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Full Length Research Paper

Some biochemical properties of guaiacol peroxidases as modified by salt stress in leaves of salt-tolerant and salt-sensitive safflower (*Carthamus tinctorius L.cv.*) cultivars

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The kinetics properties of guaiacol peroxidase (GP) and its isozymic pattern, and lipid peroxidation product were comparatively analyzed in two varieties of safflower (*cv. M-CC-190* as salt-tolerant and *cv. IL-111* as salt-sensitive cultivars) under normal and different concentrations of NaCl. The pH profile of GP activity in leaves extract of two cultivars in control and salt stressed plants showed different pattern of pH dependency with three maxima peaks at pH 4.5, 6.5 and 8 in salt-tolerant cultivar and two maxima peaks at pH 4.5 and 6.5 in salt-sensitive cultivar. Comparison of catalytic efficiency for GP between two cultivars at respective pH, showed that, salt-tolerant cultivar in both control and salt stressed condition had higher catalytic efficiency than salt-susceptible cultivar. The GP activity on the gels revealed four and two isoforms of peroxidases in salt-tolerant and salt-sensitive cultivars, respectively. GPs increased their expression with higher levels of salinity. However, in salt-sensitive cultivar GPs expression exhibited threshold behavior, with increase expressions in isoenzymes up to a certain level of salinity (25 mM NaCl), followed by decrease to a level of expressions corresponding to the control groups. The levels of lipid peroxidation as indicated by thiobarbituric acid reactive substances (TBARS) were higher in the sensitive variety than the tolerant under control and NaCl salinity. The overall results obtained in this study suggest that, oxidative stress may play an important role in salt-stressed safflower plants and that the greater protection of *M-CC-190* leaves from salt-induced oxidative damage results, at least in part, through the increase of the GPs activity, catalytic efficiency and induction of specific isoenzymes (P1 and P4).

Key words: Safflower, guaiacol peroxidase, kinetics, isoenzymes, salt stress.

INTRODUCTION

Soil salinity is one of the limiting factors for crops grown in arid and semi arid regions. Today, 20% of the world cultivated land and approximately half of all irrigated areas are affected by salinity (Zhu, 2001). The reduction in growth and yield in most crops in saline environments is known to cause an imbalance of the cellular ions resulting in hyper ionic and hyper osmotic stress in plants, leading to production of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals and metabolic toxicity (Ashraf and Harris 2004; Jaleel et al., 2007). A sudden and dramatic increase in cellular ROS production disrupt normal metabolism through oxidative damage to lipid (Fridovich, 1986; Wise and Naylor, 1987), protein (Davie, 1987; Halliwell and Harvey, 1995) and nucleic acid (Fridovich, 1986; Imlay and Linn, 1988). Scavenging of ROS in plant cells occurs by endogenous protective mechanisms

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Abbreviations: ROS, Reactive oxygen species; **GP,** guaiacol peroxidase; **PAGE,** polyacrylamide gel electrophoresis.

involving antioxidant molecules and enzymes (Jaleel et al., 2008; Turkan and Demiral, 2009).

Among the antioxidant enzymes, peroxidases (EC 1.11.1.7) are essential components of cellular detoxification system that regulated intracellular level of H_2O_2. Peroxidases belong to a large family of enzymes that are found in fungi, higher plants and vertebrates. These proteins usually contain a ferriprotoporphyrin IX prosthetic group and a monomeric, glycosylated protein, with molecular weights between 28 and 60 kDa (Hiraga et al., 2001). In higher plants, the number of isoenzymes may be extremely high, up to 40 genes corresponding to isoperoixdases for each plant and several other isoforms can be generated by posttranscriptional and post-translational modifications (De Marco et al., 1995; Welinder et al., 1996). Guaiacol peroxidase (GP) is an important group from peroxidase, which oxidize guaiacol (o-methoxyphenol) as a commonly used reducing substrate. They are found in cellular cytoplasm and appoplasm fractions and involved in range of processes related to plant growth and development. In spite of the fact that peroxidases are among the most studied enzymes in plants and are thought to participate in many physiological processes, their role in the physiology and biochemistry of plants has not being fully elucidated. In this study, we investigated the effects of salt stress on the GP activity and its isozymic pattern and lipid peroxidation in leaves of two cultivars of safflower differing in salt tolerance (*cv. M-CC-190* as salt-tolerant and *cv. IL-111* as salt-sensitive), in order to understand the role of peroxidases in conferring stress resistance in two cultivars. Kinetics characteristics, as well as sensitivity to inhibitors and thermostability of the isoenzymes were investigated and compared in two cultivars.

MATERIALS AND METHODS

Chemicals

Chemicals for electrophoresis and protein assay reagent were purchased from Sigma Chem. Co. All the other chemicals used in this work were obtained from Merck and were of reagent grade.

Plants culture and treatments

Safflower (*Carthamus tinctorius L. cv. M-CC-190* and *cv. IL-111*) seeds were provided by Seed and Plant Improvement Institute (SPII), Karaj, Iran. The plots were conducted in a growth chamber with a temperature regime of 25/18°C day/night, 14/10 h light/dark period. Surface sterilized seeds were germinated in the dark on perlite, moistened with distilled water and $CaSO_4$ at 0.05 mM. Salinity treatment started for 15 days old plants, consisting of five levels of NaCl at 0 (control), 12.5, 25, 50 and 100 mM. Composition of the nutrient solutions (pH 6.8) were used according to Hoagland modified nutrient solution (Gamborg and Wetter, 1975) for 15 days.

Extract preparation

For the preparation of the crude extract, 1 g of safflower leaves was homogenized with 3 ml of 0.1 M phosphate buffer, pH 7.2 containing 0.2% polyvinylpyrrolidone (for stabilizing the extract), in a waring blender for 3 min. The homogenate rapidly filtered through a layer of cheesecloth and centrifuged at 20000 g for 20 min at 4°C. A clear transparent supernatant termed "crude extract" was obtained and used for our studies.

Protein determination

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard.

Enzyme assays

GP activity was measured by following the H_2O_2 dependent oxidation of guaiacol at 470 nm, using an extinction coefficient of 26.6 mM^{-1} cm^{-1}. Assays were carried out at room temperature (~22 and 25°C), with an UNICO UV-2100 PC spectrophotometer. 100 µl extract were added to 0.1 M citrate-phosphate-borate buffer at given pH, containing guaiacol in final concentration of 1 to 30 mM (for K_m, V_{max} determinations) or 15 mM (for all other assays); the reaction mixture total volume was 3 ml. All reactions were started by addition of 3 mM H_2O_2 (final concentration) to the reaction mixture. One unit of GP activity was defined as the amount of enzyme that caused the formation of 1 µM of tetraguaiacol per minute.

