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Biochemical and Cellular Changes in the Root of *Lens culinaris* Grown on Crude Oil-Contaminated Soil

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ABSTRACT

Catalase (CAT) is an enzyme that decomposes hydrogen peroxide with high velocity. Under environmental stress, CAT plays an important role in the disposal of hydrogen peroxide. Spillage of crude oil into the soil can damage plants and microorganisms. Oil contamination in soil may act as a stressful element and cause damage to plants. In this experiment, the effect of crude oil-contaminated soil (5% w/w) on root of lentil (*Lens culinaris*) CAT activity and subcellular changes was studied. CAT activity was measured at different pHs and temperatures. The optimum pH was 10 and maximum activity was observed at 30°C in treated and control samples. Both K_m and V_{max} changed in treated samples. The K_m of the enzyme was 1.13 and 1.5 mM and V_{max} was 1.16 and 2 mM/min/mg protein in the treatment and control, respectively. After purification of CAT, SDS-PAGE of purified enzyme revealed a minor difference between the molecular weight of the enzyme in treated samples and the control, suggesting that a CAT isoenzyme was induced in treated samples relative to the control.

Keywords: enzyme, plant, pollution, subcellular
Abbreviations: CAT, catalase

INTRODUCTION

Lentil (*Lens culinaris*) belongs to the *Leguminosae* family and is an annual herb grown for its edible, high protein, flattened seeds (Erskine *et al*. 2009). It is one of the oldest cultivated crops and presumed to be native to southwestern Europe and temperate Asia. It is rich in protein, fiber, carbohydrates, B vitamins, magnesium, iron, and zinc (Savage 1988; Adsule *et al*. 1989). Lentil has been used in the phytoremediation of heavy metals from contaminated soil. The concentration of mercury in lentil roots was 3.5 times higher than the control (Rodriguez *et al*. 2007). Jamal *et al*. (2002) also showed that nickel (Ni) and zinc (Zn) uptake from soil by lentil was enhanced in the presence of arbuscular mycorrhizal fungi.

In oil-producing countries, the risk of soil contamination during oil extraction, transportation and refining is high. The effect of oil pollution on microorganisms and plants depends on the concentration and type of pollutant (Boethling and Alexander 1979; Minai-Tehrani 2008). Oil-contaminated soil can cause delays in germination, reduce shoot length and induce early chlorosis in plants (Minai-Tehrani *et al*. 2007; Minai-Tehrani 2008). The macroscopic effect of oil contamination on plants is well studied although microscopic damage has not been reported. Few studies have been reported on the effect of crude oil on enzyme activity in plant cells. The effect of 5.9% crude oil-contaminated soil on the activity of amylase and invertase of *Vigna unguiculata* (cow pea) seedling noted a 45 and 15% reduction in invertase and amylase activity, respectively (Anigboro and Tonukari 2008). Octane and benzene have also been shown to change the activity of glutamate dehydrogenase (GDH) and malate dehydrogenase (MDH) in *Lolium perenne* (ryegrass) and *Medicago sativa* (alfalfa). Benzene at 1-100 mM stimulated the activity of GDH and MDH by 2-3 fold in *L. perenne* while octane at 1-100 mM reduced the activity of GDH by 50 to 96% and MDH by 17 to 46% in *M. sativa* (Sadunishvili *et al*. 2009).

Most environmental stresses such as high energy radiation, drought and pathogen attack lead to an increase in the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) (Bolwell 1999; Cheeseman 2007; Mafakheri *et al*. 2011). Catalase (CAT), an enzyme that rapidly decomposes H_2O_2, plays an important role in environmental stress (Yang and Poovaiah 2002; Cheeseman 2007). When a plant encounters to different environmental stress, some ROS such as superoxide (O_2-) and hydrogen peroxide (H_2O_2) are generated. ROS can damage DNA and oxidize polyunsaturated fatty acids and amino acids in proteins. CAT degrades H_2O_2 and prevents cell damage by H_2O_2 (Cheeseman 2007; Mafakheri *et al*. 2011).

