



Research article

Physiological response of broccoli exposed to RuO₂ nanoparticle

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[Accepted: 28 November 2015]

Abstract: Nanotechnology no doubt is a boom for science but exposure of nanoparticles (NPs) in the environment is a new concern. Application of NPs in agriculture to increase crop yield is still debated. In the present study 10–40 µg.ml⁻¹ concentrations of ruthenium oxide (RuO₂) NPs were exposed to Broccoli (*Brassica oleracea* var. *italica*) seedlings in hydroponic culture. RuO₂ NPs are synthesized via Laser ablation method and hence are contamination free. UV-visible spectroscopy and field emission scanning electron microscope (FE-SEM) is used for the characterization of NPs. Carotenoids, protein and sugar content decreased with increase in concentrations of RuO₂ NPs. Total chlorophyll content increased to maximum with highest content of Chlorophyll a & b at 10 µg.ml⁻¹ of RuO₂ NPs, but no stimulatory effect was recorded at highest doses. Lipid peroxidation, was unaffected by exposure to 0–20 µg.ml⁻¹ NPs, but at 40 µg.ml⁻¹ malondialdehyde (MDA) formation and SOD activity increased by two fold. It is concluded that RuO₂ NPs significantly inhibited the seedlings growth of broccoli by impairing the metabolism of the test plants.

Keywords: RuO₂ NPs - *Brassica oleracea* var. *italica* - FESEM - Hydroponic culture - Malondialdehyde.

[Cite as: Hussain I, Singh A, Singh H, Singh SC & Singh NB (2015) Physiological response of broccoli exposed to RuO₂ nanoparticle. *Tropical Plant Research* 2(3): 246–252]

INTRODUCTION

Nanoparticles (NPs) in between 1–100 nm size act as bridge between bulk material and atomic or molecular structure (Kaushik *et al.* 2010). They possess remarkable and interesting properties due to small size, large surface area, free dangling bonds, high reactivity other than bulk material of same composition (Daniel & Astruc 2004). The use of nanomaterials in industries such as medicine (Panatarotto *et al.* 2003) and agriculture (Singh *et al.* 2014, Shekhawat *et al.* 2014) is increasing rapidly which leads to its exposure in the environment. Application of NPs in agriculture to increase crop yield is still debated. It is now widely recognized that sufficient amount of NPs exists in the soil which affects living systems. A broad and mechanistic understanding of the risks is posed by NPs in the environment, including bioaccumulation through the food chain, thus it is necessary to adequately protect human and environment.

Ruthenium (Ru) a rare earth element is known to possess useful catalytic properties and have imperative role in nuclear reactors which become source of Ru in the environment. Cowser & Parker (1958) reported Ru as radioactive waste in soil. Incorporation of Ru in by plants is already verified (Selders 1950, Klechkwosky 1956, Goss & Romey 1959). Menzel & Brown (1959) found that ruthenium at 0.0018 and 0.18 mgml⁻¹ in hydroponic solution had no effect on the metabolism of *Trifolium pratense*. Very limited studies have been done to explore the response of RuO₂ NPs on plants and the toxicity data regarding Ru and its oxides are also scarce. Ru NPs synthesized using *Gloriosa superb* L. leaf extract shows antibacterial activity against gram positive & negative bacterial strains (Kasi *et al.* 2014).

The essential processes leading to plant adaptation to any stress or toxicity include regulation of water loss through stomata, metabolic adjustment, toxic ion homeostasis, and osmotic adjustment has been studied (Hasegawa *et al.* 2000, Munns & Tester 2008). Inlay & Linn (1998) reported that the reactive oxygen species (ROS) are responsible for various stress-induced damages to tissues of an organism. Consequently the role of antioxidant enzymes *viz.* superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) are responsible for the quenching of ROS becomes very important (Bowler *et al.* 1992). Thus the present study

aimed to evaluate the potential effects of RuO₂ NPs on living systems especially on physiological metabolism of broccoli plant.

