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Research Article
Nano-titanium Dioxide-induced Synthesis of Hydrogen Sulfide and Cysteine Augment Drought Tolerance in *Eruca sativa*

Mohammad Nasir Khan and Fahad Mohammed Alzuaibr

Department of Biology, Faculty of Science, University of Tabuk, Tabuk 71491, Saudi Arabia

Abstract

Background and Objectives: In recent years nano-materials have emerged as an important tools in manipulating crop performance worldwide. Also hydrogen sulfide (H_2S) has gained substantial attention of plant biologists. Present study was planned to investigate the effect of nano-titanium dioxide ($nTiO_2$) on the synthesis of H_2S and their role in the tolerance of *Eruca sativa* plants to drought stress. **Materials and Methods:** Three week old plants of *Eruca sativa* were sprayed with 20 mg L^{-1} $nTiO_2$ and 1 mM hypotaurine (HT, an H_2S scavenger) then plants were subjected to drought stress by withholding water and nutrient supply for one week except for the control which received double distilled water (DDW) only. The treatments were given as: (1)DDW (Control), (2) $nTiO_2$, (3) Drought stress (DS), (4) $nTiO_2$+DS, (v) $nTiO_2$+HT+DS. Plants treated with DDW only were considered as control. **Results:** Results showed that drought stress induced the generation of hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substances (TBARS), electrolyte leakage (ELKG) and caused reduction in leaf relative water content (LRWC). At the same time drought-stressed plants also showed enhanced activities of antioxidant enzymes [superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT)] and accumulation of osmolytes [proline (Pro), glycine betaine (GB)]. Moreover, drought-stressed plants pre-treated with $nTiO_2$ showed further enhancement in the activities of antioxidant enzymes and accumulation of osmolytes that resulted in reduced H_2O_2 content, TBARS, ELKG and improved LRWC. Furthermore, $nTiO_2$ also enhanced the synthesis of H_2S and cysteine. Role of H_2S in drought stress tolerance was confirmed using H_2S scavenger hypotaurine (HT). **Conclusion:** Results showed that application of HT along with $nTiO_2$ to drought stressed pants suppressed H_2S content and plants showed weak tolerance against drought stress. Therefore, these results suggest that $nTiO_2$-induced synthesis of H_2S induces drought tolerance capacity of plants through enhancing the activities of antioxidant enzymes and accumulation of osmolytes.

Key words: Antioxidant enzymes, cysteine, drought stress, hydrogen sulfide, osmolytes, *Eruca sativa*

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Corresponding Author: Mohammad Nasir Khan, Department of Biology, Faculty of Science, University of Tabuk, Tabuk 71491, Saudi Arabia
Tel: +966 504102439

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Drought has been considered as one of the most damaging environmental stresses that causes severe losses to crops across the globe. Loss of water is the signature effect of drought that disrupts water relations and suppresses water use efficiency of plants resulting in osmotic stress. Lower water status causes closure of stomata, turgor loss, reduced photosynthetic activity and suppressed carbon assimilation[1,2], leading to poor dry matter accumulation in affected plants[3,4]. In addition, drought stress creates an imbalance between production and scavenging of reactive oxygen species (ROS) that causes excessive generation of ROS such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroperoxyl radical (HO_2), singlet oxygen ($^1O_2$) and hydroxyl radical ($OH^{\bullet}$). Over accumulation of ROS creates oxidative stress that causes oxidation of membrane lipids, proteins and nucleic acids[2,5]. Being sessile in nature plants are always exposed to several abiotic stresses. Under such circumstances plants are provided with various types of defense mechanisms. To counter osmotic stress plants synthesize osmolytes such as proline (Pro), glycinebetaine (GB) which provide osmotic adjustment through stabilizing biomolecules and maintaining membrane stability[6-9]. Moreover, to invalidate oxidative stress plants possess a system of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT). It is well documented that SOD dismutates $O_2^{\bullet -}$ radicals to H_2O_2, whereas POX and CAT convert H_2O_2 into water and oxygen. Activation of these defense systems, in response to abiotic stresses, is carried out by a network of signaling molecules. Of these, hydrogen sulfide (H_2S) has emerged as an important signaling molecule that mediates responses to biotic and abiotic stresses in plants. It has been studied that H_2S protects plants against abiotic stress induced oxidative and osmotic stress through enhancing the activities of antioxidant enzymes and accumulation of osmolytes[10,11]. In plants H_2S is synthesized by the degradation of cysteine (Cys), a sulfur containing precursor of various biomolecules[12]. Therefore, endogenous level of H_2S depends on the availability of Cys in the cells.

