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ORIGINAL ARTICLE

Crude Oil-polluted Soil Induces Ultrastructural and Enzyme Activity Changes in the Shoot of Lentil

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Plants are always under threat due to various biotic and abiotic stresses. They respond to such threats by an efficient antioxidative enzyme system such as catalase. The presence of petroleum in soil is a stressful environmental factor for plants. In this study, cellular and biochemical changes were investigated in the shoot of lentil grown in the crude oil-polluted soil. Transmission electron microscope micrographs showed various injuries in the level of subcellular content including nucleus, chloroplast and mitochondria. In the treated samples, catalase activity and its kinetics factors (Vmax and Km) were different from the control. In the treated, Km of the enzyme was lower than that of the control. Temperature and pH profiles of the enzyme were different in the control and treated samples. Catalase purification in both treated and control samples showed that two catalase isoforms were expressed in the treated sample in comparison to the control, in which only one type of catalase was detected.

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Key words: lentil; pollution; cell damage; catalase; isoenzyme

Abbreviations: CAT: Catalase; TEM: Transmission electron microscope

Lentil *(Lens culinaris)* belongs to the family of *Leguminosae* and is predominantly grown in south East Asia (Adsule *et al*., 1989). Lentils are excellent sources of protein and also rich in important vitamins, minerals and soluble and insoluble dietary fibers. The unsaponifiable lipid fraction of lentil is a potential source of bioactive components such as phytosterols, squalene, tocopherols and phenolic compounds with high antioxidant activity (Amarowicz *et al*., 2010).

Crude oil may leak into the soil during extraction, refining and transportation. It can be washed out from the soil by rain and enter surface or underground waters which can contaminate cultivating soil. Crude oil makes the soil unpleasant for growing plants due to reduction in the level of available plant nutrients or increase in toxic levels of certain elements such as multiple rings, aromatic compounds, long chain saturated and unsaturated aliphatics and heavy metals (Cd and Va) (Chaineau

et al., 1997). It has been reported that oil contamination causes slow rate of germination in plants (Minai-Tehrani *et al*., 2012; Minai-Tehrani 2008; Kirk *et al*., 2005; Merkl *et al*., 2005). Crude oil causes delay in germination, decreases number of germination, reduces the shoot and root length and biomass and induces early chlorosis in plants (Minai-Tehrani *et al*., 2012; Minai-Tehrani 2008). It has been reported that this consequence could be due to the oil which acts as a physical barrier for preventing or reducing access of seeds to water and oxygen. It is well established that reactive oxygen species (ROS) are involved in the response of plants to biotic and abiotic stresses (Bolwell *et al*., 2002; Gallego *et al*., 2002). The ROS includes singlet oxygen, superoxide radical, hydrogen peroxide (H_2O_2) and hydroxyl radical, which can trigger unrestricted oxidation of various cellular structures, leading to oxidative destruction of the cell. The typical catalase (CAT) (EC: 1.11.1.6) reaction is disruption of two molecules of H_2O_2 to water and O_2.

The best characterized type of haem-dependent CAT is found in diverse organisms including prokaryotes, fungi, animals and plants, which is composed of 'typical' or 'monofunctional' CATs (Chelikani *et al*., 2004). These enzymes consist of polypeptides with 50–70 kDa in mass which are organized into tetramers, with each monomer bearing a haem prosthetic group (Regelsberger *et al*., 2002). The available genomic information suggests that most animals including mammals have a single CAT gene. Multiple forms of the enzyme have been reported for many higher plants such as *Carthamus tinctorius* (Tayefi-Nasrabadi *et al*., 2011), *Picea omorika* (Bogdanovic *et al*., 2007) and *Nicotiana tabacum* (Havir and McHale 1989). In maize, three CAT isozymes have been determined (CAT-1, CAT-2 and CAT-3), each of which is expressed under various environmental conditions (Scandalios 1994; Williamson and Scandalios 1992).

In this study, effect of crude oil on the biochemical and subcellular levels of lentil shoots were investigated. Lentil was selected because of belonging to the *Leguminosae*, a group of plants with good potential for phytoremediation of oil-polluted soil. It is also an edible plant which can be a model for studying other dietary vegetations.