MATERIALS AND METHODS

Synthesis of RuO₂ NPs

Procedure for the synthesis of NPs was adopted from Singh *et al.* (2010). In the experiment, 2g of ruthenium oxide bulk powder was dispersed in 20 ml of double distilled water (DDW). Fundamental (1064 nm) output from pulsed Nd:YAG laser operating at 40 mJ pulse⁻¹ energy, 10 ns pulse width and 10 Hz rep rate was bombarded at the centre of the solution column using 25 cm focal length quartz lens for half an hour with continuous magnetic stirring. In order to avoid laser induced sedimentation or aggregation, ablation process was carried out for 30 minutes. The weight of the source was measured before and after the ablation process to estimate concentration of RuO₂ NPs. This process was repeated several times to get colloidal solution of RuO₂ NPs as stock solution.

Characterization of RuO₂ NPs

Characterization of synthesized RuO₂ NPs was carried out by UV-visible spectroscopy and field emission scanning electron microscope (FE-SEM). Lambda 35 Perkin Elmer spectrophotometer was used to record of UV-visible absorption spectra of colloidal solution of RuO₂ NPs. Scanning electron microscopic analysis was done by JEOL JXA-8230. Very few amount of precipitated powder of NPs was coated on the copper grid and allowed to magnify the grid to record morphological characters and size of NPs.

Hydroponic culture

The experiment was conducted in the month of February, 2015 in the glass house in the Department of Botany, University of Allahabad, Allahabad (24°47' and 50° 47'N latitude; 81° 91' and 82° 21'E longitude; 78 m above sea level). The different concentrations viz. 0, 10, 20, 30 and 40 µg.ml⁻¹ of RuO₂ NPs considered for C, R1, R2, R3, R4 treatments respectively were obtained by dilution of stock solution with appropriate amount of double distilled water (DDW). The nursery of the test plants was raised in nursery beds (1×1m). Twenty one days old seedlings were uprooted and washed with tap water followed by DDW until soil was totally removed from the roots. The properly washed 10 seedlings were transferred in transparent plastic boxes (23×17×9 cm) filled with 2 litre of half strength Hoagland solution (Hoagland & Arnon 1950). Aeration of medium was done with the help of bubblers for 12 h daily. The plants were allowed to establish for a week. After a week the Hoagland solution was replaced with fresh Hoagland solution and the respective concentration of NPs twice at the interval of seven days were also added. Box containing only Hoagland solution was taken as control. The boxes were covered with black paper to avoid the algal growth in the nutrient medium. The biochemical parameters were recorded after 14 days of treatment.

Pigment, Protein and Sugar content

The photosynthetic pigments viz. chlorophyll a, chlorophyll b and carotenoids from the first fully expanded leaves (10 mg) were extracted with 80% acetone and quantified following Lichtenthaler (1987). Optical density of supernatant was measured with UV-visible spectrophotometer Lambda 35 PerkinElmer at 663, 646 and 470 nm. The amount of pigments was calculated as:

$$\text{Chlorophyll a } (\mu\text{g.ml}^{-1}) = 12.21 \times A_{663} - 2.81 \times A_{645}$$

$$\text{Chlorophyll b } (\mu\text{g.ml}^{-1}) = 20.13 \times A_{645} - 5.03 \times A_{663}$$

$$\text{Carotenoid } (\mu\text{g.ml}^{-1}) = [1000 \times A_{470} - 2.27(\text{Chl. a}) - 81.4(\text{Chl. b})] / 227$$

Where, A is the observed OD

Quantitative analysis of protein was done following Lowry *et al.* (1951). The absorbance was measured at 650 nm. The amount of protein was calculated with reference to standard curve of bovine serum albumin.

The quantification of total soluble sugars was done following Hedge & Hofreiter (1962). Fresh leaf tissue (0.05 mg) was homogenized in 5 mL of 95% ethanol. After centrifugation, 1 mL of supernatant was mixed with 4 ml anthrone reagent and heated in boiling water bath for 10 min. After cooling, the absorbance was recorded at 620 nm. The amount of sugar was determined using the standard curve prepared from glucose.