Among the nanomaterials (NMs), nano-titanium dioxide ($nTiO_2$) has been shown to play significant role in growth and development of crop plants. In addition to their role in growth and development of plants, $nTiO_2$ also plays vital role in the protection of plants against various abiotic stresses such as drought, salinity, cold, heat, metal and UV radiation[13]. It has been shown that $nTiO_2$ counters drought stress[14], mimics the activities of antioxidant enzymes and scavenges ROS[13]. Drought-stressed plants treated with $nTiO_2$ exhibit improved morphological and physiological attributes[14].

Although, various studies have been carried out to investigate the role of NMs in plants, meager information is available regarding the effect of $nTiO_2$ on the level of H_2S and Cys under drought stress. Therefore, considering the important role of $nTiO_2$ and H_2S in plants the objective of the present work was to investigate whether exogenous $nTiO_2$ could affect endogenous levels of H_2S and Cys and to explore their interactive role in the activation of antioxidant defense system and osmolytes accumulation in relation to the tolerance of *Eruca sativa* plants to drought stress. To achieve the objective a pot experiment was carried out under natural environmental conditions using hypotaurine (HT) as H_2S scavenger.

MATERIALS AND METHODS

Plant materials and treatments: Seeds of arugula (*Eruca sativa* Mill.) were purchased from local market of Tabuk. Healthy and uniform seeds were surface sterilized with 1% sodium hypochlorite for 10 min, then vigorously rinsed with double distilled water (DDW). On March 14, 2018, surface sterilized seeds were sown in plastic pots (20 cm diameter and 20 cm height) containing soil/vermiculite (1:1) mixture. The plants were allowed to grow for 3 weeks under natural illuminated conditions with average day/night temperature $26/8\pm 3°C$. All the pots were supplied with 50 mL of Raukura's nutrient solution[15] daily. After three weeks (on April 3, 2018), foliar spray of $nTiO_2$ at the rate of 20 mg L^{-1} and 1 mM hypotaurine (HT, an H_2S scavenger) was given and then plants were subjected to drought stress by withholding water and nutrient supply for 1 week (from April 4 to April 10, 2018), except for the control which received DDW only. The treatments were given as: (1) Double distilled water (DDW: control), (2) $nTiO_2$, (3) Drought stress (DS), (4) $nTiO_2$+DS, (5) $nTiO_2$+HT+DS. Plants treated with DDW only were considered as control. Each treatment was replicated three times and each replicate was consisted of three plants. After one week of drought when the plants were four weeks old, the effect of $nTiO_2$ on drought stress was tested (on April 11, 2018) by measuring leaf relative water content (LRWC), electrolyte leakage (ELKG), hydrogen peroxide (H_2O_2) content, thiobarbituric acid reactive substances (TBARS) and proline (Pro) and glycine betaine (GB) content. Activities of antioxidant enzymes superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) were analyzed. Concentration of cysteine (Cys), hydrogen sulfide (H_2S) and chlorophyll (Chl-a, Chl-b, total Chl and Chl a/b ratio) was also estimated.

Measurement of physiological and biochemical parameters: Leaf relative water content (LRWC) was measured by adopting the method of Yamasaki and Dillenburg[16]. Fresh weight (FW), dry weight (DW) and turgid weight (TW) of leaves was measured and LRWC was calculated using the equation below. The values for FW, TW and DW were used to calculate LRWC using the equation below:

$$\text{LRWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Effect of drought stress on membrane permeability was assessed in term of percentage of electrolyte leakage (ELKG) by the method of Lutts *et al.*[17]. Hydrogen peroxide (H_2O_2) content was determined according to Velikova *et al.*[18]. The content of H_2O_2 was calculated based on a standard curve and was expressed as µmol g^{-1} leaf DW. Lipid peroxidation was determined by measuring the content of thiobarbituric acid reactive substances (TBARS) as described by Cakmak and Horst[19]. Content of TBARS was expressed as nmol g^{-1} DW. Proline (Pro) and glycine betaine (GB) content was determined according to the method of Bates *et al.*[20] and Grieve and Grattan[21], respectively.