The pH dependence of the enzyme activity was determined using 0.1 M citrate-phosphate-borate buffer system ranging from pH 3 to pH 9. Effect of inhibitors including sodium azide and sodium cyanide on GP activity was investigated in concentration ranges of 1 to 1000 µM. The concentration of inhibitor that causes 50% loss of enzyme activity was defined as IC_{50}.

Thermal stability assay

Thermal stability of safflower GP were studied by incubating aliquots of extract at temperature of 60°C for 40 min in thermostatic water bath and measuring their activity at room temperature after brief cooling in ice. The incubation was carried out in sealed vials to prevent change of volume of the sample and the enzyme concentration due to evaporation. The activity that was measured at 25°C was taken as 100% and activities which were measured at 60°C were compared with the activity measured at 25°C.

Lipid peroxide determination

Lipid peroxidation was determined by measuring the amount of MDA produced by the thiobarbituric acid reaction as described by Heath and Packer (1968). Aliquots of leaves were homogenized in 20% trichloroacetic acid (TCA) containing 0.67% thiobarbituric acid and incubated at 100°C in water bath for 60 min. Then, the mixture was quickly cooled in an ice bath and centrifuged at 10000 ×g for 20 min. The absorbance of supernatant was measured at 532 nm. Thiobarbituric acid reactive substances (TBARS) levels were calculated using 155 mM^{-1} cm^{-1} as extinction coefficient (Heath and Packer, 1968; Fu and Huang, 2001).

Gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed in 10% separating and 5% stacking gels according to Laemmli (1970) method, but omitting sodium dodecyl sulfate (SDS) and mercaptoethanol. 30 µl samples containing 30 µg protein were

subjected to electrophoresis at room temperature at 80 V for 18 h. Peroxidase bands were detected on the gel by submerging the gel in a staining solution containing 50 mM sodium citrate buffer, pH 4.5, 3 mM H_2O_2 and 0.6 mg/ml o-dianisidine.

RESULTS

The pH dependency of GP activity

The effect of pH on GP activity in leaves extract of salt-tolerant (*cv. M-CC-190*) and salt-sensitive (*cv. IL-111*) cultivars of safflower grown under control or salt stress (12.5, 25, 50 and 100 mM NaCl) conditions were investigated. In the two cultivars, in both control and salt stress conditions, peroxidase activity were found in pH ranging from 3.5 to 8.5 (Figure 1a and b). As shown in Figure 1, pH profile curves demonstrated a different pH dependency for GP activity between two cultivars. The pH profile in salt-tolerant cultivar (*cv. M-CC-190*) shows three optima peaks at pH 4.5, 6.5 and 8, respectively, but in salt-sensitive cultivar (*cv. IL-111*) only two optima peaks at pH 4.5 and 6.5 were observed in curve and peak at pH 8 was absent. The GP activity in leaves extract of salt stressed plants in two cultivars show the same peaks as well as in their related control plants (data not shown). As suggested by Fullbrook (1996), the presence of various pH optima for one enzymatic activity indicates the presence of distinctive isoenzymes.

Kinetic parameters of GP

In the two cultivars at respective pH (three optima pH at 4.5, 6.5 and 8 in salt-tolerant cultivar and two optima pH at 4.5 and 6.5 in salt-sensitive cultivar) and at saturating concentration of hydrogen peroxide, steady-state kinetics parameters were determined for GPs isoform in control and salt stressed plants (Table 1 and 2). As shown in Table 1, in control plants of salt-tolerant cultivar, apparent K_m, V_{max} and catalytic efficiency (V_{max}/K_m calculated per mg extract protein) of GP were different for all three pHs examined. The highest catalytic efficiency value and lowest apparent Michaelis-Menten constant (K_m) value were found for isoenzymes active at pH 6.5 and 4.5, respectively. Salt stress had no significant effect on catalytic efficiency of two isoenzymes with optima pH at 4.5 and 8, but causes a reduction in catalytic efficiency of isoenzyme active at pH 6.5 compared to the control group. The order of catalytic efficiency values for control and salt stressed plant at three pH optima was pH 6.5 > pH 8 > 4.5.

In control leaves of the salt-sensitive cultivar at respective pH (4.5 and 6.5), apparent K_m and V_{max} of GP were different, but the catalytic efficiency values were very close in two pH optima (0.04 and 0.041 at pH 4.5 and 6.5, respectively, in Table 2). In salt-stressed leaves of the salt-sensitive cultivar, at optima pH 4.5 and 6.5, the highest catalytic efficiency value was found for 25 mM NaCl treated plant. At this concentration of NaCl, Increase in catalytic efficiency was 1.25 fold for pH 4.5 and 2 fold for pH 6.5 in comparison to the control groups, respectively.

Effect of inhibitors on GP activity

Cyanide and azide are two typical peroxidase inhibitors (Mika and Luthje, 2003). The sensitivity of GP isoform to these inhibitors was studied in leave extract of safflower in controls and salt treated plants of two cultivars (Figure 2). In control groups of the salt-tolerant cultivar, at three pH optima (4.5, 6.5 and 8), azide was more efficient than cyanide in reducing GP activity of the extract samples. Order of sensitivity of GP activity to azide at three pH optima was pH 8 > pH 4.5 > pH 6.5, with IC_{50} values (the concentration of inhibitor that causes 50% loss of enzyme activity) of 24.5, 46.9 and 64.4 μM, respectively. The same order of sensitivity was found for cyanide at three pH optima with IC_{50} of 82.5, 113.3 and 183.6 μM, respectively. NaCl at different concentration (12.5, 25, 50 and 100 mM NaCl) had no significant effect on sensitivity of isoenzymes of GP active at three pH optima to the cyanide and azide.

Similar to salt-tolerant cultivar, in control groups of the salt-sensitive cultivar, at respective pH (4.5 and 6.5), azide was more efficient than cyanide in reducing GP activity of the extract samples. In contrast to the salt-tolerant cultivar, order of sensitivity of GP activity to azide and cyanide at two pH optima was pH 6.5 > pH 4.5, with IC_{50} values of 12.7 μM, 43 μM for azide and 27.3 μM, 105.5 μM for cyanide, respectively. GP activity at pH 4.5 in control groups of salt-tolerant and salt-sensitive cultivars showed the same sensitivity to azide with IC_{50} values of 46.9 μM and 43 μM, respectively. Similarly, cyanide had an undiscriminating effect on GP activity at pH 4.5 in control groups of salt-tolerant and salt-sensitive cultivars. Fifty percent loss of activity was achieved with 113.3 and 105.5 μM of cyanide, respectively.

