine, 2.25 mM NBT, 1.5M sodium carbonate, 60 μM riboflavin and enzyme extract. After mixing, the contents in the cuvette were illuminated (40 W light) for 15 min. The enzyme extract was kept in dark and served as blank, while, buffer with no enzyme extract was kept in light served as control. The absorbance was read at 560 nm against blank using UV-visible spectrophotometer. NBT reduction in the light was recorded in the presence and absence of enzyme extract. SOD activity was expressed as absorbance of control minus absorbance of the sample, giving the total inhibition. One unit of activity denotes the amount of enzyme required for 50% reduction of NBT under reactive condition.

The activities were expressed in units $\text{mg}^{-1}\text{protein}^{-1}$. Catalase (CAT) activity was determined by measuring the decomposition of hydrogen peroxide (Beers and Sizer, 1952). About 100 μl of enzyme extract was added to the reaction mixture containing 100 mM phosphate buffer (pH 7.0) and 75 mM H_2O_2 .

The decrease in the absorbance was recorded at 240 nm. CAT activity was calculated using an extinction coefficient of $39.04\text{ mM}^{-1}\text{cm}^{-1}$. One unit of CAT activity is defined as the amount required for decomposing $1\mu\text{mol}$ of H_2O_2 (per minute per milligram) protein under assay conditions. Peroxidase (POX) activity was estimated by the method of Zhang *et al.* (1999). The reaction mixture (3ml) contained 100mM potassium phosphate buffer (pH 6.1), 96mM guaiacol, and 12mM H_2O_2 , and enzyme extract was used for the estimation of enzyme activity. Oxidation of guaiacol was measured by the increase in absorbance at 470nm. The activity was calculated using an extinction coefficient of $25.5\text{ mM}^{-1}\text{cm}^{-1}$ and expressed in units. One unit of enzyme is the required necessary to decompose $1\mu\text{mol}$ of substrate (H_2O_2) per minute.

Effects of nanoparticles on the accumulation of micro and macro elements in shallots: After harvest, shallots were cut into small pieces and oven dried at 60°C for 72 hr. Dried samples were ground into powder with mortar and pestle, and 0.2 g of sample was acid-digested with 10 ml of HNO_3 and 30% H_2O_2 (Packer *et al.*, 2007; Hong *et al.*, 2015). The temperature was ramped (5 min) from 20 °C to 100 °C and held constant for 5 min. Subsequently, the temperature was ramped up to 160 °C for 5 mins and held constant for 20 min. After digestion, shallot extracts were diluted with 50 ml distilled water for quantification of Ca (317.933nm), Cu (327.393nm), Fe (238.204nm), K (766.490nm), Na (589.592 nm), and Zn (206.200 nm) elements using Inductively Coupled Plasma-optical Emission spectroscopy at their respective wavelengths (ICP-OES, PerkinElmer Optima 5300 DV, Shelton, CT).

Statistical analyses: The replicate data were collected and the results are depicted as mean $\pm$ SE using One-way ANOVA. The significant level was determined at $P \leq 0.05$, and the means were separated using Duncan's Multiple Range Test (IBM, SPSS version 20).

Results and Discussion

In the present investigation, CuONPs were synthesized from the phycocompounds of *C. recemosa*. The use of algal extracts for synthesis of metallic nanoparticles has several advantages such as simplicity, easy, low-cost and absence of organic solvents and hazardous materials. Biosynthesized CuONPs were further characterized by different techniques viz., FTIR, XRD, EDX and SEM. FT-IR measurements (Fig. 1A) were carried out to confirm the presence of possible bioactive molecules involved in the synthesis of CuONPs. Copper oxide NPs showed peaks at 3438 cm^{-1} – Alcohols, Phenols, 2979 cm^{-1} – Alkanes, 1414 cm^{-1} – Aromatics, 1027 cm^{-1} – Carboxylic acids, 655 cm^{-1} – Alkenes and 527 cm^{-1} – Alkyl halides. Similarly, the biosynthesized CuONPs showed peaks at 3885, 1585 and 9983 for alcohols, phenols, aromatic and alkene group in (Nabila *et al.*, 2018). Recently, Ahmaruzzaman (2019) reported that synthesis of copper nanoparticle from *Coccinia grandis* flower extract showed same alcohols and phenols at 3300 cm^{-1} . XRD analysis (Fig. 1B) confirmed the crystalline nature of engineered