Lipid peroxidation and SOD activity

The lipid peroxidation (LP) in leaves was measured by determining the malondialdehyde (MDA) content according to Heath & Packer (1968). The plant material (200mg) was homogenized in 5 ml of 0.1% w/v

trichloroacetic acid and centrifuged at 10,000g for 10 min. One mL of supernatant was mixed with 4 ml of 0.5% thiobarbituric acid made in 20% trichloroacetic acid. The mixture was then heated at 95 °C for 30 min followed by cooling and centrifugation. The absorbance of supernatant was measured at 532 nm and corrected by subtracting the non-specific absorbance at 600 nm. The MDA concentration was calculated using the extinction coefficient of 155 mM⁻¹ and expressed as n mol g⁻¹ FW.

The activity of SOD (EC 1.15.1.1) was estimated by the nitrobluetetrazolium (NBT) photochemical assay following Beyer & Fridovich (1987). The reaction mixture (4 ml) consisted of 20 mM methionine, 1.3M riboflavin, 0.15 mM ethylene diamine-tetra acetic acid (EDTA), 0.12 mM NBT, and 0.5ml supernatant. The test tubes were exposed to fluorescent lamp for 30 min and identical unilluminated assay mixture served as blank. One unit of enzyme was measured as the amount of enzyme which caused 50% inhibition of NBT reduction.

Statistical Analysis

Treatments were arranged in a randomized block design with three replications. Data were statistically analyzed using analysis of variance (ANOVA) by using SPSS (Ver.10; SPSS Inc., Chicago, IL, USA). The treatment means were analyzed by Duncan's multiple range test (DMRT) at $p < 0.05$.

RESULTS AND DISCUSSION

UV-visible absorption spectra of bulk as well as NPs produced after 5, 10 and 30 minutes of laser irradiations are shown in figure 1. It is evident that with the increase of time of laser irradiation, band edge absorption shifts towards the shorter wavelength side, which indicates laser induced reduction in the size of particles. Colloidal solution of NPs produced after 30 minutes of laser irradiation is highly constant in colloidal form for several months and is used for the experiment. The morphological studies of synthesized NPs have been carried out by Field Emission Scanning Electron Microscopy magnifications (FE-SEM). It is observed, NPs constructing nanostructures were in the range of 30–113 nm with spherical shape in the under 50,000 X (Fig. 2). The FE-SEM images of NPs were assembled on to the surface due to the interactions such as hydrogen bond and electrostatic interactions which is supported by the SEM images of Kathiravan *et al.* (2015).

