Full Length Research Paper

Role of Heme Oxygenase1 in Alteration of Antioxidant Defense Responses of *Brassica juncea* (L.) Czern. under Salt Stress Condition

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Abstract. Economically important crops are frequently facing many stresses, such as salt stress. They have developed an antioxidant defense network for preventing and repairing oxidative damage caused by salt stress. In this study, *Brassica juncea* seedlings were subjected to salt stress (NaCl) for time course analysis (1st-5th day) of heme oxygenase1 (HO1) activity along with other antioxidant enzymes. Treatment of salt induced lipid peroxidation and H$_2$O$_2$ content in root tissue as days of treatment increased. However, 1st and 2nd day of treatment did not modify these parameters, were similar to control values. HO1, Ascorbate peroxidase (APX), Peroxidase (POD) and Glutathione reductase (GR) activities were highest at 5th day of treatment. Gene expression pattern of HO1 was similar as shown in biochemical analysis. These results showed that HO1 along with other antioxidant enzymes play an essential protective role against salt stress in *B. juncea* seedlings.

Keywords: Antioxidant enzymes, *Brassica juncea*, heme oxygenase1, Salt stress, Time course analysis

1. INTRODUCTION

Abiotic stress is a global challenging issue at the present time for sustainable agriculture. Salt stress is major abiotic stresses concerned with restraining plant growth and yield. Salt stress has negatively affected nearly 20% agricultural land (Flowers and Yeo, 1995). It negatively affects many processes of plants specifically seed germination, development of seedlings, vegetative growth, flowering and fruit growth as well as product quality (Khan et al., 2010). Only, 10% area of agricultural field is in non-stress environment (Epstein, 1980). Salt stress affects the plants in two ways. Primarily, it generates osmotic stress by reducing the soil-water potential which further causes limited water uptake. Secondly, it causes excessive uptake of ions specially Na$^+$ and Cl$^-$ which further generates an imbalance of ions. These effects ultimately interfere with various metabolic processes of plants (Abogadallah, 2010).

In order to cope with adverse effects of abiotic stress, antioxidant network exists in plant system. In this system, enzymes such as Ascorbate peroxidase (APX), Peroxidase (POD) and Glutathione Reductase (GR) and nonenzymatic molecules such as Thiols, Proline and carotenoids has been confirmed to be accountable for the protection against toxic reactive oxygen species (ROS) (Salin, 1988). Recently, one more enzyme is introduced in this network; heme oxygenase (HO, EC 1.14.99.3) which is involved in the plants defense mechanism. HO catalyzes the oxidation of heme to biliverdin IXα (BV), CO and Fe$^{2+}$ in mammals, biliverdin IXα is further reduced to bilirubin (BR) through biliverdin reductase. Both BV and BR have strong antioxidant properties in plant and animal system, respectively (Noriega et al., 2003, 2004; Balestrasse et al., 2008; Shekhawat and Verma, 2010; Shekhawat et al., 2011). In the case of plants, recent reports have suggested the antioxidant defensive role of heme oxygenase (HO1). In recent past, an increase in BV due to HO1 induction in soybean plants under cadmium and salt stress (Balestrasse et al., 2005, 2008; Zilli et al., 2009) was thought to be an antioxidant response to oxidative stress. Earlier studies also indicated that HO substantially contributes to alleviate the effect of salt stress (Zilli et al., 2009; Xie et al., 2008 and 2011).

Keeping all the earlier reports in observation, in the present study, the function of HO in *Brassica juncea* was studied for its antioxidant potential against salt induced oxidative stress at a biochemical and molecular level in order to understand the mechanism lying behind antioxidant role of HO.
2. MATERIALS AND METHODS

2.1. Cultivation of the crop plant, salt treatment and sample harvesting

*Brassica juncea* seeds were surface sterilized with 0.5% sodium hypochlorite for 10 minutes to avoid fungal contamination and washed thoroughly with distilled water. These seeds were then germinated in a petri dish containing filter paper soaked in distilled water at 30°C under dark conditions. After 3 days the uniformly germinated seedlings were selected and transferred to plastic pots enclosing half strength Hoagland’s nutrient solution. These pots were transferred to a thermostatically controlled culture room maintained at 25±2°C temperature, 500µmol m⁻² s⁻¹ and 16 hour photoperiod. The nutrient solution was bubbled daily and changed on alternate days.

Fifteen days old acclimatized seedlings were used for further experimental work. Salt solution (150 mM) was added to the nutrient solution for different treatment days (1st-5th days). A control with half strength Hoagland’s nutrient solution without salt was used.