Fig. 1: Characterization of bioengineered CuONPs coated with phycocyanin from marine green algae *C. recemosa* extract by: (A) FTIR analysis; (B) XRD pattern; (C) EDX spectroscopy analysis; (D) SEM analysis and (E) DLs.

nanoparticles and produced strong intense peaks at a spectrum of 2θ values ranging from 20 to 80. The peaks were corresponding to the angles (110, 002, 112, 202, 113, 220 and 004) referred to the JCPDS No: 96-901-5925. Similarly, Saravanakumar *et al.* (2019) reported the synthesis of CuONPs (JCPDS. 96-901-5925) using *Trichoderma asperellum* extract. Scanning Electron Microscopy revealed (Fig. 1C) that the CuO nanoparticles were spherical and crystal shaped at $2\mu\text{m}$. Fig. 1 (D) confirmed the presence of copper element due to the surface plasma resonance of CuONPs showing strong peak at 1 and 8 keV. Dynamic Light Scattering particle size analysis of phycocyanin coated nanoparticles unveiled size ranging from 23.4 to 149.5 nm in CuONPs (Fig. 1E).

In order to evaluate the stimulated growth in nanoparticle treated shallots, various physio-chemical characteristics such as biomass, seedling shoot and root length were studied. The shoot

and root lengths increased by 123.31 and 184.47%, respectively, at 100mg NPs treated plants over control. The fresh and dry biomass of plants increased significantly with higher concentrations of CuO nanoparticles. Plants treated with CuONPs at 100 mg kg^{-1} had enhanced fresh biomass of shoot and root by 311.99 and 727% (Fig. 2B), and dry biomass of shoot and root by 485.41-636.36% (Fig. 2C), respectively, than control. Similar results were also reported earlier by Prasad *et al.* (2012) in *Arachis hypogaea*, where a significant increase in root length, seed germination percentage, seedling vigour index and yield of NPs were observed. In contrast, Adams *et al.* (2017) reported that CuONPs inhibited root elongation at 10, 30, and 300 mg Cu kg^{-1} sand. Recent study have shown that *A. hypogaea* seedlings grown under various doses of NPs treatment showed highest germination percentages at 300 ppm treatment (Rajiv and Vanathi, 2018). Interestingly, nanoparticles have been found to inhibit bacterial infection and promote growth in rice (Ibrahim *et*

Fig. 2: Effects of CuO nanoparticle with different doses on: (A) shoot and root length; (B) Fresh weight and (C) dry weight of shallots. Values are mean of three replicates $\pm$ SE. Means following within each row having same letter are not statistically significant to new Duncan's Multiple Range Test.

al., 2019). It was observed that the CuONPs treated plants showed a significant decrease in MDA contents with increasing concentrations of 100 mg CuONPs. Venkatachalam *et al.* (2017) demonstrated that exposure of NPs in *L. leucocephala* decreased the lipid peroxidation level in roots. Recent studies have shown that CuO NPs at different concentrations can inhibit MDA contents (Wang *et al.*, 2019). Photosynthetic pigments play an important role in plant growth and crop productivity. NPs exposure had significantly affected the chlorophyll a, chlorophyll b and carotenoid contents and the findings were compared to the untreated control (Fig. 3B).

The increased percentage of photosynthetic pigment was directly correlated to the CuONPs concentrations used in the experiment. In 100mg kg⁻¹ NPs treated plants, the chlorophyll a, chlorophyll b and carotenoid contents was 277.24%, 301.42% and 104.81%, respectively. Earlier studies have indicated that metal oxide nanoparticles were presumed to be involved in

energy transfer of isolated reaction centres, consequently altering the photosynthetic efficiency, photochemical fluorescence and quantum yield in plants (Rico *et al.*, 2015). Faizan *et al.* (2017) reported that NPs treatments at different durations enhanced chlorophyll content in tomato plants. Sujatha and Vijayalakshmi (2013) reported the positive role of *Caulerpa racemosa* extract as evidenced by increase in dry weight, leaf area index, total chlorophyll and yield attributes against unsprayed and water sprayed plants. In addition, exposure to 3% *Caulerpa racemosa* extract highly accelerated the growth that attributes increased seed yield (74%) compared to control in black gram.

