RESEARCH ARTICLE

***EsHO 1* mediated mitigation of NaCl induced oxidative stress and correlation between ROS, antioxidants and HO 1 in seedlings of *Eruca sativa*: underutilized oil yielding crop of arid region**

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Received: 22 November 2018 / Revised: 12 February 2019 / Accepted: 29 March 2019 / Published online: 5 April 2019
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Abstract Study have focused on NaCl induced HO 1 production and its co-relation to ROS and antioxidant regulation in *Eruca sativa*. Seedlings were subjected to NaCl stress ranges from 10 to 150 mM. After 96 h of treatment, plants samples were harvested to evaluate the cellular equilibrium and salt tolerance mechanisms through morphological, stress parameters, non enzymatic and antioxidant enzymes. The HO 1 activity was found to be highest at 75 mM NaCl in leaves and roots which were 2.49 and 2.02 folds respectively. The expression of *EsHO 1* was also observed and the higher expression was recorded in roots than leaves. The overall activity of other antioxidants (APX and proline) was also found to be higher at 75 mM concentration. The highest HO 1 activity with other antioxidants indicates the decline in LPX and ROS at 75 mM NaCl. The present study concluded that HO 1 helps in amelioration of NaCl stress by working within a group of antioxidants that create the defense machinery in seedlings of *E. sativa* by manipulating various physiological processes of plants. These findings for the first time suggest the protective role of HO 1 in scavenging ROS in *E. sativa* under salinity stress.

Keywords Heme oxygenase 1 · *Eruca sativa* · Salinity stress · Antioxidants · Reactive oxygen species

Abbreviations

HO 1 Heme oxygenase 1
CAT Catalase

APX Ascorbate peroxidase
GPX Guaiacol peroxidase
SOD Superoxide dismutase
EtBr Ethidium bromide
ROS Reactive oxygen species
TBA 2-Thiobarbituric acid
TCA Trichloroacetic acid

Introduction

Plants are sedentary organisms and are strained to experience various environmental changes. When these changes are intense and rapid, plants perceive them as stress. Plant stress may be either abiotic (drought, salinity, cold, heat, heavy metal stress and extremes of temperature) or biotic that negatively affects plant growth and productivity. Among the stress, abiotic stress is the severe one as it is the major reason of crop yield failure worldwide which normally reduces yields of agricultural crops and food by approximately 50% and further leads to economic loss (Shanker and Venkateswarlu 2011). Tolerance or susceptibility towards abiotic stress is a complex phenomenon, which depends on the type of plant species, developmental stage of plant, type of stress as well as on the duration of stress.

Salinity stress is the principal abiotic stress which has a decisive influence on plant physiology. It limits the productivity of agricultural crops, by affecting germination, plant vigour and crop yield (Munns and Tester 2008). High salt accumulation results in decreased osmotic potential of soil solution eliciting water stress in plants and further interactions of the salts with minerals cause nutrient imbalance and deficiencies, oxidative stress which eventually lead to plant death as a result of physiological

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changes, metabolic damage and growth arrest (Sehrawat et al. 2013). Soil salinization is noticeably intensifying process accelerated by crop irrigation. The overall effect of irrigation in the saline environment is that it “imports” huge amount of new salts to the soil that were previously not present (Munns 2002). Among the various sources of salinity stress, irrigation and poor drainage is the most severe, as it represents loss of once productive agricultural land (Zhu 2007). The irrigation water contains calcium (Ca^{2+}), magnesium (Mg^{2+}), and sodium (Na^+). When the water evaporates, Ca^{2+} and Mg^{2+} precipitate into carbonates, leaving Na^+ dominant in the soil (Serrano et al. 1999). High concentrations of Na^+ in the soil solution may lower nutrient-ion activities and produce extreme ratios of $\text{Na}^+/\text{Ca}^{2+}$ or Na^+/K^+ (Grattana and Grieveb 1999) that can prevent or reduce the influx of water into the root resulting in water deficit. Additionally Na^+ causes soil aggregates to break down, increase bulk density, make the soil more compact and decrease total porosity, thus hindering soil aeration. Therefore, plants in saline soils not only suffer from high Na^+ levels, but are also affected by some degree of hypoxia (Singh and Chatrath 2001).