Assay of antioxidant enzymes: A crude enzyme extract was prepared prior to determination of antioxidant enzyme activities. Fresh leaves were homogenized with three volumes (w/v) of an ice-cold extraction buffer (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM $MgCl_2$ and 1.5% (w/w) polyvinylpyrrolidone). The homogenate was centrifuged at 15,000 g for 20 min at 4°C. The supernatant was used as the crude extract for the assay of activities of superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT).

Activities of superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POX; EC 1.11.1.7) and catalase (CAT; EC 1.11.1.6) were determined by the method of Beauchamp and Fridovich[22], Upadhyaya *et al.*[23] and Cakmak and Marschner[24], respectively.

Measurement of hydrogen sulfide (H_2S) and cysteine (Cys) content: The method of Nashef *et al.*[25] was adopted to estimate the concentration of H_2S. Concentration of H_2S was expressed in n mol g^{-1} DW. Cysteine content was determined according to Gaitonde[26] as described by Riemenschneider *et al.*[27] with slight modifications. The amount of Cys was calculated using pure Cys as standard and the result was expressed as nmol g^{-1} DW.

Estimation of chlorophyll (Chl) content: Chlorophyll (Chl) content was estimated using the method of Lichtenthaler and Buschmann[28]. The optical density of the pigment solution was recorded at 662 and 645 nm to determine Chl a and Chl b, respectively using a spectrophotometer.

Statistical analysis: Analysis of variance (ANOVA) was performed to evaluate the significance of the treatment means. The data were expressed as the Mean±standard error and the data were analyzed statistically using SPSS ver. 17 statistical software (SPSS Inc., Chicago, IL, USA). Treatment means were statistically compared by Duncan's Multiple Range Test (DMRT) at $p<0.05\%$ level. Each treatment was replicated three times and each replicate was consisted of three plants.

RESULTS

Effect of $nTiO_2$ and drought stress on LRWC and membrane permeability: Hydration level of plants was assessed in term of LRWC. Perusal of the data showed that under drought stress plants exhibited 56.7% LRWC which was lower as compared with the control. However, drought-stressed plants pre-treated with $nTiO_2$ showed 78.5% LRWC which was significantly higher than drought-stressed plants (Fig. 1a).

Effect of drought and $nTiO_2$ on membrane permeability was tested by measuring ELKG. Drought stress caused a significant increase in ELKG compared to the control (Fig. 1a). However, pre-treatment with $nTiO_2$ alleviated the effect of drought and caused reduction in the level of ELKG as compared with the drought-suffered plants (Fig. 1a).

Effect of $nTiO_2$ and drought stress on H_2O_2 content and lipid peroxidation: The results exhibited that exposure of plants to drought caused a significant increase in H_2O_2 content than the control. However, exposure of $nTiO_2$-treated plants to drought ($nTiO_2$+DS) showed a considerable reduction in H_2O_2 content than drought-stressed plants not treated with $nTiO_2$ (Fig. 1b).

The results showed that drought stress instigated peroxidation of membrane lipids as reflected by increased level of TBARS (Fig. 1c). Value of TBARS in drought-stressed plants was about two-folds higher than the control plants. Nevertheless, drought-stressed plants pre-treated with $nTiO_2$ ($nTiO_2$+DS) exhibited a decrease in the synthesis of TBARS than the stressed plants (DS) (Fig. 1c).

Effect of $nTiO_2$ and drought stress on Pro and GB content: It was evident from Fig. 1d and e that drought stress enhanced the accumulation of Pro and GB content. Moreover, drought-stressed plants supplemented with $nTiO_2$ ($nTiO_2$+DS) showed a further increase in Pro and GB content as compared with the drought-suffered plants (Fig. 1d and e).