The sensitivity of GP activity in salt-stressed leaves of the salt-sensitive cultivar to azide and cyanide at pH 4.5 and 6.5 was altered by increasing NaCl concentration to 100 mM. By increasing NaCl concentration, IC_{50} values for both azide and cyanide was increased in comparison to the control groups.

Effect of temperature on GP activity

Thermal stability of GP isoenzymes in the salt-tolerant and salt-sensitive cultivars of safflower were studied by incubating aliquots of extract at temperature of 60°C for 40 min in thermostatic water bath and measuring their activity at room temperature after brief cooling in ice. In salt-tolerant cultivar when the extract was incubated at

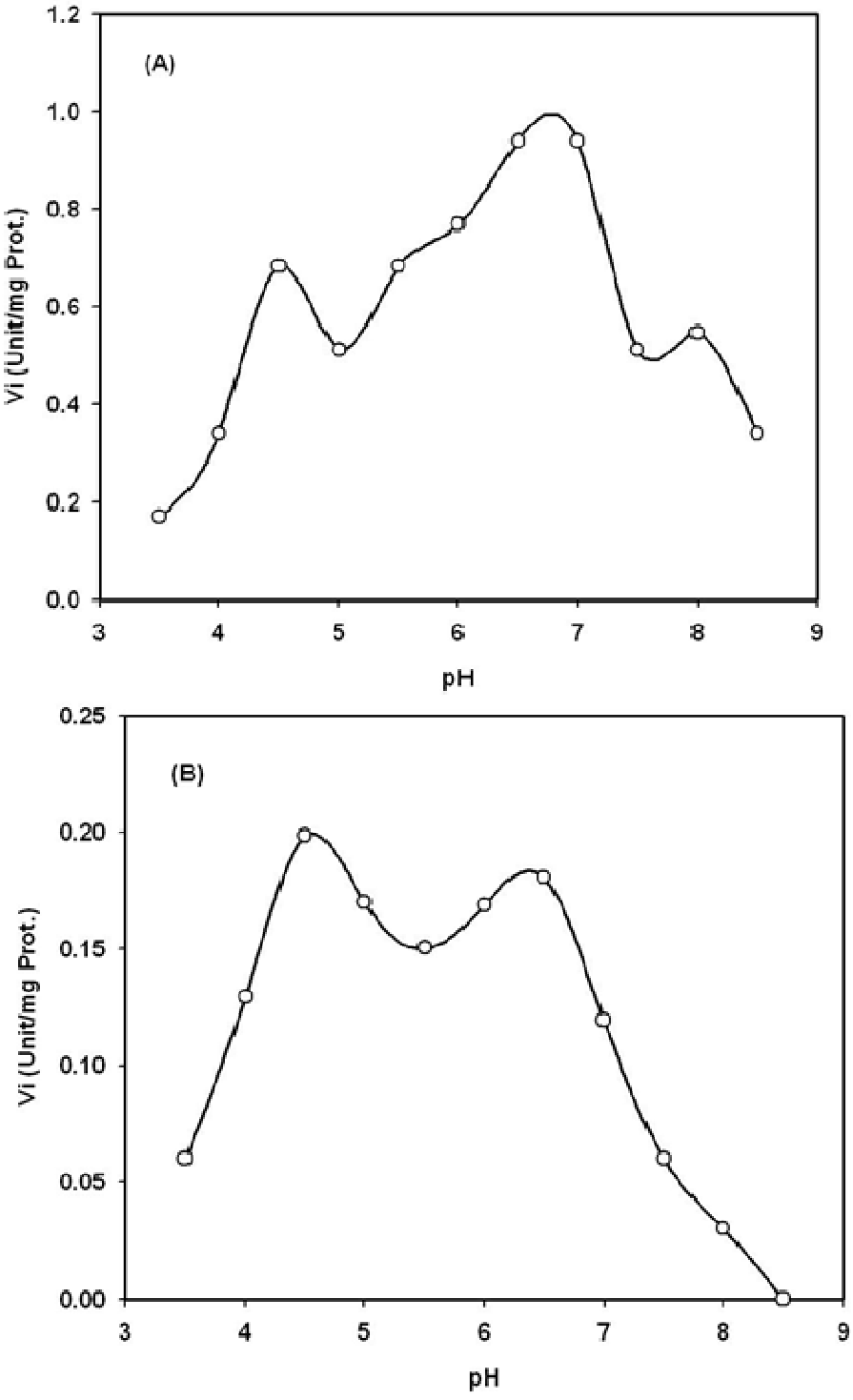

Figure 1. pH dependency of GP activity in leaves extract of: (A) Salt-tolerant (*cv. M-CC-190*); (B) salt-sensitive (*cv. IL-111*) cultivars of safflower grown under control conditions. Guaiacol oxidation was determined in 0.1 M citrate-phosphate-borate buffer system in the presence of 15 mM guaiacol and 3 mM H_2O_2.

60°C for 40 min, GP activity lost about 75, 50 and 40% of the original activity at pH 4.5, 6.5 and 8, respectively. Therefore, the order of thermostability of three GP iso-enzymes from salt-tolerant cultivar extract at three pH optima was pH 8 > pH 6.5 > pH 4.5. In salt-sensitive cultivar after incubation of the extract at 60°C for 40 min,

Table 1. Kinetics parameters of GP activity in leaves extract of salt-tolerant (*cv. M-CC-190*) cultivar of safflower in response to different concentration of NaCl at three pH optima.

NaCl (mM)	pH optima								
	4.5			6.5			8		
	K_m (mM)	V_{max} (U/ml)	V_{max}/ K_m*	K_m (mM)	V_{max} (U/ml)	V_{max}/ K_m*	K_m (mM)	V_{max} (U/ml)	V_{max}/ K_m*
0	3.0	0.3	0.1	7.0	2.7	0.38	23.7	3.5	0.15
12.5	10.3	1	0.1	8.3	2.3	0.28	8	0.88	0.11
25	26.6	2.4	0.09	9.7	2.5	0.26	7.8	1.1	0.14
50	36.0	4.0	0.11	10.0	2.0	0.20	11.9	1.9	0.16
100	38.2	3.8	0.1	20.0	3.6	0.18	6.2	1.0	0.16

Calculated per mg extract protein..

Table 2. Kinetics parameters of GP activity in leaves extract of salt-susceptible (*cv. IL-111*) cultivar of safflower in response to different concentration of NaCl at two pH optima.

NaCl (mM)	pH optima					
	4.5			6.5		
	K_m (mM)	V_{max} (U/mL)	V_{max}/ K_m*	K_m (mM)	V_{max} (U/mL)	V_{max}/ K_m*
0	3	0.12	0.04	4.9	0.2	0.041
12.5	14	0. 1	0.01	7.1	0.3	0.042
25	8.6	0.43	0.05	5.3	0.41	0.08
50	6.1	0.24	0.04	12.2	0.4	0.03
100	22.8	0.64	0.03	20.1	0.6	0.03

Calculated per mg extract protein..