Plant exposure to salinity stress increases the production of reactive oxygen species (ROS) like singlet oxygen ($^1\text{O}_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\text{OH}^\cdot$) (Mahawar and Shekhawat 2016). ROS can be enormously harmful to organisms at high concentrations as it can oxidize proteins, lipids, pigments and nucleic acids, often leading to changes in cell structure, thus affecting cell viability (Foyer et al. 1997). To defend the cell from oxidative damage, surplus ROS should be neutralized. Generally, antioxidant enzymes including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (GPX) and non-enzymatic antioxidants played this role. Besides these well known molecules, HO (Heme oxygenase) is recently identified as an antioxidant and play crucial role in defense mechanism as (1) HO products possess antioxidant properties; (2) if Heme oxygenase performance is improved by HO inducers, the activity of other enzymatic antioxidants also enhanced to reduce ROS stress (Ling et al. 2009); and (3) Heme oxygenase is triggered by various stress-provoking stimuli, namely heavy metals, UV radiation, salinity, hydrogen peroxide, nitric oxide and hypoxia.

Heme oxygenases are the universal and highly active family of enzymes which catalyzes the oxidative degradation of Fe(III) protoporphyrin IX (heme) to biliverdin, ferrous ion and carbon monoxide in the presence of reducing equivalents (Shekhawat et al. 2011; Shekhawat and Verma 2010). The enzyme was originally identified in the animal system where it plays a role in production of bilirubin (Tenhunen et al. 1968). Now the genes encoding Heme oxygenase has been isolated from various living system including animals, higher

plants, algae, cyanobacteria, cryptophyta and pathogenic bacteria. In higher plants HO 1 among various HO isoforms (HO 1, HO 2, HO 3 and HO 4) is the foremost stress response enzyme (Dixit et al. 2014). HO 1 performs antioxidant activity under various abiotic stress including UV-B stress (Yannarelli et al. 2006; Santa-Cruz et al. 2017) and cadmium stress (Mahawar et al. 2018a). Reports on heme–Heme oxygenase 1 (heme–HO 1), a novel compound which act as an antioxidant and involve in regulation of tolerance towards ammonium was investigated in rice *HO 1* (*OsSE5*). Results showed that *OsSE5* RNAi transgenic rice plants exhibited enhanced tolerance to NH_4Cl with impaired antioxidant defense which helps in improving plant tolerance to excess ammonium fertilizer (Xie et al. 2015). Additionally the product BV IX α (biliverdin) is reduced to P ϕ B (phytychromobilin) that play a role in photomorphogenesis (Verma and Shekhawat 2012; Zhu et al. 2017; Mahawar and Shekhawat 2018) under light signaling using ferredoxin and ferredoxin NADP $^+$ oxidoreductase as a reducing equivalents. Apart from these, HO 1 role has also been reported in adventitious root formation in *CsHO 1* (HO gene in Cucumber) (Lin et al. 2014), *BnHO 1* (HO gene in *Brassica napus*) and lateral root formation in tomato which is induced by salt osmotic and cobalt chloride stress (Cao et al. 2011; Xu et al. 2011).

Eruca sativa (Taramira) is an important oil yielding crop of arid and semiarid region, mainly grown in Indian Thar Desert. The crop is known for its drought tolerance capacity which is due to its efficient root system. The oil content of taramira ranges from 32 to 37% and being considered as a protein meal supplement (Fagbenro 2004). The seed oil is used as lubricant, vesicant, illuminant, hair oil, for massage and pickling. Oil cakes serve as cattle feed and powdered seeds are reported to possess antibacterial activity (Jakhar et al. 2002). Salinity is one of the major problems which reduce production potential of the crop. Thus, the present investigation has been executed to evaluate various physiological and molecular changes in *E. sativa* when introduced to increase levels of NaCl. The utility of the research is to explore the probable roles of HO 1 in different plant parts as well as to ensure the HO 1 role in biological defense against salinity stress. Consequently the current study is mainly emphasized on the physiological application of HO 1 in crop plant exposed to NaCl stress and the correlation of HO 1 with other antioxidants and growth parameters.