Plants, unlike animals, have multiple CAT enzyme forms (or isozymes). Among the various plant species containing multiple CAT isozymes are *Carthamus tinctorius* (Tayefi-Nasrabadi *et al*. 2011), *Picea omorika* (Bogdanović *et al*. 2007), *Nicotiana tabacum* (Havir and McHale 1989), *Pinus taeda* (Mullen and Gifford 1993), and sunflower (*Helianthus annuus*) (Eising *et al*. 1989). In maize, three CAT isozymes were discovered (CAT-1, CAT-2 and Cat-3), each expressing under different environmental conditions. CAT-2 was the dominant isozyme in maize when plants were exposed to high temperature (40°C) (Scandalios 1994). In the presence of a fungal toxin, cercosporin, CAT-3 levels increased in maize leaves (Williamson and Scandalios 1992).

In this report the effect of crude oil-contaminated soil on the root CAT activity of lentil was studied, and subcellular changes were also investigated. Lentil was chosen for this study because it belongs to the *Leguminosae*, a family that has shown strong potential for bioremediation of oil-contaminated soil. Other legumes such as *Medicago sativa* (alfalfa) was examined for light crude oil-contaminated soil

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(1-10% w/w) bioremediation (Shahriari *et al*. 2007), *Trifolium hirtum* (rose clover), *Trifolium repens* (white clover) and *Vicia villosa* (hairy vetch) have also been used for bioremediation of 2.5% petroleum-contaminated soil (Kulakow *et al*. 2000). Lentil is also an edible plant which can be a model to study other nutritional plants.

MATERIALS AND METHODS

Chemicals

All the chemicals used for buffer preparation and enzyme assay were of reagent grade and were purchased from Merck Co. (Darmstadt, Germany).

Soil preparation

Garden soil, which was used to cultivate the lentils, was dried and passed through a 4 mm mesh. Lentil (cv. 'Ziba') was provided by the Research Institute of Forests and Rangelands (R.I.F.R) of Iran. Light crude oil (American Petroleum Institute (API) gravity = 40) was obtained from the Sarkan region in the west of Iran. To obtain oil-contaminated soil, 500 g of soil and 5% (w/w) crude oil were added to a bucket. After fastening the lid of the bucket tightly, the bucket was shaken firmly by hand for approx. 10 min until homogeneity was achieved (Minai-Tehrani 2008). Ten seeds were planted in each bucket. The control was also prepared by planting 10 seeds/bucket containing soil without oil contamination. Tap water was used to moisten the samples and 3 g of animal manure was sprinkled on top of each sample as fertilizer. Plants were grown under natural light behind the windows of a laboratory. The temperature and relative humidity were about 30°C and 45%, respectively. Control group and treated samples consisted of 7 replicates each.

Harvesting the plant

At the end of the experiment (i.e., 30 days, allowing sufficient time for shoots and leaves to develop, and allowing chlorosis to be observed in contaminated soil), the plants were removed from the soil and the roots were washed with water to eliminate excess soil adhering to the roots. Roots were separated from the shoots and divided to two groups. In one group, the roots from treated and control samples were fixed in 2.5% glutaraldehyde for a subcellular study while the other group was kept at -20°C for biochemical experiments.

Thin section preparation

The roots of treated samples and the control group were cut into small pieces (0.5-0.7 mm) and fixed in 2.5% glutaraldehyde for 48 h. These samples were then washed with 0.1 M phosphate buffer (pH = 7) and then immersed in 1% osmic acid for 30 min. After washing several times with distilled water, samples were dehydrated in an ethanol gradient. The specimens were embedded in epoxy resin and sectioned by an ultramicrotome (Reichert OMU3). Uranium acetate and lead citrate were used to stain the thin sections. Five sections were prepared from each sample. Images were taken by a transmission electron microscope (TEM) (Zeiss EM-109, Carl Zeiss GmbH, Munich, Germany).

Cell-free extract preparation

The roots of lentil, both control and treated samples, were cut into small pieces and dissolved in 0.1 M phosphate buffer (pH = 7). The cells were lysed by ultrasound (Sonics Vibracell VCX130PB) at 4°C and 20 KHz. The suspension was centrifuged at 4000 × *g* for 10 min to remove intact cells. The supernatant was used as a cell-free extract for the enzyme assay and purification.