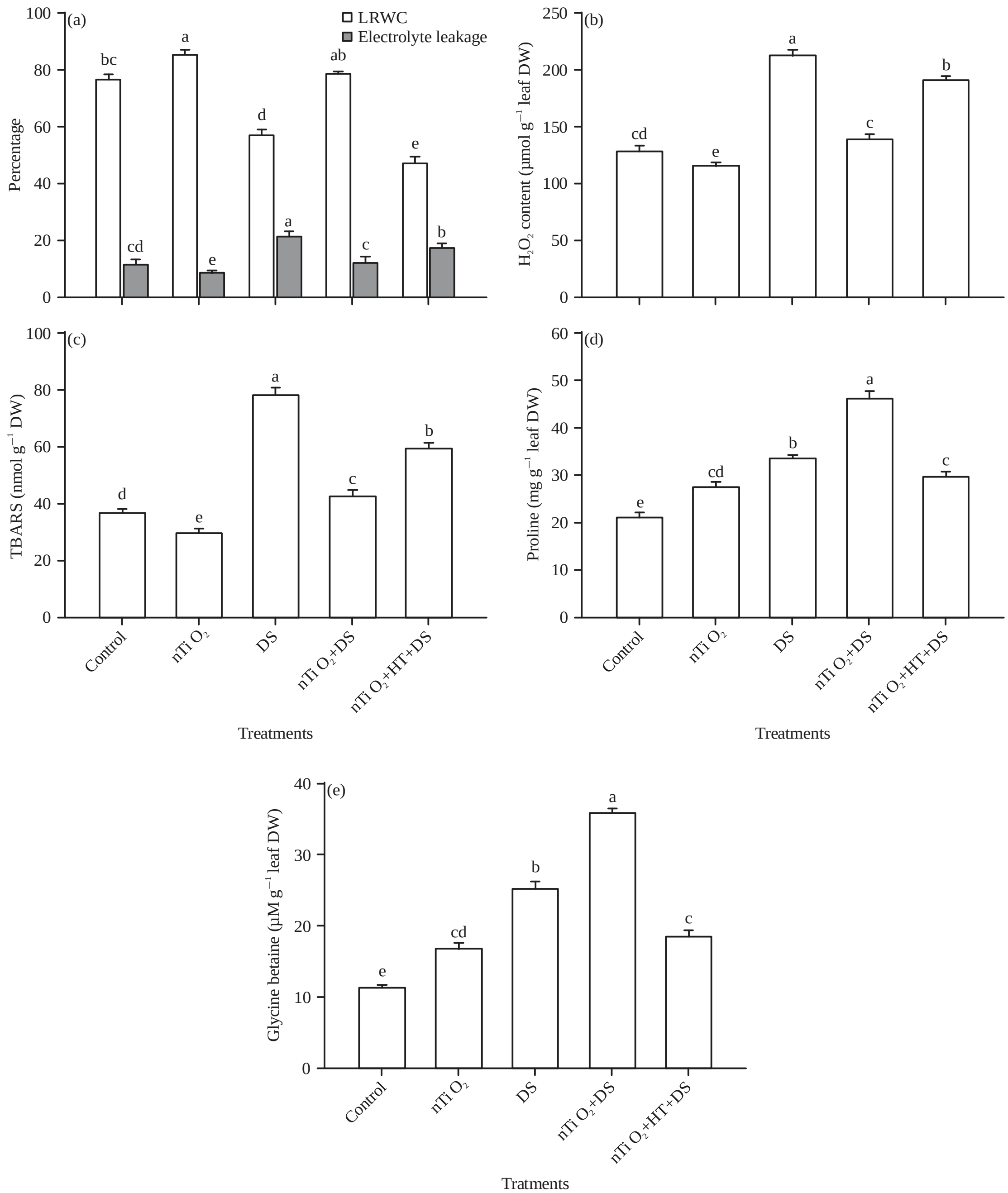

Fig. 1(a-e): Effect of nano-TiO_2 on (a) LRWC and electrolyte leakage, (b) H_2O_2 content, (c) TBARS, (d) Pro content and (e) GB content of *Eruca sativa* under drought stress. Average of three determinations is presented with bars indicating S.E. Bars followed by the same letter do not differ statistically at p<0.05 (Duncan Multiple Range Test). DDW: Control, $nTiO_2$: 20 mg L^{-1} nano-TiO_2, DS: Drought stress, HT, an H_2S scavenger: 1 mM hypotaurine

Effect of $nTiO_2$ and drought stress on the activities of antioxidant enzymes: Analysis of the data showed that plants under drought stress exhibited higher activities of antioxidant enzymes (SOD, POX and CAT) than the control plants (Fig. 2a, b). In addition, application of 20 mg L^{-1} of $nTiO_2$ further enhanced the activities of these antioxidant enzymes than drought-stressed plants grown without $nTiO_2$ (DS) (Fig. 2a, b).