Materials and methods

Plant material and its growth conditions

Eruca sativa var. *DPY BKP 98* seeds were collected from NBPGR (National Bureau of Plant Genetic Resources),

Jodhpur (Rajasthan) and surface sterilized with 1000 ppm of HgCl_2 for one to two minutes and rinsed thoroughly with deionized water 4–5 times to remove the residuals of mercuric chloride. Disinfected seeds were germinated in sterilized glass Petri-plates (10 cm) containing Whatmann filter paper No. 1 soaked with autoclaved distilled water (15–20 ml) at 20 °C in a seed germinator (vaiometra) under dark conditions. Germinated seeds were transferred in thermostatically controlled conditions maintained at 25 ± 2 °C and 50% relative humidity. After a week, the hydroponic cultures were successfully established from the uniformly germinated seedlings grown in petri-plates under the above mentioned conditions. Ten seedlings were planted in each plastic pot (12 cm $\times$ 12 cm) containing 1 l of Hoagland medium (pH 6.8–6.9, Elico LI 120 pH meter). The nutrient rich medium was bubbled twice a day using glass rod to provide ample amount of oxygen and mixing of nutrients. The Hoagland medium was replaced from the fresh hydroponic medium on alternate day to avoid nutrient scarcities in *Eruca sativa* seedlings.

NaCl treatment in nutrient medium

The half month (15 days) old hydroponically adapted seedlings were subjected to NaCl stress at varying concentrations ranges from 10 to 150 mM with an interval of 25 mM (10 mM, 25 mM, 50 mM, 75 mM, 100 mM, 125 mM and 150 mM) (Fig. 1). A Hoagland nutrient solution without salt was considered as a control and used to compare the effect of varying NaCl concentrations on *E. sativa*. After 96 h the treated plants were harvested to evaluate different physiological parameters (Mahawar et al. 2018a, b).

Fig. 1 Effect of NaCl on morphology of *E. sativa* seedlings treated with NaCl at various concentrations (10, 25, 50, 75, 100, 125, 150 mM) for a period of 96 h

Growth parameters

Growth parameters were determined by measuring root length, leaf water content, tolerance index, fresh and dry weight. For measuring the root length treated as well as control seedlings were harvested and immediately washed with distilled water. Root length of control and treated seedlings were measured (in cm) to examine the effect of NaCl on the crop plant. For calculating the dry weight, fresh tissue of both the treated and control seedlings were placed at 65 °C in a hot air oven (Nsico Hicon Oven) for dehydration and the weight (Adair Dutt 125 A SCS) of dried tissue was calculated. The leaf water content was calculated by using the formula (Fresh weight – Dry weight/Fresh weight) $\times$ 100. Tolerance index (TI) of plants was estimated from the formula (Fresh weight of treated tissue/Fresh weight of control tissue) $\times$ 100 (Wilkins 1978).

Estimation of lipid peroxidation and H_2O_2 content

Lipid peroxidation was determined in nmol MDA (malondialdehyde) g^{-1} fresh weight of plant tissue according to the procedure given by De Vos et al. (1989). ROS production in terms of H_2O_2 was determined spectrophotometrically by Alexieva et al. (2001) method.

Proline content

Estimation of proline was done according to the procedure of Bates et al. (1973). Fresh plant tissue (0.5 g) was homogenized in 5000 μl of 3% sulfosalicylic acid. Homogenate was centrifuged at $3000\times g$ for 20 min at room temperature. The reaction mixture contains 2 ml supernatant, 2 ml acid ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) and 2 ml glacial acetic acid. For the reaction to perform mixture was kept in a water bath at 100 °C for 1 h and further transfer on ice to stop the reaction. 4 ml of toluene was added to the terminated reaction mixture and spin for one min. Absorbance of the colored organic layer was recorded at 520 nm. The proline content was estimated by means of a standard curve prepared by L-proline and was calculated in $\mu\text{g g}^{-1}$ fresh weight of tissue.

Antioxidant enzyme assays

For antioxidant enzyme extraction 500 mg of fresh plant tissue was homogenized in 5000 μl of 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged in cold conditions at $5000\times g$ for 20 min. The supernatant was stored at -20 °C for the antioxidant enzymes assay.

APX (EC 1.11.1.11) activity was estimated by the procedure given by Chen and Asada (1989). The reaction mixture consists of 50 mM phosphate buffer (pH 7.0) containing 0.6 mM ascorbic acid. Reaction was initiated by the addition of 10 μ l of 10% H_2O_2 . Oxidation rate of ascorbic acid was recorded by the decrease in absorbance at 290 nm for three minutes (extinction coefficient $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$).

Activity of CAT (EC 1.11.1.6) was measured by the method of Aebi (1974). The assay mixture consists of 50 mM phosphate buffer (pH 7.0), 9 mM H_2O_2 and a suitable aliquot of enzyme in the final volume of 3 ml. Rate of H_2O_2 decomposition was recorded by the decrease in absorbance at 240 nm. The molar extinction coefficient of H_2O_2 was taken as $0.039 \text{ mM}^{-1} \text{ cm}^{-1}$.