Enzyme assay

The reaction was started by adding 100 µl of the cell-free extract which contained CAT (EC 1.11.1.6) to test tubes containing 0.1 M phosphate buffer (pH = 7) and different concentrations of H_2O_2 as substrate (0.4, 0.6, 0.8, 1, 1.2 mM). The final volume in each test tube was always 2.2 ml. The catalytic activity of CAT was monitored by following the decrease in absorption at 240 nm using a UV-visible spectrophotometer (Shimadzu 1240, Shimadzu, Osaka). CAT activity was measured using the extinction coefficient of 43.6 M^{-1} cm^{-1} for H_2O_2 at 240 nm (Bienert *et al*. 2007).

The reaction rate of the enzyme was also examined at different pHs (3, 4, 5, 6, 7, 8, 9, 10 and 11) and temperatures (0, 10, 25, 30, 40, 50, 60, 70, 80, 90, and 100°C). Different buffer systems, including glycine, phosphate, acetate and Tris were used to obtain 0.1 M buffer in which pH varied from 3 to 11. The results of enzyme assays were the average of at least three separate experiments and expressed as the mean ± standard deviation (±SD) using GraphPad Prism 5 program for statistical analysis.

The amount of protein was measured by the Lowry method (Robyt and White 1987), using different concentrations of casein for drawing the standard curve.

Enzyme purification

The cell-free extract was brought to 50% ammonium sulfate saturation. The precipitate was collected by centrifugation at 5000 × *g* for 10 min and dissolved in 0.1 M phosphate buffer (pH = 7). The suspension was dialyzed against phosphate buffer overnight. All the above procedures were performed at 4°C. The dialyzed suspension was loaded onto a DEAE cellulose column equilibrated by 50 mM Tris buffer (pH = 8). Elution was performed by increasing NaCl concentration at a flow rate of 1 ml/min. The fractions were monitored for the amount of protein (at 280 nm absorption) and enzyme activity was also detected. The fractions with maximum activity were selected for electrophoresis. Enzyme purification was performed three times and the best result was reported.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 7.5% separating gel and a 3.5% stacking gel according to the Laemmli method (1970). After electrophoresis, the proteins on the gel were detected by silver nitrate staining. A protein marker and bovine serum albumin (BSA) were used as molecular weight standards.

RESULTS

Morphological and subcellular changes

Fig. 1 shows the roots in treated and control samples. The tap root system of the control was longer than that of the treated samples. Rootlets and root hairs were also more developed in the control. TEM images from a normal cell in the cortex region of the root shows that it contains a vacuole that filled most of the cell volume, a nucleus to one side, and mitochondria with normal shape that appeared to be active and are in condensed form and a Golgi complex (**Fig. 2**). Some of the cells in the cortex region of root in treated samples were normal, but some had been damaged: the nucleus had lost its membrane unity, and the mitochondria had become autolytic (**Fig. 3**).

Effect of pH and temperature

CAT activity was studied at different pHs and different temperatures in both control and treated samples. **Fig. 4** shows the effect of various pHs on CAT activity. In both the control and treated samples, there were two peaks of activity. In the control, the peaks were observed at pH 7 and 10, while in treated samples the peaks were at pH 8 and 10. The optimum pH was 10 in both samples. No activity was seen at pH 11.

Maximum activity was detected at 30°C in both samples (**Fig. 5**). Increasing the temperature above 30°C decreased the activity. No activity was detected at 90°C in the control group while in the treated samples, the enzyme showed minor activity at 90°C and it completely lost its activity at 100°C.

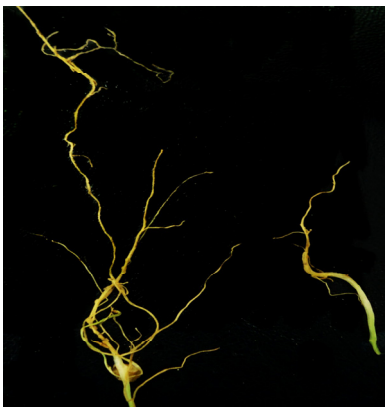

Fig. 1 Comparison of the roots in the control (left) and treated samples (right).