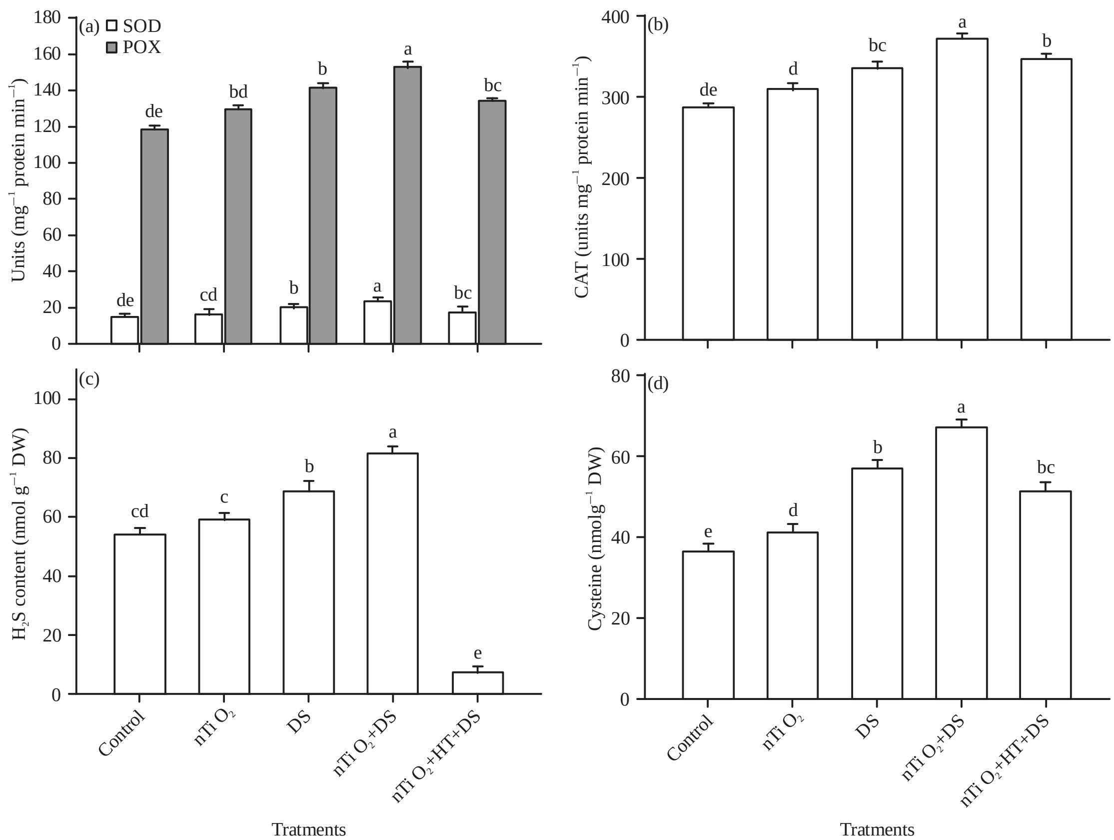

Fig. 2(a-d): Effect of nano-TiO_2 on (a) SOD and POX activities, (b) CAT activity, (c) H_2S content and (d) Cysteine content of *Eruca sativa* under drought stress. Average of three determinations is presented with bars indicating S.E

Bars followed by the same letter do not differ statistically at p<0.05 (Duncan Multiple Range Test). DDW: Control, $nTiO_2$: 20 mg L^{-1} nano-TiO_2, DS: Drought stress, HT, an H_2S scavenger: 1 mM hypotaurine

Table 1: Effect of nano-TiO_2 on chlorophyll content of *Eruca sativa* under drought stress

	Parameters			
Treatments	Chl-a (mg g^{-1} FW)	Chl-b (mg g^{-1} FW)	Total Chl (mg g^{-1} FW)	Chl a/b ratio
Control	1.76 ± 0.054^{ab}	0.85 ± 0.016^{b}	2.61 ± 0.043^{b}	2.07 ± 0.070^{b}
$nTiO_2$	1.82 ± 0.096^{a}	0.93 ± 0.0096^{a}	2.75 ± 0.054^{a}	1.96 ± 0.036^{bc}
DS	1.46 ± 0.026^{d}	0.76 ± 0.0082^{d}	2.22 ± 0.063^{d}	1.92 ± 0.071^{d}
$nTiO_2$+DS	1.64 ± 0.072^{bc}	0.82 ± 0.0310^{bc}	2.46 ± 0.040^{bc}	2.00 ± 0.059^{bc}
$nTiO_2$+HT+DS	1.32 ± 0.060^{e}	0.55 ± 0.0180^{e}	1.87 ± 0.026^{e}	2.40 ± 0.034^{a}