Guaiacol Peroxidase (GPX) (EC 1.11.1.7) was estimated by the procedure given by Putter (1974). The reaction mixture comprises of 50 mM phosphate buffer (pH 7.0), 20 mM guaiacol, 3.7 mM H_2O_2 and an appropriate aliquot of enzyme in the final volume 3 ml. Increase in absorbance was recorded at 436 nm for 3 min (extinction coefficient $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$). Activity of GPX was expressed as unit $\text{mg}^{-1} \text{ protein}^{-1}$.

Estimation of Heme oxygenase 1 activity

Heme oxygenase 1 (HO 1) activity (EC 1.14.99.3) was measured by the method of Balestrasse et al. (2005). Fresh plant tissue (300 mg) was ground in 4000 μ l of homogenization buffer (ice cold solution of 250 mM sucrose containing 1000 μ M PMSF (Phenylmethylsulfonyl fluoride), 200 μ M EDTA (Ethylenediaminetetraacetic acid) and $50 \times 10^3 \mu$ M potassium phosphate buffer [7.4]). Homogenate was centrifuged at $20,000 \times g$ for 20 min at 4 °C, and supernatant was utilized for analyzing HO 1 activity. The assay mixture comprises of 50 mM potassium phosphate buffer (pH 7.4), 0.06 μ M NADPH, 250 μ l HO 1 extract (500 μ g of protein), and 0.2 μ M hemin to a final volume of 500 μ l. Reaction mixture was incubated at 37 °C for 1 h, and absorbance was recorded at 650 nm. HO 1 activity was estimated by calculating the biliverdin formation (extinction coefficient $6.25 \mu\text{M}^{-1} \text{ cm}^{-1}$).

Transcriptional level analysis of Heme oxygenase 1 in crop plant

RNA Isolation and cDNA synthesis

Total RNA was extracted from fresh tissues of *E. sativa* (100 mg) by grinding in liquid nitrogen followed by the treatment of RibozolTM RNA extraction reagent (Biotechnology Grade, AMRESCO) according to the manufacturer's instruction. The concentration of isolated RNA was

determined by measuring the absorbance at 260 nm and its purity was checked by the ratio of absorbance at A_{260}/A_{280} . High quality RNA should be in between 1.6 and 1.8. One microgram of total RNA was treated with RNase free DNase I (Promega) as described by the manufacturer. 20 ng of treated RNA was reverse transcribed into cDNA in a 20 μ l reaction mixture containing $5 \times$ cDNA synthesis buffer, dNTPs (10 mM), RT Enhancer, Verso enzyme mix (Genei) for 30 min at 42 °C and 2 min at 92 °C.

Semi-quantitative RT-PCR

Primers (Forward 5'AGGAGATGAGGTTTGTGGCG 3' and Reverse 5' CCACTCCCCTGCAACTTTGT 3') were designed to amplify the 536 bp region of the *E. sativa* HO 1 cDNA (GenBank accession No. XM 009105300.1); Forward (5' GTTGGGATGAACCAGAAGGA 3') (Ac No. 1458691/2), Reverse (5' GAGGAGCCTCGGTAA-GAAGA 3') (Ac No. 1458692/4) primers amplifying a 196 bp region of actin. For standardization of results, the relative profusion of actin was analyzed and used as the internal standard. A reaction mixture of 25 μ l containing $10 \times$ PCR buffer, dNTPs (10 mM), MgCl_2 , forward primer (10 μ M), reverse primer (10 μ M), Taq polymerase ($3 \text{ U } \mu\text{l}^{-1}$) and cDNA template. Cycling conditions were as follows 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing 64.3 °C for 1 min, extension 72 °C for 2 min and final extension of 72 °C for 5 min. The amplified transcripts were visualized on 1.2% agarose gel under UV light with the use of EtBr.

Statistical analysis

Experimental data were statistically examined by SPSS 16 version software. Results were considered as mean ($\pm$ standard error) of individual replicates ($n = 3$) of every test conducted independently. The significance of difference amongst control and each treatment were interpreted using one way ANOVA (Analysis of Variance) at 0.05% level of significance according to DMRT (Duncan's multiple range test).