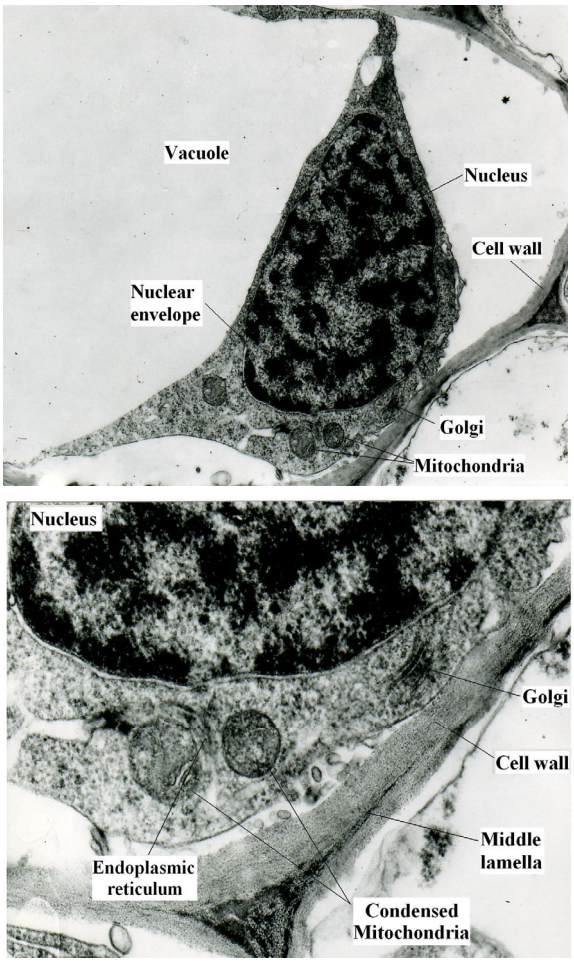

Fig. 2 A normal cell in cortex region of the root. Most of the cell volume has been occupied by vacuole. Mitochondria are active and are in condensed form. (top 7000 ×), (bottom 12000 ×)

Kinetic parameters

A double reciprocal plot was drawn to determine the kinetic parameters of the enzyme in the control and treated samples (**Fig. 6**). Both K_m and V_{max} changed in treated samples in comparison with the control. The K_m of the enzyme was 1.13 and 1.5 mM and V_{max} was determined to be 1.16 and 2 mM/min/mg protein in treated and the control samples, respectively.

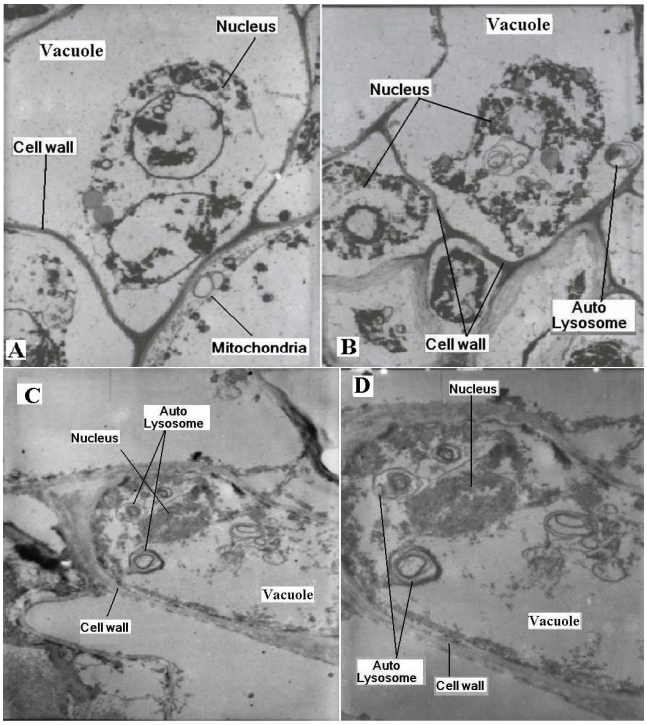

Fig. 3 Damaged cells from cortex region of the root in treated samples. The nucleus has lost its integrity. Mitochondria have entered autolysis. (A and D 12000 ×) (B and C 7000 ×)