GP activity lost about 75 and 80% of the original activity at pH 4.5 and 6.5, respectively. Order of thermostability of these isoenzymes in salt-sensitive cultivar at two pH optima was pH 4.5 > pH 6.5.

Analysis of the gauaiacol peroxidase activity on non-denaturing polyacrylamide gel electrophoresis

GP isoforms were detected in the leave extracts of both control and NaCl treated safflower of both cultivars by using 10% native PAGE (Figure 3). Since the same amount of soluble proteins from each preparation was loaded on gel, the intensity of isoform bands reflects the induction patterns of individual peroxidase isoforms, in both cultivars.

The GP activity on the gels revealed four isoforms of peroxidase designated P1, P2, P3 and P4 in salt-tolerant cultivar (*cv. M-CC-190*) and two isoperoxidases (P2 and P3) in salt-sensitive (*cv. IL-111*) cultivar, respectively. In the control group of salt-tolerant cultivar, zymogram revealed four GP isozymes in leaves, two were faint (P1 and P4) and the other two were prominent (P2 and P3, (Figure 3). The activity of these isoperoxidases increased under NaCl stress and was intensified specially at 100 mM NaCl concentration (Figure 4).

As shown in Figure 4a, the activity of isoperoxidase representation of P4 (expressed by pixel intensity), drastically increased at 12.5 mM NaCl concentration followed by little change in expression up to 100 mM salt concentration. Meanwhile, the intensities of the two bands, P2 and P3, were increased by salt at 12.5 mM, followed by little change in expression up to 50 mM, thereafter a significant increase in activities were observed at 100 mM NaCl concentration. While that of band P1, pixel intensity increased in a roughly linear fashion from 0 mM NaCl up to 100 mM NaCl concentration.

In salt susceptible cultivar (*cv. IL-111*), GP activity on the gels revealed only two isoforms of peroxidase (P2 and P3) and bands P1 and P4 were absent (Figure 3). However, the activity of isoperoxidases in 12.5 mM NaCl was very close to the detection limit (no band was observed). In the control group, 50 and 100 mM salt treated plants, two bands P2 and P3 were very faint, but in 25 mM salt treated plant, these bands were prominent. As shown in Figure 4b, P2 and P3 exhibited threshold behavior, with increase expressions up to a certain level of salinity (25 mM NaCl), followed by decrease to a level

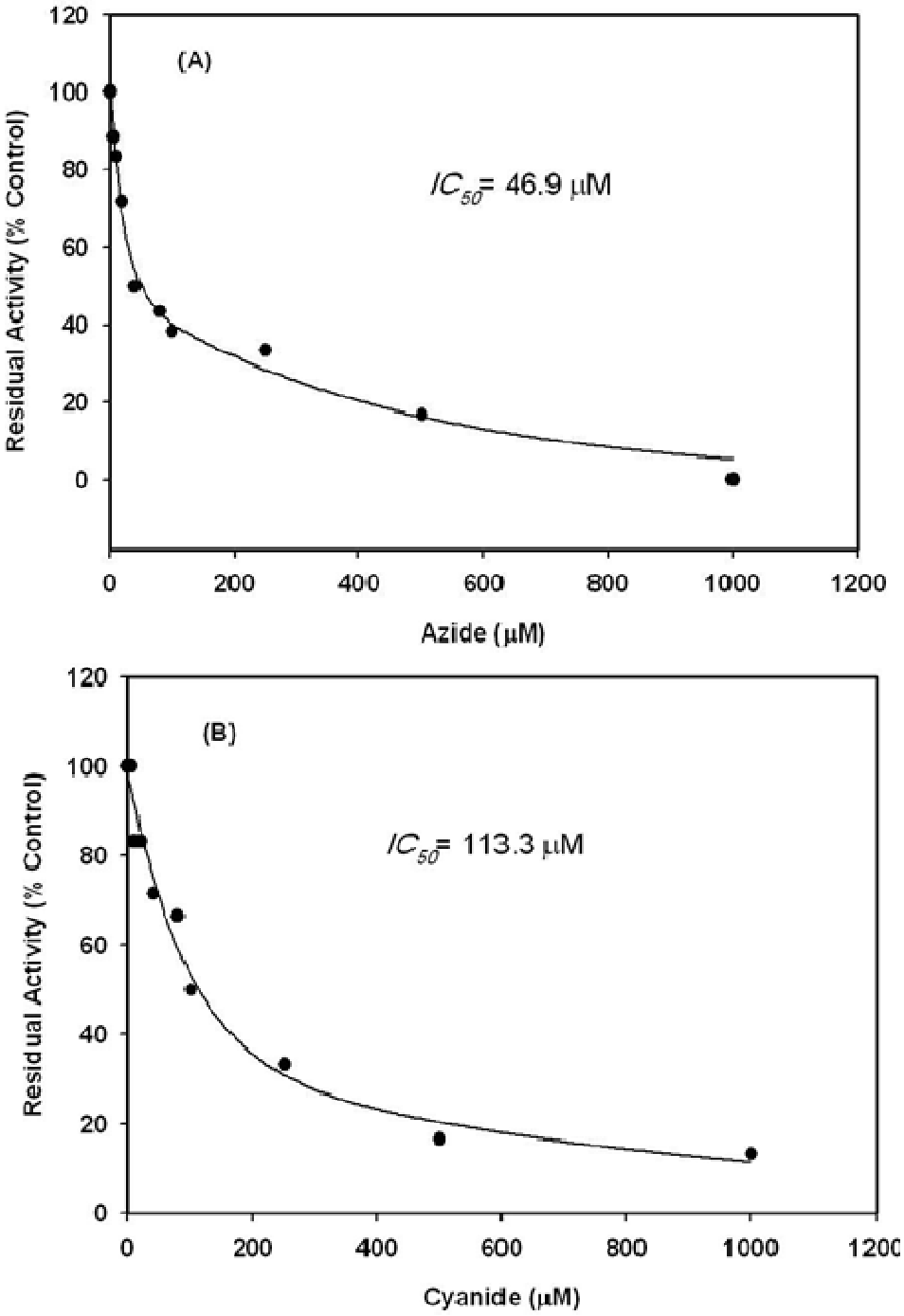

Figure 2. Inhibition by azide (A); cyanide (B), against GP at pH 4.5 in salt-tolerant cultivar (*cv. M-CC-190*) of safflower; inhibition by azide (C); cyanide (D), against GP at pH 4.5 in salt-susceptible cultivar (cv. IL-111) of safflower.

of expressions corresponding to the control groups.