Results

Effect of NaCl on growth parameters

Growth parameters of *E. sativa* were evaluated by calculating root length, fresh weight, dry weight, leaf water content and tolerance index. As depicted in Table 1 increasing concentration of NaCl significantly decreases the dry as well as fresh weight up to 50 mM concentration. However at 75 mM significant increase in fresh and dry

Table 1 Effect of NaCl at different concentrations on fresh weight, dry weight, root length, leaf water content and Tolerance index in seedlings of *E. sativa*

S. no.	Conc. of NaCl (mM)	Fresh weight (g)	Dry weight (g)	Root length (cm)	Leaf water content (%)	Tolerance Index (%)
1.	Control	0.37 ± 0.014 ^d	0.16 ± 0.008 ^b	13.90 ± 0.208 ^e	56.47 ± 3.05 ^b	100.0 ± 0.00 ^c
2.	10	0.30 ± 0.012 ^e	0.12 ± 0.008 ^c	17.00 ± 0.115 ^c	58.54 ± 4.57 ^{ab}	80.13 ± 6.21 ^d
3.	25	0.23 ± 0.015 ^f	0.10 ± 0.008 ^{cd}	13.73 ± 0.120 ^e	54.11 ± 7.24 ^c	61.45 ± 5.78 ^e
4.	50	0.17 ± 0.018 ^g	0.07 ± 0.015 ^d	9.67 ± 0.172 ^g	53.87 ± 7.72 ^c	44.04 ± 3.04 ^e
5.	75	0.61 ± 0.019 ^a	0.20 ± 0.011 ^a	19.83 ± 0.203 ^a	67.42 ± 1.29 ^{ab}	163.06 ± 4.67 ^a
6.	100	0.55 ± 0.018 ^b	0.16 ± 0.008 ^b	17.77 ± 0.145 ^b	71.25 ± 2.09 ^{ab}	145.86 ± 9.50 ^a
7.	125	0.45 ± 0.017 ^c	0.13 ± 0.007 ^c	15.27 ± 0.145 ^d	71.77 ± 1.77 ^a	119.96 ± 7.51 ^b
8.	150	0.22 ± 0.020 ^f	0.08 ± 0.003 ^d	11.00 ± 0.115 ^f	60.88 ± 3.70 ^{ab}	58.11 ± 7.63 ^e

weight was observed which were 1.63 and 1.23 times higher than control. Further increase in concentration results in decrease in both fresh and dry weight. Effects of NaCl on root length and leaf water content were also recorded. Root length on exposure to NaCl increases at initial concentration and then decreases till 50 mM NaCl treatment. A progressive increase in root length was observed at 75 mM NaCl which was 1.43 folds higher in comparison to control. Similar results were obtained for leaf water content. Moreover tolerance index was found to decrease with increase in concentration of NaCl up till 50 mM but a significant increase was recorded at 75 mM concentration which was 63.06% higher in comparison to control. Further increase in concentration result in decrease in tolerance index (Table 1).

Effect of salt on stress parameters (MDA and H₂O₂)

A significant ($p < 0.05$) increase in MDA content was recorded in leaves and roots subjected to increase concentrations of sodium chloride after 96 h. Roots treated with 50 mM NaCl represents 134.78% increase in malondialdehyde content with respect to control. After 50 mM NaCl concentration a gradual decrease was recorded at 75 mM NaCl but the measured MDA content at this concentration was higher than control. While in leaves, an increase in MDA content was recorded at initial concentration from control to 25 mM NaCl and then decreases. The highest MDA content in leaves was observed at 150 mM NaCl concentration which is 2.21 folds higher than control (Fig. 2). The increased accumulation of lipid peroxides is an indication of surplus production of reactive oxygen species like hydrogen peroxide. Increase in H₂O₂ content was mainly observed in leaves and roots of NaCl treated seedlings of *E. sativa* (Fig. 2). The level of H₂O₂ gradually decreases from 202.29% (at 50 mM NaCl) to 71.76% (at 75 mM NaCl) over a period of 4 days (96 h) and increases thereafter but the increase in H₂O₂ level at

Fig. 2 Effect of NaCl on MDA and ROS formation (H₂O₂ production) in *E. sativa* seedlings at various treatments. Values are mean ± SE (n = 3) and are statistically significant according to DMRT test ($p < 0.05$). Data points marked with the same letters show insignificant differences ($p < 0.05$) within treatments

high salt concentration was recorded to be insignificant with respect to control in roots. At 50 mM NaCl, H₂O₂ content increase by 1.91 and 3.02 folds as compared to control in leaves and roots respectively (Fig. 2).