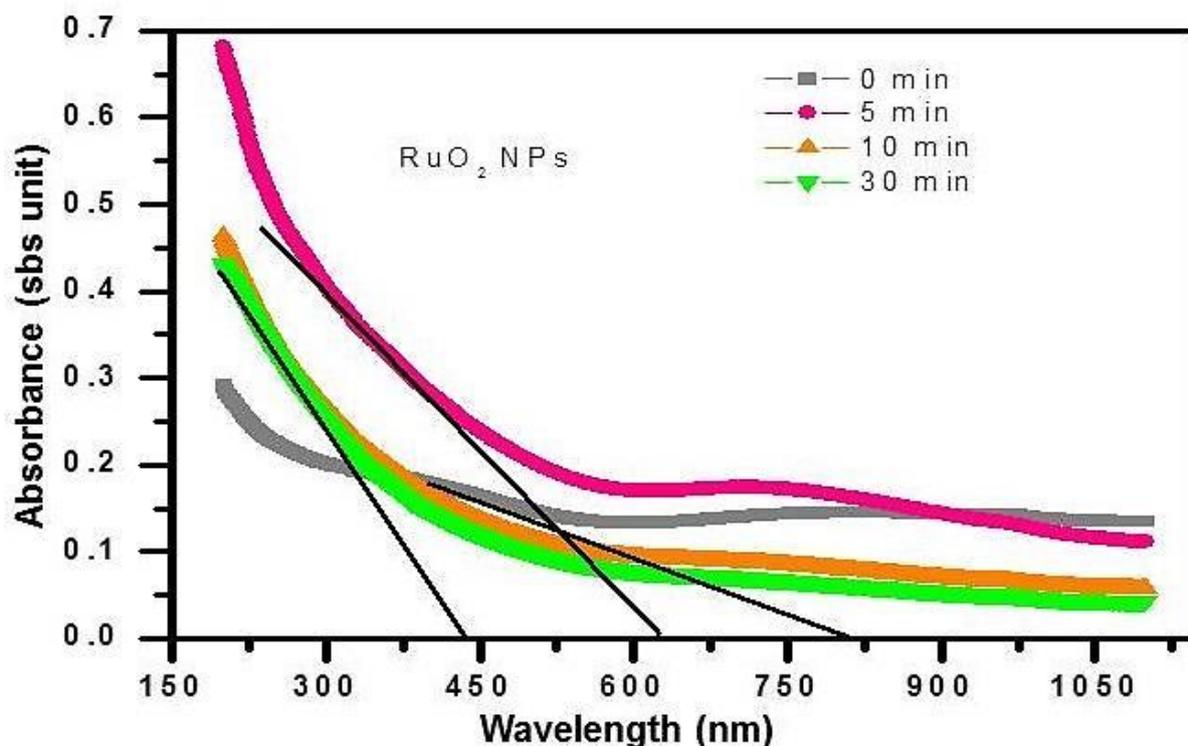


Figure 1. UV-Visible spectra of ruthenium oxide NPs at time in interval of 0, 5, 10 and 30 minutes.

Ru exhibited adverse effect on both physiological and biochemical parameters of crop plants. Amounts of chlorophyll a, chlorophyll b, carotenoids, protein and MDA content and SOD activity were considered as indicators for the estimation of effects of different concentrations of ruthenium oxide NPs on the health of the plant.