Lipid peroxidation

Salt stress (12.5, 25, 50 and 100 mM NaCl) caused a significant increase in the levels of thiobarbituric acid reactive (TBARS) content in both the cultivars and the degrees of elevation in the TBARS were salt concentration dependent. However, the degree of accumulation was more in the salt susceptible than in the salt tolerant cultivar indicating a high rate of lipid peroxidation in the

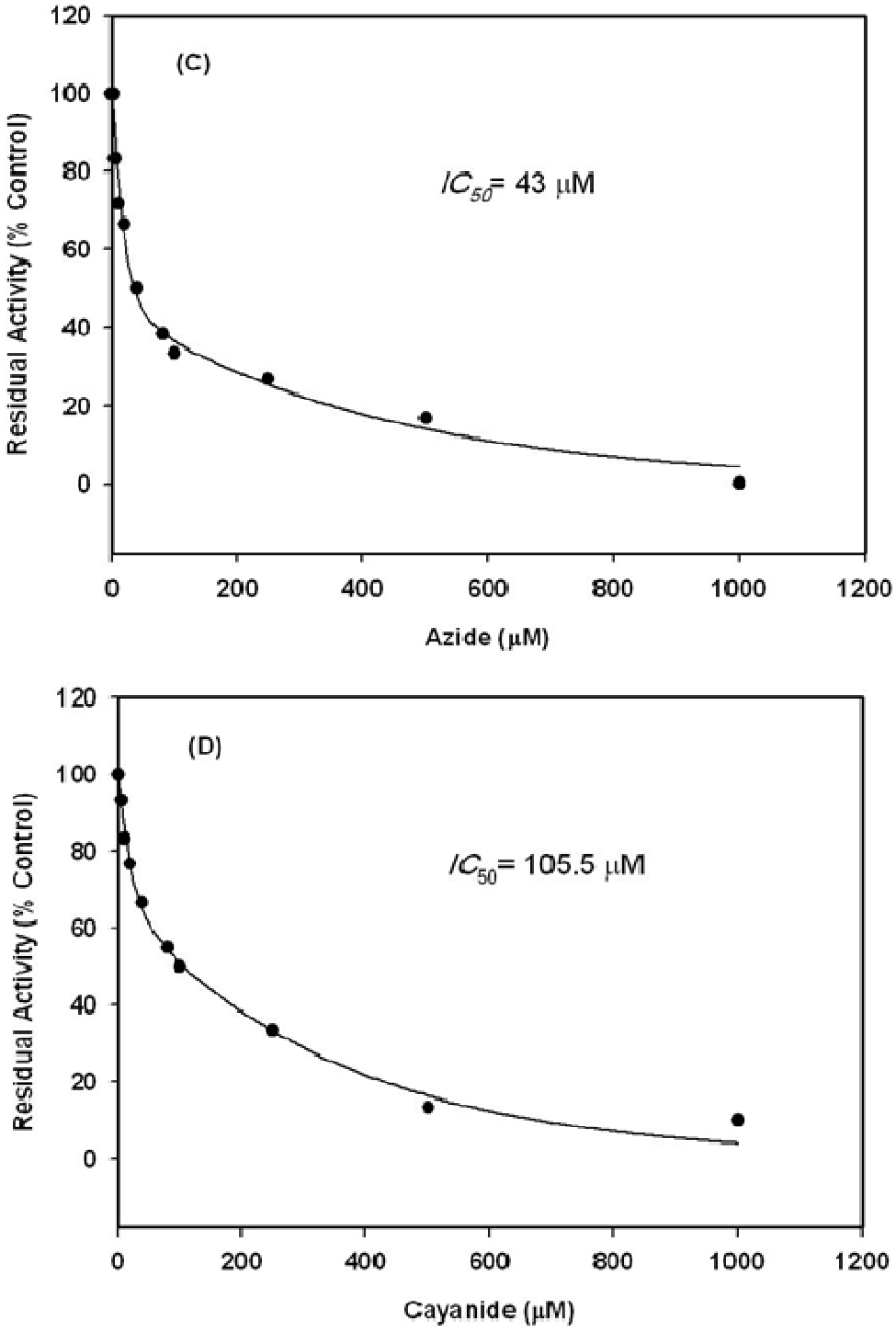

Figure 2. Contd.

susceptible cultivar due to salt stress (Figure 5).

DISCUSSION

Peroxidases (EC 1.11.1.7) are the most studied enzymes in plants and the fact that they have been found in all major divisions of plants denotes the functional importance of these proteins. Numerous biochemical and physiological functions have been attributed to plant isoperoxidases. These include growth, cell formation, fruit development, ethylene biosynthesis, suberization and