Average of three determinations is presented with ±indicating standard error. Values with the same letters with in a column do not differ statistically at p<0.05 (Duncan Multiple Range Test). DDW: Control, $nTiO_2$: 20 mg L^{-1} nano-TiO_2, DS: Drought stress, HT, an H_2S scavenger: 1 mM hypotaurine

Effect of $nTiO_2$ and drought stress on H_2S and Cys content: Plants exposed to drought for one week synthesized more H_2S and Cys than the control plants (Fig. 2c, d). Moreover, drought-stressed plants treated with $nTiO_2$ showed further enhancement in H_2S and Cys levels than drought stressed plants (Fig. 2c, d).

Effect of $nTiO_2$ and drought stress on Chl content: Results showed that exposure of plants to drought stress caused a significant reduction in Chl-a, Chl-b and total Chl content and Chl a/b ratio than the control (Table 1). However, drought-suffered plants pre-treated with $nTiO_2$ ($nTiO_2$+DS) countered detrimental effects of drought and showed an increase in Chl-a, Chl-b and total Chl content and Chl a/b ratio than drought-stressed plants that did not receive $nTiO_2$ (Table 1).

DISCUSSION

Loss of water in the plants is hallmark of drought stress which was assessed in terms of LRWC. Perusal of the data showed that under drought stress plants exhibited lower

LRWC (Fig. 1a). It is well established that drought stress adversely affects water relations that lead to reduction in leaf water potential, turgor loss and stomatal closure. All these together reduce water uptake capacity of plants[29,30] as witnessed by lower LRWC. Maintenance of optimum water status of plants is highly desirable for normal functioning of cellular system under abiotic stresses. To cope with deprived water status, plants accumulate osmolytes such as Pro and GB that maintain normal hydration level of plants[31,32]. Drought stress enhanced the accumulation of Pro and GB content (Fig. 1d, e). But in spite of increase in the level of these osmolytes, a decrease in LRWC was noticed under drought stress. It indicates that increased concentration of Pro and GB content was not sufficient to counter drought stress-induced decrease in LRWC. However, drought-stressed plants treated with $nTiO_2$ showed a further increase in Pro and GB concentration that possibly increased osmotic pressure resulting in the enhancement of water uptake capacity of treated plants as shown by improved LRWC (Fig. 1a). Furthermore, it was cleared from the results that $nTiO_2$ accelerated the synthesis of H_2S which had been shown to induce the activity of Pro-synthesizing enzymeΔ^1-pyrroline-5-carboxylate synthetase and reduces the activity of Pro degrading enzyme, Pro-dehydrogenase[33] that resulted in enhanced accumulation of Pro. H_2S also enhances the activity of betaine aldehyde dehydrogenase, a key enzyme in the biosynthesis of betaine which induces GB synthesis that stabilizes biological membranes and protects the plants against adverse effects of abiotic stress[34,35]. Involvement of H_2S in osmotic adjustment of stressed plants was further confirmed when H_2S scavenger HT was applied that decreased Pro and GB content to the level recorded from drought-stressed plants.

Onset of drought stress induces generation of ROS such as H_2O_2 that creates oxidative stress[36,37]. To cope with oxidative stress, plants possess a system of antioxidant enzymes which continuously scavenge ROS and maintain the normal level of ROS. However, under suppressed activities of antioxidant enzymes the rate of ROS production exceeds the rate of ROS scavenging which results in over production of ROS. Excessive accumulation of ROS causes peroxidation of membrane lipids and leakage of electrolytes[38,39] (Fig. 1a, c). The results showed that plants under drought stress enhanced the activities of antioxidant enzymes (SOD, POX and CAT) but a parallel increase in H_2O_2 content was also noticed. It shows that increase in plants' antioxidant defense system was not efficient to counter oxidative stress. However, drought-stressed plants pre-treated with $nTiO_2$ showed a further increase in the activities of antioxidant enzymes to a level which was effective in scavenging ROS as witnessed by decreased levels of H_2O_2 content coupled with reduced ELKG and TBARS (Fig. 1a-c). It confirms that $nTiO_2$ can regulate the activities of antioxidant enzymes to the level required to counter ROS and can also modulate ROS dependent signaling pathways[40] leading to significant enhancement in plant growth[41,42]. These results also corroborated the findings of Khan[43]. Moreover, $nTiO_2$ enhanced the synthesis of H_2S which has been shown to induce antioxidant defense system of plants[10,44]. On the contrary, application of H_2S scavenger HT suppressed the activities of antioxidant enzymes and an increase in the generation of H_2O_2 content was noticed which galvanized the leakage of electrolytes and levels of TBARS (Fig. 1a-c). It validates the role of H_2S against oxidative stress.