2.2. Physiological parameter: root elongation rate

Physiological parameters were calculated in terms of root elongation rate.

Root length was measured before and after salt treatment. The following formula was used for measurement of root elongation rate:

\[
\text{Root elongation rate (cm day}^{-1}\text{)} = \frac{\text{Mean final longest RL} - \text{Mean initial longest RL}}{\text{t}_2 - \text{t}_1}
\]

(RL: root length; t₁: last day of treatment; t₂: initial day of treatment)

2.3. Estimation of lipid peroxidation and H₂O₂ content

Lipid peroxidation was determined in root tissue of NaCl treated seedlings after 1st-5th day by the estimation of malonaldehyde (MDA) according to De Vos et al. (1989). Tissues were extracted in 10 mL of 0.25 % (w/v) TBA made in 10 % (w/v) Trichloroacetic acid (TCA). The mixture was incubated at 95 °C for 30 minutes and then cooled quickly on ice bath. The resulting content was centrifuged at 10,000 x g for 15 minutes. The absorbance of the supernatant was recorded at 532 and 600 nm. The non- specific absorbance at 600 nm was subtracted from the absorbance at 532 nm. The concentration of MDA content was estimated using the extinction coefficient of 155 mM⁻¹ cm⁻¹.

H₂O₂ production in plant roots was determined spectrophotometrically as described by Jana and Choudhuri (1981). H₂O₂ was extracted by homogenizing plant material in 3 mL of 50 mM phosphate buffer (pH 6.8). Homogenate was centrifuged at 6000g for 25 minutes. To determine H₂O₂ levels, 3 mL of the above extracted supernatant was mixed with 1 mL of 0.1 % titanium chloride (in 20 % [v/v] H₂SO₄) and the reaction mixture was centrifuged at 6000 x g for 15 minutes. The intensity of yellow color developed was measured at 410 nm. H₂O₂ was calculated by using the extinction coefficient of 0.28 µmol⁻¹ cm⁻¹.

2.4. Biochemical assay for checking activity of antioxidant enzymes

2.4.1. Extraction of antioxidant enzymes

For preparation of extracts to determine APX, POD and GR activities, root tissue was homogenized under ice cold condition in 3ml of extraction buffer. This mixture was centrifuged; Resulting supernatant was further used for the assay of different antioxidant enzymes.

2.4.2. Enzyme assays

The activity of APX is the rate of H₂O₂-dependent oxidation of ascorbic acid. Its activity was measured in a reaction combination that enclosed 50 mM phosphate buffer (pH-7.0), 0.6 mM ascorbic acid and enzyme extract (Chen and Asada, 1989). The reaction was started by the addition of 10 µL of 10% H₂O₂ and the oxidation rate of ascorbic acid was estimated by following the decrease in absorbance 290 nm for 3min (extinction coefficient 2.8 mM⁻¹ cm⁻¹). The enzyme activity was expressed as unit mg⁻¹ of protein.

The GR activity was assayed by following the methods of Smith et al. (1988).The reaction mixture contained 0.1 M phosphate buffer, pH 7.5, 0.5 mM EDTA, 0.75 mM 5,5'-dithiobis (2-nitrobenzoic acid), 0.1 mM NADPH and 1 mM GSSG. The components were added in the order listed above directly to a cuvette and the reaction was started by the addition of GSSG. Increase in absorbance was observed for 5 minutes at 412 nm (extinction coefficient 6.2 mM⁻¹ cm⁻¹). The enzyme activity was expressed as unit mg⁻¹ of protein.

POD assay was carried out in the reaction mixture enclosed enzyme extract, 50 mM potassium phosphate buffer (pH 7.0), 3.4 mM guaiacol and 0.9 mM H₂O₂ (Putter,1974). POD activity was determined by monitoring the formation of tetra guaiacol from initial substrate guaiacol in the presence of H₂O₂ at 436 nm (extinction coefficient, 6.39 mM⁻¹ cm⁻¹).
Table 1: Changes in root elongation rate in Brassica juncea at different days of NaCl concentration (1st-5th day)

<table>
<thead>
<tr>
<th>Days of Salt treatment (at 150mM)</th>
<th>Root elongation rate (cm/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.53 ± 0.15 *b,c,d,e,f</td>
</tr>
<tr>
<td>1st day</td>
<td>1.46 ± 0.05 *a,c,d,e</td>
</tr>
<tr>
<td>2nd day</td>
<td>1.45 ± 0.17 *a,b,c,d</td>
</tr>
<tr>
<td>3rd day</td>
<td>1.16 ± 0.15 *a,b,e,d</td>
</tr>
<tr>
<td>4th day</td>
<td>0.60 ± 0.1 *a,b,c,d,e</td>
</tr>
<tr>
<td>5th day</td>
<td>0.56 ± 0.15 *a,b,c,d,e</td>
</tr>
</tbody>
</table>

Data presented are mean ± S.D. (n = 3) and *Significant mean difference among all groups at P <0.05 according to tukey test. a- represent control, b- represent 1st day , c- represent 2nd day , d- represent 3rd day , e- represent 4th day and f- represent 5th day.