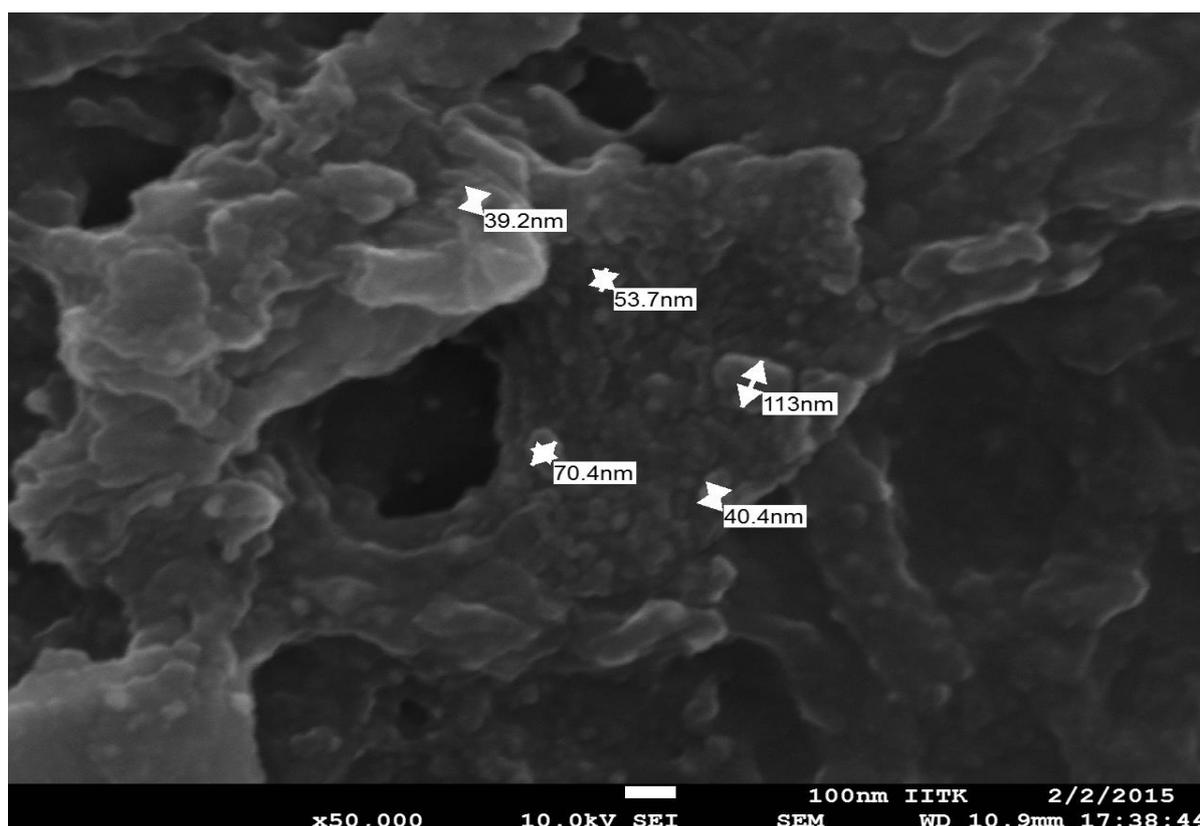


Figure 2. FE-SEM image of ruthenium oxide NPs taken at 50,000 X, showing NPs in the range of 30-113 nm.

The present study confirms that the increase in RuO₂ NPs concentrations on broccoli seedlings appeared to have negative impacts, primarily which is evident from a reduced sugar and protein content and the increase in MDA formation and SOD activity by 2 fold at highest concentration. A decrease in sugar and protein under unfavourable conditions allows the conservation of energy, thereby launching the appropriate defence response and also reducing the risk of damage. RuO₂NP did not significantly influence the chlorophyll content of plant seedlings but in all treatments carotenoid content decreased (Table 1). The total chlorophyll content improved under lower concentrations of RuO₂ NPs. The seedlings treated with 10µg.ml⁻¹ of RuO₂ NPs exhibited maximum

Table 1. Effects of Ruthenium Oxide NPs on pigment content of broccoli seedlings.

Treatments	Chlorophyll a (mg.g ⁻¹ FW)	Chlorophyll b (mg.g ⁻¹ FW)	Total Chlorophyll (mg.g ⁻¹ FW)	Carotenoids (mg.g ⁻¹ FW)
C	2.26±0.088 ^b	0.56±0.032 ^c	2.83±0.120 ^c	0.52±0.017 ^a
R1	2.93±0.088 ^a	0.85±0.034 ^a	3.78±0.070 ^a	0.44±0.008 ^b
R2	2.72±0.109 ^a	0.68±0.037 ^b	3.41±0.140 ^b	0.33±0.017 ^c
R3	1.91±0.075 ^c	0.45±0.033 ^d	2.36±0.041 ^c	0.25±0.014 ^d
R4	2.16±0.094 ^{bc}	0.40±0.006 ^d	2.56±0.100 ^c	0.20±0.005 ^e

Note: C= 0 µg.ml⁻¹; R1= 10 µg.ml⁻¹; R2= 20 µg.ml⁻¹; R3= 30 µg.ml⁻¹; R4= 40 µg.ml⁻¹.

Mean ± SE values followed by same letters within each column are not significantly different at 0.05 (ANOVA and Duncan's multiple range test) n=3.

content of chlorophyll a and b, while no effect was recorded at the highest dose of NPs. This might be due to increase in efficiency of photosystems by Ru NPs and the role of Ru in photosystem is in agreement with the work of Xiaojun *et al.* (2004). They found that Ruthenium tris-bipyridine complex act as photosensitizer, that plays the role of the p680 chlorophyll in psII. The protein content of leaves decreased significantly, reaching the minimum value in the plant treated with 30µg.ml⁻¹ of RuO₂ NPs (Table 2). The decrease of sugar was higher under low treatments as compared to that of treatments with increased concentration. RuO₂ at 20 µg.ml⁻¹ concentration caused maximum decrease in sugar content over control (Table 2). Our results on biochemical parameters are in agreement with the findings of Khuzihko *et al.* (2011). According to their report RuO₂ in

lower concentration enhanced the photocatalytic activity by taking part in oxygen evolution reaction (OER) while in excess amount exhibited adverse effect on photocatalytic activity. Moreover, RuO₂ also acts as a water oxidation catalyst (Trasatti & Buzzanca 1971).