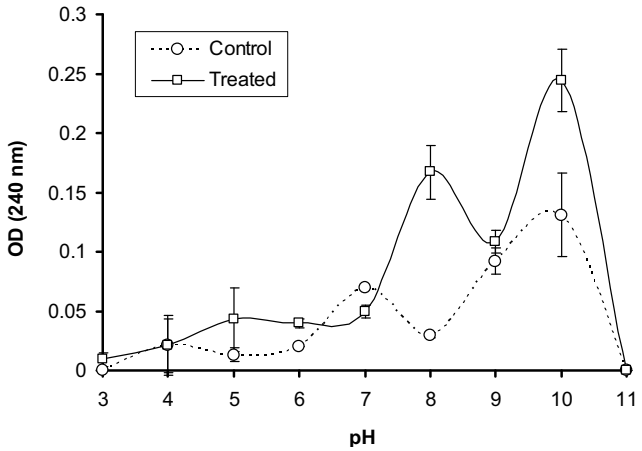

Fig. 4 The effect of different pH on the catalase activity in the control and treated samples. Average values given ± standard deviation (n = 3).

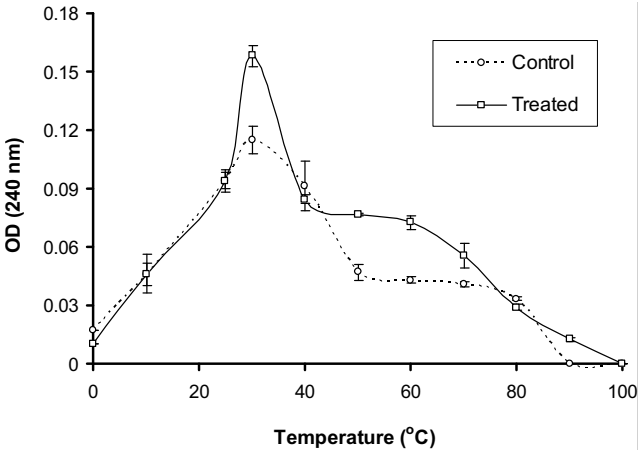

Fig. 5 The effect of different temperature on the catalase activity in the control and treated samples. Average values given ± standard deviation (n = 3).

Table 1 Purification details of the control group.

Purification step	Volume (ml)	Total activity (U)	Total Protein (mg/ml)	Specific activity (U/mg protein)	Yield (%)	Purification factor
CFE[a]	2	0.03	0.11	0.027	100	1
AS[b]	1.5	0.021	0.09	0.23	70	8.5
IEC[c]	1	0.013	0.02	0.65	61	24.1

[a]CFE= cell-free extract.
[b]AS= Ammonium sulfate.
[c]IEC= Ione exchange chromatography

Table 2 Purification details of treated samples.

Purification step	Volume (ml)	Total activity (U)	Total Protein (mg/ml)	Specific activity (U/mg protein)	Yield (%)	Purification factor
CFE	2	0.04	0.28	0.014	100	1
AS	1.5	0.032	0.13	0.24	80	17.1
IEC	1	0.022	0.0.3	0.73	68	52

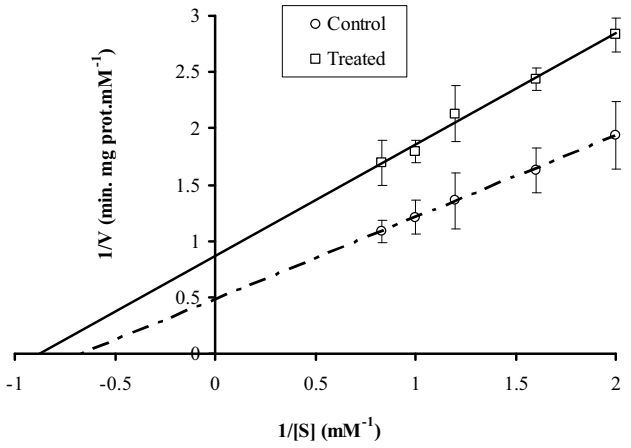

Fig. 6 Lineweaver plot shows that both K_m and V_{max} have changed in treated samples. The error bars indicate ± standard deviation (n = 3).