Detoxification of NaCl induced oxidative stress by non enzymatic antioxidants

To neutralize the ROS toxicity in response to a varying concentration of salinity stress, plant has the ability to stimulate their antioxidant defense system. Proline content in seedlings of *E. sativa* on exposure to NaCl increase significantly in all the three tissues viz leaves, shoots and roots with increasing concentration of NaCl (Fig. 3). A noticeable increase in proline content was recorded at 75 mM NaCl treatments which were 7.62, 2.58 and 1.28

Fig. 3 Proline content in seedlings of *E. sativa*. Seedlings were treated with 10, 25, 50, 75, 100, 125, 150 mM NaCl for a period of 4 days. Values are mean $\pm$ SE of three replicates ($n = 3$) and are statistically significant according to DMRT test ($p < 0.05$). Data points marked with the same letters show insignificant differences ($p < 0.05$) within treatments

folds higher in comparison to control in leaves, shoots and roots respectively.

Activity of ROS quenching enzymes (CAT, APX, GPX) under salinity stress

Changes in the activity of antioxidant enzymes were observed in the seedlings of *E. sativa* treated with sodium chloride for a period of 96 h. Activity of catalase increased significantly with increasing concentration of NaCl up to 25 mM in leaves and shoots. Catalase activity considerably decreases with further increase in salt concentration (50 mM) which is approximately equal to that of control. After 50 mM NaCl treatment no significant change in the CAT activity were observed in both the tissues (leaves and shoots). However in the roots, CAT activity was found to increase with increase concentration of NaCl till 50 mM and then decreases with elevated concentration (Fig. 4). While the effect of NaCl on GPX activity was mainly recorded in roots. In roots GPX activity increases

Fig. 4 **a** Catalase activity, **b** Ascorbate peroxidase activity, **c** activity of Guaiacol peroxidase, **d** Heme oxygenase 1 activity in seedlings of *E. sativa*. Seedlings were treated with 10–150 mM NaCl for a period of 4 days. Values are mean $\pm$ SE of three replicates ($n = 3$) and are

statistically significant according to DMRT test ($p < 0.05$). Data points marked with the same letters show insignificant differences ($p < 0.05$) within treatments

progressively at initial salt concentration till 25 mM and then decreases with further increase in concentration (up to 50 mM). At 25 mM NaCl treatment GPX activity was 1.38 times higher in comparison to control. No significant increase in GPX activity was recorded at higher salt concentration after 50 mM (Fig. 4).

Consequences of NaCl treatment on APX activity was observed in all the three tissues (leaves, shoots and roots) of *E. sativa* seedlings. At preliminary NaCl concentration till 25 mM no change in the APX activity was observed but on further increase in concentration APX activity progressively increase up to 75 mM which were 283.54%, 284.09% and 565.88% with respect to control in leaves, shoots and roots. After 75 mM no significant change in APX activity was observed in all the three tissues (Fig. 4).

HO 1 activity in response to NaCl stress

Role of HO 1 in alleviation of NaCl induced oxidative stress in seedlings of *E. sativa* was evaluated at varying salt concentration. HO 1 activity decreases in leaves and roots with increasing concentration of NaCl from control to 50 mM and increases significantly thereafter. The maximum activity of HO 1 was recorded at 75 mM which were 2.49 and 2.02 folds higher than control in leaves and roots respectively (Fig. 4). Following that point HO 1 activity gradually decreases and further increases at higher concentration. No significant effect of NaCl on HO 1 activity was observed in shoots.

Transcription levels of gene encoding *EsHO 1*

Expression of HO 1 mRNA was analyzed in *Eruca* tissues (leaves and roots) in response to 75 mM NaCl treatment (as maximum HO 1 activity was observed at this concentration). Semi-quantitative RT-PCR reveals that the highest induction of HO 1 gene was observed in roots of 75 mM NaCl. However the level of actin which is used as an internal standardization was unaffected throughout the experiment (Fig. 5). The results demonstrated that NaCl treated *E. sativa* seedlings over expressed the HO 1 gene

Fig. 5 HO 1 mRNA expression analyzed by semi-quantitative RT-PCR in *E. sativa* seedlings at control (CL leaves, CR roots) and 75 mM NaCl (75L: leaves, 75R: Roots). Confirmation of equal loading of cDNA and RT efficiency from actin bands

transcript than that of control and the highest expression was observed in roots than leaves (Fig. 5).