Fig. 1: Shows decrement in root elongation rate in Brassica juncea treated with NaCl for different days of treatment (1st - 5th day). Data presented are mean ± S.D. (n = 3) and *Significant mean difference among all groups at P <0.05 according to tukey test. (a- represent control, b- represent 1st day , c- represent 2nd day , d- represent 3rd day , e- represent 4th day and f- represent 5th day)

2.5. Analysis of heme oxygenase through biochemical assay

Heme oxygenase activity was determined by method of Balestrasse et al. (2005). Homogenization of root tissue was done in sucrose solution containing phenylmethyl sulphonyl fluoride, EDTA and potassium phosphate buffer. Homogenized solution was centrifuged and used for activity determination. The assays (1 ml final volume unless otherwise indicated) contained, HO (0.5 mg protein), hemin, bovine serum albumin, spinach ferredoxin, spinach ferredoxin-NADP+ reductase. Adding NADPH to a final concentration of 100µl started the reaction, incubation of all the samples at 37°C during 30 min was done and BV formation was calculated by measuring the absorbance change at 650 nm (extinction coefficient: 6.25 mM⁻¹ cm⁻¹).

2.6. Transcriptional level analysis of HO1 in Brassica juncea

Semi quantitative RT PCR was performed to check the transcript level of HO1 in the root part of Brassica juncea to analyze on which day of treatment, it showed maximum response. We isolated total RNA by using Trizol reagent, then gave treatment with RNase-free DNase I (Promega). Then isolated RNA was reverse transcribed into cDNA using random hexamers and Superscript II, RT PCR reactions was
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carried out using root parts of *B. juncea* (L.) Czern.

The reverse-transcribed material was amplified by use of a specific gene primer pair through PCR. Primers used for practical were as follows: for *B. juncea* HO1 (accession number JX275832.1), forward (5'-ATGGCTTACTCAGCTCCCATATCTCCATCCCT-3') and reverse (5'-TTAAGCTTTGAAGCAAATAAGAG-3'), amplifying an 811-bp fragment; for actin (accession number AF111812), forward (5'-CTGGAATGGTGAAGGCTGGGTT-3') and reverse (5'-CGGAGGATAGCGTGAGGAAGAG-3'), amplifying a 293 bp. Each primer set was amplified using an optimized number of PCR cycles to ensure the linearity requirement for semi-quantitative RT-PCR analysis. Amplified transcripts were scanned on Ethidium bromide stained gels, and the level of HO1 mRNA in root tissue on different days of treatment was analyzed to check the expression level.

2.7. Statistical analysis

Data were recorded for three independent experiments and represented as mean ± SE. Data was analyzed for one-way analysis of variance (ANOVA) (SPSS version 16). Significance of difference (P<0.05) was detected using Tukey’s multiple range tests.

![Fig. 2: Shows (a) MDA content level (b) H$_2$O$_2$ content of *Brassica juncea* treated with NaCl for different days of treatment (1$^{st}$-5$^{th}$ day). Data presented are mean ± S.D. (n = 3) and *Significant mean difference among all groups at P <0.05 according to tukey test. (a- represent control, b- represent 1$^{st}$ day , c- represent 2$^{nd}$ day , d- represent 3$^{rd}$ day , e- represent 4$^{th}$ day and f- represent 5$^{th}$ day)]](image)

3. RESULTS AND DISCUSSIONS

3.1. Root elongation rate

Root elongation rate of *B. juncea* decreased as days of treatment increases (Figure 1, Table1). The root elongation rate of 5$^{th}$ day treated seedlings declined significantly (p<0.05) as compared to control, 1$^{st}$, 2$^{nd}$, 3$^{rd}$ and 4$^{th}$ day treated seedlings. 3$^{rd}$ day treated seedling showed significant (p<0.05) alteration in comparison to control, 1$^{st}$, 2$^{nd}$, 4$^{th}$ and 5$^{th}$ day treated seedlings. Similarly, Eyidogan and Oz (2007) also reported that stress duration has also a negative effect on the growth in chickpea seedlings.