Exposure of plants to drought caused a significant increase in H_2S content (Fig. 2c). It has been already observed that drought up-regulates the expression levels of H_2S-synthesizing genes that induce production of H_2S and tolerance to drought stress[45]. Although, enhanced concentration of H_2S improved the activities of antioxidant enzymes and Pro and GB content but at the same time an increase in H_2O_2 content, ELKG and TBARS was also noticed with an antiparallel decrease in LRWC and Chl content. It shows that enhanced level of H_2S was not sufficient to provide complete protection against drought-induced impairments. However, application of $nTIO_2$ further enhanced the accumulation of H_2S to a level required to boost the activities of antioxidant enzymes and accumulation of Pro and GB that resulted in reduced H_2O_2 content, ELKG and TBARS and increased LRWC. In order to maintain uninterrupted synthesis of H_2S under stress conditions, there should be continuous supply of Cys. Application of $nTIO_2$ not only increased the synthesis of H_2S but also of Cys and thus Cys pool was maintained that assisted the plants to synthesize more H_2S for proper functioning of cellular system under stressful conditions. These results were in agreement with the findings of Khan *et al.*[10] who observed that osmotic stress enhanced the activities of H_2S and Cys-synthesizing enzymes that contributed to enhanced levels of H_2S and Cys, respectively. Based on these observations, it can be speculated that $nTiO_2$ might had enhanced H_2S and Cys synthesis by accelerating the activities of H_2S and Cys-synthesizing enzymes.

Drought stress also caused a significant reduction in Chl content (Table 1). As mentioned earlier that drought stress induced synthesis of ROS (H_2O_2 content). Excessive accumulation of ROS causes lipid peroxidation, leakage of electrolytes and photo-oxidative damage to chlorophyll[46,47], instability of protein complexes and increase in the activity of Chl-degrading enzyme chlorophyllase[48] leading to destruction of Chl. It is noteworthy here that drought stress caused higher decrease in Chl-a than Chl-b which indicates that Chl-a was more sensitive to drought. However, drought-stressed plants pre-treated with $nTiO_2$ showed higher concentration of Chl. Khan *et al.*[43] also observed that $nTiO_2$ induces Chl content under salt stress. Moreover, $nTiO_2$ also improved the synthesis of H_2S which causes decline in H_2O_2 content, ELKG and TBARS and increase in LRWC through enhancing the activities of antioxidant enzymes and accumulation of Pro and GB. All these together might have contributed to the alleviation of photo-oxidative damage and reduction in the activity of chlorophyllase that resulted in improved Chl concentration. These results are supported by the findings of Zhang *et al.*[49] and Wei *et al.*[50] who observed that H_2S plays an active role in suppressing Chl degradation.

CONCLUSION

The results showed that, $nTiO_2$ induced the synthesis of H_2S and Cys in drought stressed plants. Improved level of H_2S and Cys together with $nTiO_2$, alleviated drought stress by inducing the activities of antioxidant enzymes viz. SOD, POX and CAT and accumulation of Pro and GB content. Activated antioxidant enzymes significantly countered oxidative stress by suppressing the generation of H_2O_2 that resulted in the reduction of lipid peroxidation and electrolyte leakage. Similarly, enhanced synthesis of Pro and GB maintained normal osmotic pressure that facilitated drought-stressed plants to uptake more water that was reflected in the form of increased LRWC and Chl content.

SIGNIFICANCE STATEMENT

This study was carried out to explore the interactive role of $nTiO_2$, H_2S and Cys in the protection of *Eruca sativa* plants against drought stress. The results showed that application of $nTiO_2$ to drought stressed plants enhanced the synthesis of H_2S and Cys. Improved level of H_2S gave protection to the plants against detrimental effects of drought stress through enhancing the activities of antioxidant enzymes and accumulation of osmolytes. Role of H_2S in the tolerance of plants to drought stress was confirmed by the application of H_2S scavenger HT.

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