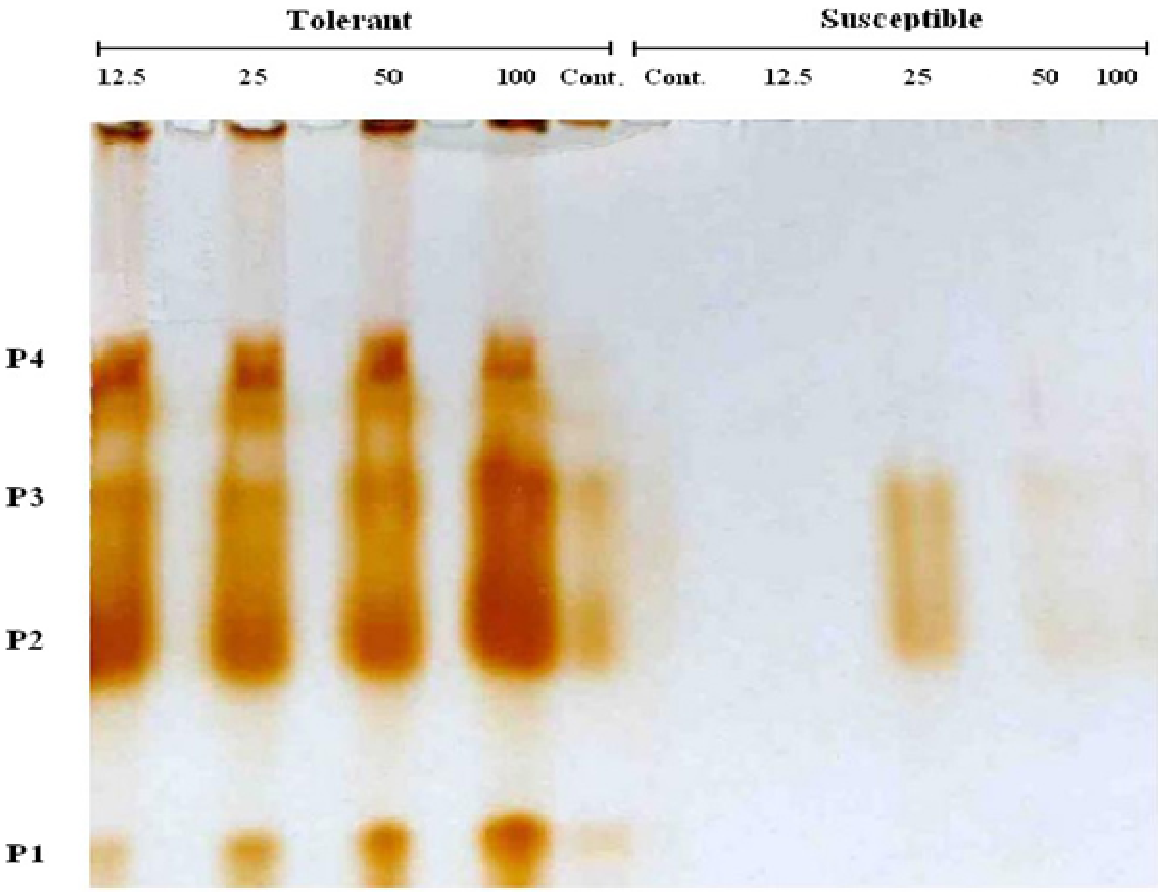

Figure 3. Activity stain of GP on non-denaturing gel of salt-tolerant (*cv. M-CC-190*) and salt-susceptible (*cv. IL-111*) cultivars of safflower grown under control and NaCl (12.5, 25, 50 and 100 mM) conditions. P1, P2, P3 and P4 presented different GP isoform.

lignification, defense against pathogens, cross linking of cell wall proteins, H_2O_2 detoxification, stress response, senescence and salt tolerance (Yamasaki et al., 1997; De Marco et al., 1999; Bernards et al. 1999; Bolwell et al., 2002; Matamoros et al., 2003; Liszkay et al., 2004; Sasaki et al., 2004; Ben Amor et al. 2005).

Safflower (*C. tinctorius L.cv*) is a member of the family compositae that is cultivated mainly for its seed, which is used as edible oil and bird seed. Traditionally, the crop was grown for its flowers which have some applications in medicine, coloring and flavoring food (Mass, 1986; Elias et al., 2002; Siddiqi et al., 2009). In this study, some biochemical properties and changes in isoform of guaiacol peroxidases and lipid peroxidation product under normal and salt stress conditions in leaves of two cultivar of safflower with differences in salt tolerance (*cv. M-CC-190* as salt-tolerant and *cv. IL-111* as salt-sensitive), were investigated.

Kinetics studies for identification of various types of GP in two cultivars at control and salt stressed plants were based on the following criteria as suggested by Schulz (1994): (1) Variation in activity as a function of pH; (2) variation in activity as a function of substrate concentration (K_m and V_{max}); (3) effect of inhibitors on activity (differential effects of azide and cyanide); (4) effect of temperature on activity.

The pH profile of GP activity in leaves extract of two cultivars in control and salt stressed plants showed different pattern of pH dependency with three maximum peaks at pH 4.5, 6.5 and 8 in salt-tolerant cultivar and two maximum peaks at pH 4.5 and 6.5 in salt-sensitive cultivar (Figure 1a and b). It has been suggested by Fullbrook (1996) and De Marco (1999) that, variation in pH can change the relative contribution of different isoperoxidases to total activity and this is manifested with the emergence of different pH optima in pH activity curves. When the leaves extracts were subjected to non-denaturating PAGE, followed by activity staining for GP, the presence of four isoenzymes of GP designated by P1, P2, P3 and P4 in salt-tolerant cultivar (*cv. M-CC-190*) and two isoenzyme of GP denoted by P2 and P3 in salt-sensitive cultivar (*cv. IL-111*) were confirmed (Figure 3). The presence of 4 bands in the gel and existence of 3 optima peaks in pH profile curve of salt-tolerant cultivar (Figure 1a) suggested that, probably one isoenzyme of GP in the leaves of salt-tolerant cultivar has an optimum