Discussion

For improved agronomic productivity and quality of *E. sativa*, an important oil yielding crop of Indian Thar Desert their tolerance towards salinity stress is a subject of concern. Salt stress has emerged as a serious problem that negatively affects yield, distribution and survival of the crop (Wang et al. 2017). One of the serious outcomes of salinity stress is the overproduction of ROS which has been reported in different plant parts of many plants (Barbieri et al. 2011; Shan and Liu 2017; Liu et al. 2017) but the equilibrium between generation and degradation of reactive oxygen species is essential. Thus the current work is primarily focused on the physiological relevance of antioxidants including HO 1 in hydroponically grown seedlings of *E. sativa*. The research is imperative, as it is helpful in understanding the tolerance level of NaCl as well as several metabolic adaptation process that exist in crop plant which will be of great importance in producing salt tolerant varieties via in vitro approaches in near future.

Plants when primarily exposed to salinity experiences water stress resulting in reduction of expansion of leaves, hindering stomatal closure, cell division and cell expansion (Munns 2002; Demiral and Turkan 2005). Excess sodium mainly NaCl has the ability to change metabolic enzymes activity which result in cell enlargement, lessen energy production and physiological variation. Thus all the chief processes like protein synthesis, photosynthesis, energy and lipid metabolism were affected (Parida and Das 2005). *E. sativa* predominantly responds to NaCl stress by affecting its growth related to fresh weight, dry weight root length and tolerance index. In the present investigation seedlings of crop plant treated with different concentrations of NaCl (10, 25, 50, 75, 100, 125, 150 mM) have an adverse effect on overall growth (Table 1). This might be due to decline in photosynthetic rate by partial stomatal closure, reduction in water uptake and inhibition of food reserves hydrolysis from storage tissue which results in the interruption in nutrients translocation to the emergent axis (Eyidogan and Oz 2007; Khan and Panda 2008). Leaf water content is an effective indicator for evaluating plant tolerance towards salinity stress as leaf water reduction frequently occurs under saline condition. Leaf water content progressively decreases with increasing concentration of sodium chloride which might be due to the turgor pressure failure that results in scarce accessibility of water for cell expansion processes (Katerji et al. 1997). Tolerance index quantify the inhibitory effect of NaCl on the physiology of plant. The progressive increase in tolerance index

at 75 mM concentration signifies the greater tolerance of crop towards NaCl at this concentration which is probably due to the increase activity of antioxidants including HO 1 at 75 mM (Verma et al. 2015; Huang et al. 2018).

Peroxidation of lipid is an indicator of membrane destruction under salinity stress (Katsuhara et al. 2005). The level of MDA content, one of the chief TBA active metabolites, increased with increasing NaCl concentration up to 150 mM. The higher MDA content at 50 mM NaCl in *E. sativa* seedlings specified higher destruction to membrane in comparison to 75 mM salt concentration (Fig. 2). Salt stress induces ion leakage, enhances reactive oxygen species in plants, which results in the elimination of hydrogen from unsaturated fatty acid, leading to the development of different reactive aldehydes and lipid radicals which is the basis of lipid bilayer and proteins destruction of the membrane (Xu et al. 2011). Roots illustrate improved quenching of reactive oxygen species and defense towards membrane destruction than leaves, which specifies greater level of antioxidant enzymes in roots (Abogadallah 2010).

Proline accretion in the current investigation has been found to be comparable as previously described by Shankar et al. (2016) in chickpea exposed to NaCl. Accumulation of proline has been found to increase with increasing concentration of NaCl (Fig. 3). The enhancement of resistance towards oxidative stress was likely to correlate with ROS detoxifying ability of proline (Tripathi et al. 2013; Mahawar et al. 2018b).

In the current study the ROS level (H_2O_2) produced during salinity stress increases with increase in NaCl concentration up to 50 mM but a decline was noticed at 75 mM NaCl in leaves and roots. Though, H_2O_2 content further increase at salt treatment above 75 mM in leaves and roots (Fig. 2). Diminution in H_2O_2 level may be due to scavenging of ROS through HO 1 and APX, because their activity were maximum at 75 mM NaCl in both the tissues (leaves and roots) (Fig. 4). Similar results for APX was obtained in chickpea, in which the APX activity increases with decrease in H_2O_2 content (Eyidogan and Oz 2007). Zilli et al. (2009) conducted a histochemical study of H_2O_2 in *Glycine max* leaves under salinity stress and reported a fall in H_2O_2 level at 100 mM, though other antioxidant enzymes activity including HO 1 was highest at this concentration. The decrease in the activity of APX and HO 1 at elevated salt treatment might be due to inactivation of enzymes by hydrogen peroxide, formed in different cell organelles and from various enzymatic and non enzymatic routes in cells (Luna et al. 1994).