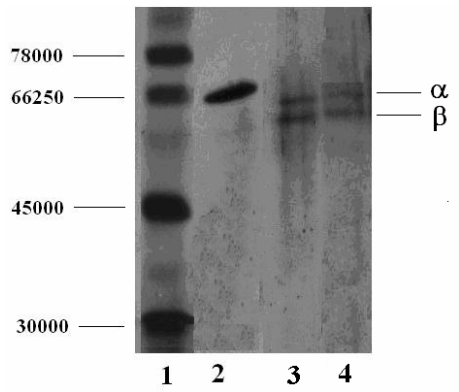

Fig. 7 Silver nitrate staining of SDS-PAGE of active fractions in both the control and treated samples. Lane 1: Protein markers. Lane 2: Bovine serum albumin (MW = 66450). Lanes 3 and 4 are treated samples and control group, respectively.

Enzyme purification

To purify CAT, an ammonium sulfate and DEAE cellulose column were used. Measurements of the amount of protein and enzyme activity were performed for each fraction. A summary of CAT purification in the control and treated samples is provided in **Tables 1** and **2**.

SDS-PAGE was performed for the highly active fractions (**Fig. 7**). Two bands were observed in the gel for both groups. The two subunits in the control group had a molecular weight (MW) of 64,000 and 65,000 Daltons (Da) while the bands of treated samples showed a MW of 63,000 and 64,000 Da.

DISCUSSION

Stress-inducing environmental elements such as salinity, drought, heat, heavy metals and organic pollutants can influence plant physiology and morphology (Cobbett 2000; Parida and Das 2005; Hatami *et al*. 2012; Mohsenzadeh *et al*. 2012; Zhou *et al*. 2012). There are many reports on the effect of organic pollutants such as crude oil and its derivatives on plant morphology. For example, it was found that crude oil-contaminated soil (10,000 mg/kg) was phytotoxic to corn and red beans (*Phaseolus nipponesis*) (Baek *et al*. 2004), Merkl *et al*. (2005) reported that *Brachiaria brizantha* (palisade grass) and *Cyperus aggregatus* (inflated scale flatsedge) showed thicker roots in heavy crude oil-polluted soil compared to the control. A greenhouse study showed that in the presence of Venezuelan heavy crude oil-contaminated soil, biomass and plant height of vetiver (*Vetiveria zizanioides*) were significantly reduced (Brandt *et al*. 2006). Minai-Tehrani (2008) also showed that heavy crude oil in the soil at a 1-15% (w/w) could delay germination and reduce the number of seeds that germinated, and the length and width of leaves and also decrease the biomass of *Poa trivialis* (rough meadow-grass). Light crude oil-contaminated soil with a concentration greater than 3% could decrease root and shoot biomass, leaf length and germination percentage of sorghum (*Sorghum bicolor*) (Minai-Tehrani *et al*. 2012). This report on lentil focused on the effect of crude oil on the activity of CAT and also subcellular changes of root cells in crude oil in oil-contaminated soil.

Our results showed that in oil pollution imposed morphological and cellular changes in lentil roots. In treated samples, the tap root was shorter than the roots of control plants and had less developed rootlets, demonstrating that oil pollution alters root growth. The effect of crude oil-contaminated soil on the root of *Poa trivialis* and *Festuca arundinacea* (tall fescue) has been previously reported to inhibit the growth of roots in contaminated soil (Minai-Tehrani *et al*. 2007; Minai-Tehrani 2008). It was shown that in the presence of 5% (w/w) of heavy crude oil in the soil, the dry biomass of root in *P. trivialis* was reduced to half of the control (Minai-Tehrani 2008). The dry biomass of root in *F. aurandinacea* decreased by about 75% in the light crude oil-contaminated soil (5% w/w) (Minai-Tehrani *et al*. 2007). Our observations on lentil at the subcellular level also showed that although some normal cells were observed in the treated roots, the number of damaged cells was quite significant. Damage was observed in the nucleus, cell wall, mitochondria and nuclear envelope, confirming that the damaged cellular structures were due exclusively to oil contamination. The formation of many autolysosomes suggests that oil contamination had a toxic effect on the cells and led to autolysis.