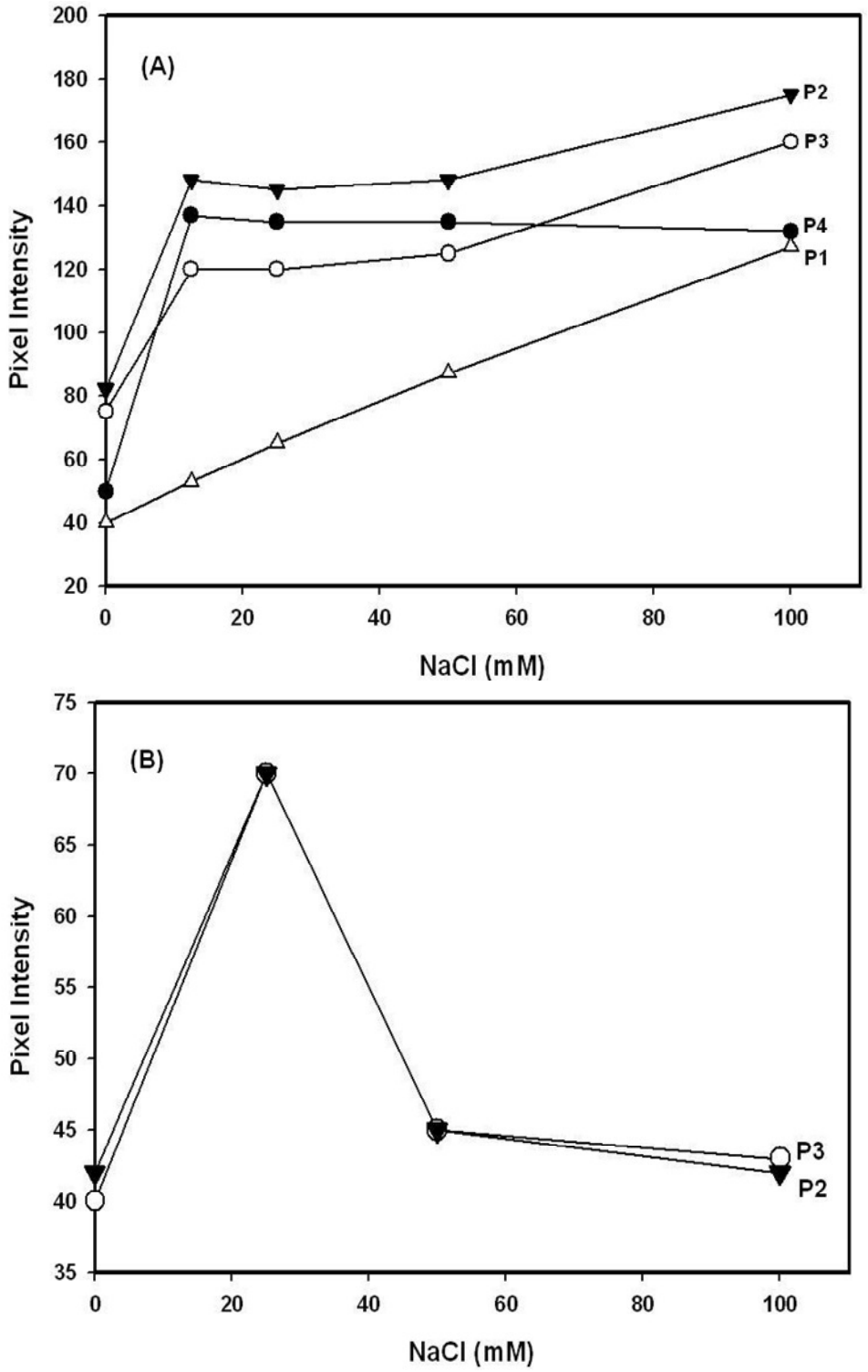

Figure 4. Changes in expression (pixel intensity) of GP isoform (P1, P2, P3 and P4) in leaves of (A) salt-tolerant (*cv. M-CC-190*); (B) salt-susceptible (*cv. IL-111*) cultivars of safflower due to increasing levels of salinity. Analyses were performed by totalLab (TL120, v2009) software.

pH value very close to one of the other isoenzymes that is present in the leave. In salt-sensitive cultivar for both control and salt treated plants, only two bands on the gel and two optima peak (pH 4.5 and 6.5) were observed in the pH profile curve (Figures 1 and 3). These results suggest that, in contrast to salt-tolerant cultivar which has

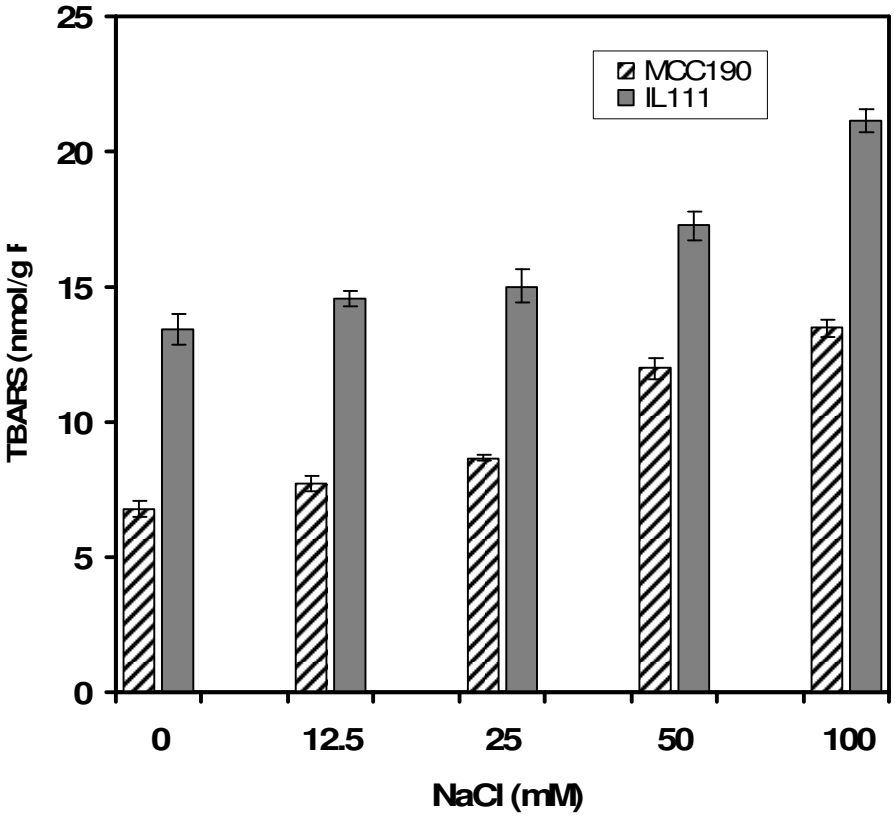

Figure 5. Lipid peroxide content (nmol/g FW) in leaves extract of salt-tolerant (*cv. M-CC-190*) and salt-susceptible (*cv. IL-111*) cultivars of safflower in response to different concentration of NaCl.

4 isoforms of GP, only two soluble of GP are active in salt-sensitive cultivar during growth under control and salt stress conditions. The peroxidase system of higher plants exists in multiple isoforms that are developmentally regulated and highly reactive in response to exogenous stimuli (Gaspar et al., 1982; Mohan et al. 1993; Bakardjieva et al. 1996; Klotz et al. 1998; Passardi et al. 2004).

When kinetics parameters were measured at three pH optima in control plants of salt-tolerant cultivar (*cv. M-CC-190*), apparent K_m, V_{max} and catalytic efficiency (V_{max}/K_m, calculated per mg extract protein) of GP were different (Table 1). Highest catalytic efficiency values were found for isoenzyme with pH optima at 6.5, thereafter for pH 8 and 4.5, respectively. The same order of catalytic efficiency was found in salt stressed plant at three pH optima. In addition, the effect of two typical peroxidase inhibitors namely, azide (NaN_3) and cyanide (NaCN) on GP activity in three pH optima (pH 4.5, 6.5 and 8) show that, isoenzymes have very different sensitivity to azide and cyanide. The order of sensitivity to both azide and cyanide was pH 8 > pH 4.5 > pH 6.5. Therewith, after heating the extract at 60°C for 40 min, GP activity lost about 75, 50 and 40% of the original activity at pH 4.5, 6.5 and 8, respectively. In salt-sensitive cultivar, order of stability to temperature was pH 4.5 > pH 6.5. It has been reported that, isoenzymes are usually different in their sensitivities to temperatures (Keyhani et al., 2002; Bardales et al., 2004; Tayefi-Nasrabadi, 2008). The results demonstrated that, GP isoenzymes in the leaves extracts of two cultivars have distinctive kinetics properties. Their distinctive kinetics properties proposed possible different metabolic roles for each isoenzyme in the leaves of plant (De Marco et al., 1999; Tayefi-Nasrabadi, 2008).