Excluding ascorbate peroxidase, other H_2O_2 quenching enzymes are catalase and guaiacol peroxidase. In the recent study, activity of catalase initially increases and then decreases continuously on increasing NaCl concentration

from 75 to 150 mM (Fig. 4). While no noticeable effect of salinity stress was observed on GPX activity. The decrease in CAT activity may be associated with the destruction caused by peroxisomal proteases or might be because of photo inactivation of enzymes (Sandalio et al. 2001). Consequently catalase and ascorbate peroxidase represent disparate level of reactions since both enzymes are working on the similar substrate, hydrogen peroxide that shows greater activity with APX. Hence, increased activity might elucidate the detoxification of H_2O_2 chiefly take place through APX. Lower accessibility of substrate might reduce catalase activity. The reduction in the catalase activity has been remunerated by increasing ascorbate peroxidase activity, which has been used to diminish H_2O_2 to H_2O .

Previous reports demonstrated that HO 1 plays an imperative role in antioxidant protection towards various abiotic stresses (Balestrasse et al. 2005; Yannarelli et al. 2006; Verma et al. 2015; Santa-Cruz et al. 2017; Mahawar et al. 2018a). Zilli et al. (2009) verified HO 1 stimulation under salinity stress in *Glycine max* leaves. Roots explained the highest stimulation of HO 1 activity amongst studied *E. sativa* parts as roots are the primary tissue that comes in contact with the salinity stress through hydroponic medium or it might be due to translocation of Heme oxygenase 1 from its source (chloroplast) to the roots due to elevated levels of reactive oxygen species. At 150 mM NaCl, HO 1 activity decreases than 75 mM NaCl (Fig. 4). Similar decrease in HO 1 activity at higher NaCl concentration was also reported (Zilli et al. 2009). At 75 mM NaCl highest HO 1 activity along with other antioxidants (APX, proline) was found in different plant parts which show a linear correlation of HO 1 with APX and proline (Fig. 6).

The semi-quantitative RT PCR study demonstrates the higher expression of *EsHO 1* in treated crops than that of control which confirmed the antioxidant role of HO 1 in response to salinity stress. However at higher NaCl treatment (150 mM) the drop in HO 1 activity might be due to the enhanced ROS production which results in decrease in HO 1 transcript level or an increase in mRNA degradation or both (Fig. 5). On the basis of former outcomes, HO 1 might work within an assembly of antioxidant enzymes that construct the defense machinery for the plant's existence in which some antioxidants positively correlate with HO 1 while other shows negative co-relation with HO 1 (Fig. 6).

Conclusion

From the current study it may be concluded that NaCl treatment at initial concentrations inhibits the growth of *E. sativa* seedlings by disrupting various physiological and

Fig. 6 Linear correlation among the growth parameters, stress parameters (a) and the activity of various enzymes and non enzymatic molecules (b) with Heme oxygenase 1. The mean data (n = 3) of the

biochemical processes. However 75 mM NaCl concentration favors the growth of the crop plant. At this concentration the antioxidant enzymes activity including HO 1 was highest which results in declining H₂O₂ level. Hence the total biomass increases at this concentration. Recent findings specify an elementary plant response through antioxidant enzymes (APX, CAT and GPX) that is prevalent to the majority of stress response pathways and an exact response of HO 1 that was formerly unexplored in *E. sativa*. HO 1 plays a significant role in series of events responsible for salt tolerance by modulating *Eruca* antioxidants stimulation or reduction (Fig. 6). HO 1 activation shows that Heme oxygenase 1 has an important function in the defense system towards salinity stress. The study may enhance our understanding towards the complexity of the defense network, including HO 1 mediated improvement of salinity stress in *E. sativa* which will be helpful in developing salt-tolerant varieties in the near future. Additionally the contemporary research will be valuable as the information on HO 1 utility has been overlooked in *E. sativa*, a salt tolerant crop.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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measured values in stress crop plants were used for correlation analysis. The correlation values are significant at 0.01 level

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