In the present study, the protein content (Fig. 3C) showed significant increase up to 100mg kg⁻¹ of CuONPs (178.49-163.35%) concentration in shallot plants over the control. Priyanka and Venkatachalam (2016) reported that exposure of NPs caused oxidative stress in cotton, which was evidenced by increased protein level, ranging from 126 – 165.4% in the treated

Fig. 3: Effects of CuO nanoparticle with different doses on: (A) MDA content; (B) Photosynthetic pigment (Chl a, Chl b and carotenoids); (C) Protein content in leaves of shallots. Values are mean of three replicates $\pm$ SE. Means following within each row having same letter are not statistically significant to new Duncan's Multiple Range Test.

plants. Tarafdar *et al.* (2014) reported that the synthesized nanoparticles had significantly increased the total soluble leaf protein content (38.7%) in pearl millet. To overcome stress, different antioxidative enzymes were synthesized to quench excess ROS species. The perusal of results showed that SOD activity (Fig. 4A) in shoot and root tissues increased to 152.17-119.42%, 161.64-139.76%, 197.51-166.86% and 165.84-125.79% treated with 25, 50, 100, 200 mg kg^{-1} CuONPs. Exposure of NPs significantly enhanced the CAT and POX activities in leaves and root of shallots. Seed treated with 100 mg kg^{-1} CuONPs enhanced the CAT activity (Fig. 4B) by 234.06 and 317.35%, while it decreased at higher doses.

Peroxidase activity in plants treated with 100 mg kg^{-1} CuONPs showed enhanced activity by 197.51 and 166.86 % (Fig. 4 (C)). Similarly, CuO NPs exposure enhanced the antioxidant activities in *Stevia rebaudiana* (Javed *et al.* 2017). Expression of Enzyme related genes such as Cu, Zn-SOD, CAT and APX were

Table. 1: Nutritional elements (micro and macro nutrients) altered shallots (*A. ascalonicum*) in soil amended with 100mg CuONPs

	Concentration ($\text{mg kg}^{-1} \text{ d.wt. tissue}$)	
	Control ($\text{mg kg}^{-1} \text{ soil}$)	100mg CuONPs ($\text{mg kg}^{-1} \text{ soil}$)
Macronutrients		
Ca	16.8833 $\pm$ 0.08838	48.01 $\pm$ 0.1267
K	8.8073 $\pm$ 0.40045	34.068 $\pm$ 0.0816
Na	10.74 $\pm$ 0.18330	30.9133 $\pm$ 0.16586
Macronutrients		
Cu	0.1377 $\pm$ 0.00769	14.9667 $\pm$ 0.9135
Fe	1.4103 $\pm$ 0.01146	3.522 $\pm$ 0.5474
Zn	0.0137 $\pm$ 0.00498	0.0213 $\pm$ 0.00669

Values are mean of three replicates $\pm$ S.E. Means following within each row having same letter are not statistically significant to new Duncan's Multiple Range Test

Fig. 4: Impact of CuO nanoparticle with different doses on antioxidative enzymes of shallots: (A) SOD; (B) CAT and (C) POX. Values are mean of three replicates $\pm$ S.E. Means following within each row having same letter are not statistically significant to new Duncan's Multiple Range Test.

regulated in CuONPs-treated plants of chickpea (Nari and Chung, 2015). Cu and Zn-nanoparticles had increased the activity of antioxidative enzymes (Taran *et al.*, 2017). Recently, nanoparticles have been found to reduce oxidative stress in *Capsicum chinense*, wheat and tomato plants, (Israel García-López *et al.*, 2018; Hussain *et al.*, 2018; Quiterio-Gutiérrez *et al.*, 2019). López-Vargas *et al.* (2019) observed that SOD and CAT enzymes showed a significant increase in Cu NPs treated tomato.

Nutrient accumulation in control and nanoparticle treated plants were estimated and found to be comparatively high in treated plants. Micro and macro elements are essential components of functional and structural molecules of living organisms. Plants accumulate these nutrients, mainly through roots, from the soil or growth medium nutrient. Earlier studies suggest that copper NPs have altered the mineral nutritional levels in many crop plants; CuONPs increased Cu, P and S by 100%, 50% and 20%, respectively, in alfalfa plants (Hong *et al.*, 2015).

Results reveal that foliar application of NPs treatments had significantly increased the nutrients content (N, P, K, Fe, Zn and Cu) in the leaves of sweet basil as compared to control leaves (El-Kereti *et al.*, 2013). In conclusion, the results of this study suggest that lower concentration CuO NPs can be used as nanofertilizer for enhancing plant growth and biomass of economically important agricultural crops.

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