Salinity negatively affects plant's ability to absorb water, which causes diminutive growth with metabolic changes alike with the water stress (Munns, 2002; Bybordi et al., 2010c). High osmotic potential and ion toxicity of Na$^+$ and Cl$^-$ ions limit water and nutrient supply for plant roots (Al-Karaki, 1997; Bybordi et al., 2010a). This study revealed that increase in NaCl concentration and its period negative influences on the growth of *B. juncea* seedlings. Similarly, Eyidogan and Oz (2007) also reported that stress duration has also a negative effect on the growth in chickpea seedlings.
Fig. 3: Shows (a) changes in activity of APX (b) changes in activity of GR (c) changes in POD activity in *Brassica juncea* treated with NaCl for different days of treatment (1\textsuperscript{st} - 5\textsuperscript{th} day). Data presented are mean ± S.D. (n = 3) and *Significant mean difference among all groups at P <0.05 according to tukey test. (a- represent control, b- represent 1\textsuperscript{st} day, c- represent 2\textsuperscript{nd} day, d- represent 3\textsuperscript{rd} day, e- represent 4\textsuperscript{th} day and f- represent 5\textsuperscript{th} day)

### 3.2. MDA content

MDA content increased in *B. juncea* seedlings as days of treatment increased (Figure2a). 4\textsuperscript{th} day treated seedling showed significant (p<0.05) increment as compared to control, 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd} treated seedlings. MDA content of 5\textsuperscript{th} day treated seedlings increased significantly (p<0.05) as compared control, 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} day treated seedlings on the other hand control, 1\textsuperscript{st}, 2\textsuperscript{nd} treated seedlings showed significant alteration in comparison to 4\textsuperscript{th} and 5\textsuperscript{th} day treated seedlings.

Lipid peroxidation is a first indicator of membrane breakage due to salt induced oxidative stress. It is calculated through the level of MDA content in that particular stressed tissue. This MDA content is resultant of polyunsaturated fatty acids in the membrane (Katsuhara et al., 2005). Many reports confirmed about enhancement of lipid peroxidation under salt stress (Khan and Panda, 2002, 2008; Panda and Khan, 2003). Pattern of MDA content was similarly reported in chickpea seedlings by Eyidogan and Oz (2007). They found out that MDA content in 4\textsuperscript{th} day treated seedlings at all salt concentrations was higher than 2\textsuperscript{nd} day treated seedlings.
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**3.3. H\textsubscript{2}O\textsubscript{2} content**

H\textsubscript{2}O\textsubscript{2} content increased up to 4\textsuperscript{th} day of treatment in *B. juncea* seedlings (Figure 2b). 3\textsuperscript{rd} and 4\textsuperscript{th} day treated seedlings showed significant (p<0.05) increment as compared to control, 1\textsuperscript{st} and 2\textsuperscript{nd} treated seedlings. On the other hand, the H\textsubscript{2}O\textsubscript{2} content of 5\textsuperscript{th} day treated seedlings slightly decreased as compared 4\textsuperscript{th} day treated seedlings.

Significant increment (p<0.05) in APX activity was recorded in 1\textsuperscript{st}, 2\textsuperscript{nd} treated seedlings in comparison to control on the other hand; they showed significant alteration in comparison to 4\textsuperscript{th} and 5\textsuperscript{th} day treated seedlings (Figure 3a). 3\textsuperscript{rd} and 4\textsuperscript{th} day treated seedlings showed significant (p<0.05) increment as compared to control, 1\textsuperscript{st} and 2\textsuperscript{nd} treated seedlings. APX activity of 5\textsuperscript{th} day treated seedlings increased significantly (p<0.05) as compared to control, 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} day treated seedlings.

Reduction in level of H\textsubscript{2}O\textsubscript{2} at 5\textsuperscript{th} day treatment may sourced by scavenging through APX because APX activity was recorded highest in at this day of treatment. Same pattern of result was reported by Eyidogan and Oz (2007) in which APX activity increased as decrement in H\textsubscript{2}O\textsubscript{2} content was occurring in chickpea.