Incubation of the extract at 60°C for 40 min caused equal reduction (75% of the original activity) in GP activity at pH 4.5 in control groups of salt-tolerant and salt-sensitive cultivars. This result suggested that, isoenzyme with optimum pH at 4.5 in two cultivars have equal thermostability. Moreover, GP activity at pH 4.5 in control groups of salt-tolerant and salt-sensitive cultivars showed the same sensitivity to azide with IC_{50} values of 46.9 and 43 µM and to cyanide with IC_{50} of 113.3 and 105.5 µM, respectively. Also, the K_m value for GP of two extract samples at pH 4.5 was the same. These results strongly confirm that, both isoenzymes with optima pH at 4.5 in two cultivars are identical. In contrast, isoenzymes active at pH 6.5 in two cultivars are different, since they have different thermostability, sensitivity to azide and cyanide and k_m value for guaiacol. All GP isoenzyme in this work,

showed K_m values for guaiacol in a millimolar range.

Similar results were reported for tomato GP, garlic GP, corn root plasma membrane GP and soluble peroxidases from kiwi fruit, with K_m values of 10, 9.5, 12.2 and 7.4 mM, respectively (Soda et al., 1991; Nair and Showalter 1996; Loukili et al. 1999; Mika and Luthje, 2003; Marzouki et al., 2005).

Comparison of catalytic efficiency for GP between two cultivars at respective pH (4.5 and 6.5), show that salt-tolerant cultivar (*cv. M-CC-190*) in both control and salt stressed condition have higher catalytic efficiency than salt-susceptible cultivar (*cv. IL-111*). Further, a significant elevation in the expression (Pixel intensity) of GP isoenzymes in the leaves of two cultivars of safflower due to increasing levels of salinity was observed. Furthermore, the degree of elevation in expression (pixel intensity) was relatively high in the salt tolerant cultivar (*cv. M-CC-190*) when compared with the susceptible one (*cv. IL-111*) (Figure 4). Similar to these results, it has been reported that, elevated antioxidant levels could be associated with salt tolerance of plants (Gossett et al., 1994; Hernandez et al., 1995; Wang et al., 1997). In tolerant plant species, peroxidase activity was found to be higher, to enable the plants protect themselves against oxidative stress (Scalet et al., 1995; Muscolo et al., 2003; De Azevedo et al., 2006), where as such high activity was not observed in sensitive plants (Sreenivasulu et al., 1999; Sreenivasulu et al., 2000; Jbir et al., 2001). High levels of salinity create stress for the plant cells that disrupts electron transport systems releasing large amounts of reactive oxygen species that can cause significant damage to nearby cells (Yoshimura et al., 2000; Mittler, 2002; Tuteja, 2007; Tanou et al., 2009). Peroxidases play important roles in eliminating these harmful reactive oxygen species, by catalyzing reactions where a phenolic substrate is oxidized while the active oxygen species are reduced to a much less harmful form. Besides their role in removal of reactive oxygen species, peroxidases are involved in lignification by catalyzing the oxidation of cinnamyl alcohols, which is the final catalytic step for the creation of lignin (Quiroga et al., 2000). Plants increase lignin synthesis during NaCl stress to prevent water loss through the cell walls (Garcia et al., 1997). Therefore, increased activity and catalytic efficiency of GP isoenzyme in two cultivars after salinity treatment can promote or increase lignification processes caused by salinity, thereby preventing water loss through the cell walls. In two cultivars, guaiacol peroxidases increased their expression with higher levels of salinity, as has been reported in many other investigations (Sreenivasulu et al., 1999; Sudhakar et al. 2001). However, in salt-sensitive cultivar (*cv. IL-111*) unlike the salt-tolerant cultivar (*cv. M-CC-190*), peroxidases expression exhibited threshold behavior, with increase expressions in GP isoenzymes, designated by P2 and P3, up to a certain level of salinity (25 mM NaCl), followed by decrease, to a level of expressions corresponding to the control groups (Figure 4b). This type of trend indicates that, the physical environment plays a large role in P2 and P3 expression in salt-sensitive cultivar (*cv. IL-111*). The peak of expression around 25 mM NaCl must be the ionic concentration at which the GP is most stable and thus most be expressed. Beyond this level of salinity, P2 and P3 lose stability and denatures resulting in significantly decreasing concentration (Figure 4b). It seems like high ionic concentrations of salinity increase the denaturation of P2 and P3 in the same way that, peroxidases in pea plants exhibited significant conformational changes due to similar salt stresses (Weimberg, 1970). The structural changes of GP isoform in different concentration of NaCl was further evidenced by the difference in V_{max} and K_m values and different sensitivity to various inhibitors, namely azide and cyanide in salt stressed plants in comparison to the control groups (Table 2).

Salt stress is known to result in extensive lipid peroxidation, which has often been used as indicator of salt-induced oxidative damage in membranes (Hernandez and Almansa, 2002). Parallel to these observations, we found lower TBARS content in NaCl stressed tolerant cultivar (*cv. M-CC-190*) when compared with the salt-sensitive cultivar (*cv. IL-111*) (Figure 5). The lower level of lipid peroxidation in *M-CC-190* suggests therefore that, salt tolerant plants are better protected from oxidative damage under salt stress. The lower level of lipid peroxidation in salt-tolerant cultivar and greater increase in GP activity and catalytic efficiency indicates involvement of peroxidases in cell membrane integrity. Similar results correlating lipid peroxidation to GP activity were also reported by other researchers (Sreenivasulu et al. 1999; Yazici et al. 2007).

In the present study, NaCl stress caused an increase in expression of isoperoxidases, namely P1, P2, P3 and P4 in salt-tolerant (*cv. M-CC-190*) cultivar and P2 and P3 in salt-susceptible (*cv. IL-111*) cultivar of safflower, respectively. Similar results, with increase in five GP isoenzymes in salt tolerant cultivar and four isoperoxidases in salt sensitive cultivar were reported in fox-tail millet associated with resistance to NaCl and were assigned a role in cell membrane integrity and regulation of early seedling growth under salt stress conditions (Sreenivasulu et al., 1999). Therefore, expression of such isoform of GP can be expected at least in part, to have some role in stress tolerance of safflower under NaCl stress. In the present study, occurrence of a specific GP isoenzyme (P1 and P4) only in the tolerant cultivar in comparison to the salt sensitive cultivar proved this finding. Therefore, oxidative stress may play an important role in salt-stressed safflower plants and that the greater protection of *M-CC-190* leaves from salt-induced oxidative damage results, at least in part, through the increase of the GPs activity, catalytic efficiency and induction of specific isoenzymes (P1 and P4). Besides their role in removal of reactive oxygen species and lignification, the presence of

multiple isoforms of GP in safflower leave extract with distinctive kinetic properties and different sensitivity to temperature and inhibitors (azide and cyanide) suggest that, each isoform plays a different role. Further experiments should clarify the overall roles of each of isoforms in environmental stresses.

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