Other reports regarding organic-derived pollutants such as benzene focused on the deterioration of chloroplasts, mitochondria and cell walls of leaves in maize (*Zea mays*) and alfalfa (*Medicago sativa*) exposed to 0.4 mM benzene

vapor (Sadunishvili *et al*. 2009). 3-4 benzopyrene at a concentration of 2.6×10^{-8} µg/ml inhibited DNA synthesis in nuclei in maize (*Zea mays*) root cells (Buadze *et al*. 1998).

CAT is an important enzyme for removing H_2O_2, which is harmful to plant cells (Chen *et al*. 2012; Mhamdi *et al*. 2012). CAT activity, which decreases the risk of oxidative stress, changes when organisms encounter stressful conditions in the environment (Mittler 2002; Shim *et al*. 2003; Attar *et al*. 2009). The amount of salicylic acid increased in rice seedlings stressed by NaCl treatment, it was inversely correlated with the decrease in the CAT activity (Shim et al. 2003). Chronic exposure to high temperature (40ºC) induced higher CAT activity in germinating seeds of maize (Scandalios 1994). In the leaves of cowpea (*Vigna unguiculata*), CAT activity decreased in response to salt stress (200 mM NaCl) (Cavalcanti *et al*. 2007). Therefore, CAT was chosen in this study to perceive the biochemical effects of crude oil on lentil roots. Some factors such as temperature, pH and enzyme activity were considered to compare the behavior of CAT in treated samples and in the control group. The temperature curve pattern of the enzyme was almost similar in both samples (**Fig. 5**) with maximum activity at 30°C. CAT in treated samples was active at 90°C but not in the control group. These results demonstrate that lentil CAT is heat-resistant and does not completely lose its activity at a temperature as high as 90°C. The thermal stability of CAT has also been reported in other plants such as spinach (*Spinacia oleracea*) and saffron (*Crocus sativus* L.) (Sapers and Nickerson 1962; Keyhani *et al*. 2002).

There was a difference between the CAT pH curve in the control group and in treated samples. In the control there were two peaks at pH 7 and 10, while in treated samples, peaks were observed at pH 8 and 10 (**Fig. 4**). This result showed that CAT of lentil roots is more active in alkaline than in acidic conditions. In dormant saffron corms, the activity of three CAT isozymes was detectable over a wide range of pHs (5-11.5) with maximum activity found at pH 6 to 11 (Keyhani *et al*. 2002).

The kinetic measurements revealed that both K_m and V_{max} of CAT changed in treated samples in comparison with the control (**Fig. 6**). The reduced K_m of CAT in treated samples demonstrates that the affinity of the enzyme for the substrate increased. The specific CAT activity in treated samples also increased. These differences suggest that a CAT isozyme may be expressed in lentil roots under stress induced in the presence of crude oil.

Kinetic comparisons of 5 cottonseed (*Gossypium hirsutum*) CAT isozymes indicated that they had similar K_m values and thermostability (Ni and Trelease 1991). CAT activity changes in different environmental conditions such as light and CO_2 level. In barley (*Hordeum vulgare*) leaves, there were two CAT isoforms with a 53 and a 57 kDa subunit, the first induced and the second repressed by light (Holtman *et al*. 1998). In high CO_2 (1% CO_2/21% O_2), total CAT activity decreased by 50% in leaves of tobacco seedlings (*Nicotiana sylvestris*, *Nicotlana tabacum*) (Havir and McHale 1989).

CAT may appear in plants as various isozymes in response to different environmental conditions. Only a single bond was reported for purified CAT of loblolly pine (*Pinus taeda*) (Mullen and Gifford 1993), cottonseed (Kunce *et al*. 1988), and potato (*Solanum tuberosum*) (Esaka and Asahi 1982). However, in our experiment, SDS-PAGE of purified enzyme indicates that lentil CAT consisted of two subunits (α and β) which could be monitored with two distinct bands in the gel. A minor difference was observed between the mobility of these two bands in treated and the control samples. This suggests that a CAT isoform is expressed in treated samples. Castor bean (*Ricinus communis*) endosperm CAT also showed two subunits in SDS-PAGE (Ota *et al*. 1992; Holtman *et al*. 1998).

In conclusion, our results showed that the presence of crude oil in soil can impose sub-cellular and biochemical changes in lentil roots as a result of their direct contact with oil-polluted soil.

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