**3.4. APX activity**

Significant increment (p<0.05) in APX activity was recorded in 1\textsuperscript{st}, 2\textsuperscript{nd} treated seedlings in comparison to control on the other hand; they showed significant alteration in comparison to 4\textsuperscript{th} and 5\textsuperscript{th} day treated seedlings (Figure 3a). 3\textsuperscript{rd} and 4\textsuperscript{th} day treated seedlings showed significant (p<0.05) increment as compared to control, 1\textsuperscript{st} and 2\textsuperscript{nd} treated seedlings. APX activity of 5\textsuperscript{th} day treated seedlings increased significantly (p<0.05) as compared to control, 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} day treated seedlings.

The result of this study showed that as days of treatment increased, activity of APX was also increased. This result indicates that APX played important role in scavenging of H\textsubscript{2}O\textsubscript{2}. Some reports confirmed active participation of APX, and its over expression under salt stress (Savoure et al., 1999; Kawasaki et al., 2001). In some reports, APX activity was high at tolerant pea cultivars (Hernandez et al., 1999), tomato (Rodriguez-Rosales et al., 1999), mulberry (Sudhakar et al., 2001) under salt stress which confirm its role in salt tolerance mechanism.

**3.5. GR and POD activity**

GR activity of 1\textsuperscript{st}, 2\textsuperscript{nd} treated seedlings increased significantly (p<0.05) as compared to control where as it showed significant alteration in comparison to 3\textsuperscript{rd}, 4\textsuperscript{th} and 5\textsuperscript{th} day treated seedlings. 3\textsuperscript{rd} and 4\textsuperscript{th} day treated seedlings showed significant (p<0.05) increment in GR activity as compared to control, 1\textsuperscript{st} and 2\textsuperscript{nd} treated seedlings. GR activity of 5\textsuperscript{th} day treated seedlings highly increased significantly (p<0.05) as compared to control, 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} day treated seedlings (Figure 3b).
Same pattern of result was recorded in case of POD activity. POD activity of 1st, 2nd treated seedlings significantly (p<0.05) increased as compared to control. 3rd and 4th day treated seedlings showed significant (p<0.05) increment in POD activity as compared to control. 1st and 2nd treated seedlings. POD activity of 5th day treated seedlings highly increased significantly (p<0.05) as compared to control. 1st, 2nd, 3rd and 4th day treated seedlings (Figure 3c).

As known, GR convert oxidized glutathione (GSSG) into reduced glutathione (GSH) and keeping a high GSH/GSSG ratio. POD enzyme plays actively for scavenging of H₂O₂ produced during dismutation of O₂ catalyzed by SOD. The activity of both enzymes was recorded highest in root samples at 5th day of treatment. According to some reports GR activity increased in mulberry (Sudhakar et al., 2001), rice (Lin and Kao, 2000) and soybean (Comba et al., 1997) under salt stress conditions. Hossain et al. (2004) found out increased GR activity in the roots of Chrysanthemum morifolium under increased NaCl concentration.

Earlier studies have already mentioned about increased POD activity caused by salinity (Gosset et al., 1996; Sudhakar et al., 2001; Lin and Kao, 2002). Abogadallah (2010) reported in his review that POD, APX and GR have magnificent tight control upon H₂O₂ concentration.

**3.6. HO activity**

As shown in figure 4 (a), HO activity was highly increased (p<0.05) in 5th day treated seedlings as compared to control, 1st, 2nd, 3rd and 4th day treated seedlings. 3rd and 4th day treated seedlings showed significant (p<0.05) increment in HO activity as compared to control, 1st and 2nd treated seedlings. The expression of HO1 mRNA of control, 1st, 2nd, 3rd, 4th and 5th day of salt treatment (NaCl) observed. Semi quantitative RT PCR results show that HO1 gene was highly induced in root at 5th day of salt treatment (Figure 4b).

Many studies reported that HO1 actively participate as an antioxidant in antioxidant defense mechanism against different abiotic (Balestrasse et al., 2005, 2008; Noriega et al., 2004; Yannarelli et al., 2006). Zilli et al. (2009) also reported that HO1 has induced under salt stress in soybean leaves. So, HO1 play actively against salt induced oxidative stress too. The Time course analysis has not been done in case of HO1 yet. As Eyidogan and Oz (2007) reported in their report that antioxidant enzymes played more actively in chickpea seedlings at 4th day treatment than 2nd day of treatment.

**4. CONCLUSION**

This study indicates that more days of treatments cause more induction in antioxidant enzymes of plant system and make plant more tolerant against salt stress. In this study, antioxidant enzymes as well as HO1 highly induced at 5th day of treatment. Through biochemical and molecular analysis, it can be concluded that HO1 play actively under salt stress conditions. More days of treatment may be induces more enhancements of antioxidant enzymes. Further researches are required for more explanation about this.

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