Table 2. Effects of Ruthenium Oxide NPs on protein and sugar content, lipid peroxidation and SOD activity of broccoli seedlings.

Treatments	Protein (mg.g ⁻¹ FW)	Sugar (mg.g ⁻¹ FW)	lipid peroxidation (n mol.g ⁻¹ FW)	Superoxide dismutase (EU.g ⁻¹ FW)
C	26.30±0.502a	86.23±0.648a	11.80±0.357c	46.05±0.138d
R1	24.39±0.265b	77.39±1.243b	12.98±0.193c	43.27±0.177e
R2	21.22±0.451c	53.55±1.442d	12.39±0.582c	48.76±0.280c
R3	19.78±0.232d	67.34±1.368c	21.56±0.238b	85.34±0.490b
R4	20.14±0.098d	77.32±0.779b	27.62±0.285a	99.79±1.015a

Note: C= 0 µg.ml⁻¹; R1= 10 µg.ml⁻¹; R2= 20 µg.ml⁻¹; R3= 30µg.ml⁻¹; R4= 40 µg.ml⁻¹.

Mean ± SE values followed by same letters within each column are not significantly different at 0.05 (ANOVA and Duncan's multiple range test) n=3.

In our study, H₂O₂ in connection with other signal molecules may contribute to the control of plant growth and development at specific checkpoints of the cell cycle (Xiong *et al.* 2002). Malondialdehyde (MDA) production, a measure of lipid peroxidation, was unaffected by exposure to 0–20 µg.ml⁻¹ NPs, but at 40 µg.ml⁻¹ MDA formation was increased by more than two fold (Table 2). Increase in H₂O₂ was observed only at higher concentrations conditions which are in agreement with the significant increase in H₂O₂ observed in cultivated tomato (Mittova *et al.* 2002) and pea plants (Hernandez *et al.* 2001) under stresses. The seedlings treated with the lower concentrations R1 and R2 treatment of RuO₂ NPs exhibited minimum lipid peroxidation while the highest dose of RuO₂ NPs exhibited maximum 128% of lipid peroxidation.

SOD activity significantly enhanced under higher concentrations of RuO₂ NPs (Table 2). Because of the significant damage likely resulting from ROS production and associated toxicity of NPs exposure, the SOD content of plant leaves was determined. Quantification of SOD activity confirms the results that at the 10 µg.ml⁻¹ exposure level had no impact but exposure at 40 µg.ml⁻¹ NPs resulted in significantly greater SOD activity. SOD activity directly modulates the amount of ROS same from what was reported by Gómez *et al.* (2004) which found an increase in all SOD isoenzymes of pea chloroplasts under stress. Deficiency of micronutrients such as Mn and Zn also affects SOD activities in plants (Yu & Rengel 1999) but we provide appropriate hogland solution as reported earlier (Singh *et al.* 2014). Thus in our results the activity of SOD under stress depends on the toxicity level.

CONCLUSION

We have successfully demonstrated the toxic effect of RuO₂ NPs on broccoli plant. Purity of laser produced RuO₂ NPs with chemical contamination free surfaces helps in the real investigation of NPs effects on the biological systems. The NPs at 40 µg.ml⁻¹ resulted in drastic increase of antioxidant enzyme (SOD) and MDA content and decrease in carotenoid, protein and sugar content in hydroponic culture. The decrease in sugar content and protein content is due to diverting of maximum energy towards plants defence mechanism. Based on findings we conclude that higher doses (>20 µg.ml⁻¹) of RuO₂ NPs are toxic. Further study is required to explore the minimum dose of RuO₂ NPs which have least impact on biological system that ensures safe environment release.

ACKNOWLEDGEMENTS

The authors are thankful to the CSIR and UGC, New Delhi, India for providing financial assistance to Intiyaz Hussain and IIT Kanpur for providing SEM facility.

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