MATERIALS AND METHODS

Chemicals.

All the chemicals used for buffer preparation and enzyme assay were purchased from Merck Company.

Soil Collection.

The soil used in this study was garden soil. The soil was dried and passed through 4 mm mesh. Light crude oil was obtained from Sarkan Oil Processing Company and added to the soil. The oil was mixed with the soil by shaking the mixture to result in homogenous contaminated soil. The final weight of soil was always 500 g with oil concentration of 5% (w/w).

Germination.

Ten lentil seeds were planted in the control soil and in the contaminated soil which received oil. About 5 g of animal manure was added to each sample as the fertilizer. The required light for the growth of the plants was obtained from the sun. The samples were put behind the glass windows of the laboratory and received the sun light during the experiment. Tap water was used to moisten the control and treated samples. After thirty days, the plants were removed from the soil in both treated and control samples. The shoots were separated from the roots and divided into two fractions. One

fraction was cut to small pieces and fixed in glutaraldehyde (2.5 %) for microscopic studying and another fraction was preserved at -20°C for further biochemical experiments.

Transmission electron microscope (TEM) preparation.

The fixed samples were washed by 0.1 M phosphate buffer pH=7 and then immersed in 1% osmium tetroxide for 30 min. After washing with distilled water, dehydration was done by ethanol gradient. The specimens were embedded in epoxy resin and sectioned by ultramicrotome (Reichert OMU3). Uranium acetate and lead citrate were used to stain the thin sections. The images were taken by TEM (Ziess EM-109).

Cell-free extract preparation.

The frozen samples were cut into small pieces and suspended in a phosphate buffer 0.1M, pH=7. The cells were broken by ultrasonic equipment at 4°C. The suspension was centrifuged at 4500 ⨀ *g* for 15 min to precipitate the intact cells. The supernatant was used as cell-free extract for enzyme assay.

Enzyme assay.

CAT activity was detected by mixing 100 µl H_2O_2 (0.4, 0.6, 0.8, 1, 1.2 mM) in 0.1 M phosphate buffer pH 7.0 and 100 µl of the cell-free extract suspension. The final volume in each test tube was always 2.2 ml. CAT activity was monitored by decreasing the absorbance of H_2O_2 at 240 nm using Shimatzu 1240 UV-Visible spectrophotometer. The kinetic parameters were calculated by linear regression from double-reciprocal plots.

Effect of pH and temperature.

The effect of pH on CAT activity was determined in different pH values (pH 3.0-11.0). Various buffer systems including glycine, phosphate, acetate and Tris were used to obtain 0.1 M buffer with different pH. The effect of temperature on CAT activity was obtained at different temperatures (0-100°C).

Catalase purification.

The cell-free extract was fractionated by 50% ammonium sulfate. The suspension was centrifuged (5000 ⨀ g for 10 min at 4 °C). The pellets were dissolved in 0.1 M phosphate buffer, dialyzed against 0.05 M phosphate buffer pH=7 (overnight at 4°C) and loaded to DEAE cellulose column. The fractions were monitored for the amount of protein (at 280 nm) and enzyme activity. The fraction with the highest activity was selected for electrophoresis. SDS-PAGE was performed with 3% stacking and 7.5% separating gels. Silver nitrate was used to stain the gel. CAT activity was measured using the extinction coefficient of 43.6 M^{-1} cm^{-1} for H_2O_2 at 240 nm.

RESULTS

Ultrastructural changes.

TEM was used to compare ultrastructural changes between the normal and injured cells. In the normal shoot cell, the nucleus was resided in the corner of the cell. Chloroplasts had concave shape and were also located close to the cell wall. Mitochondria were seen near the nucleus and chloroplasts and most volume of the cell was occupied by a vacuole (Fig. 1). In the treated samples, there were normal as well as injured cells. In the injured cells, the nucleus lost its integrity and moved to the center of cell. The chloroplasts changed to spherical shape and some of them were surrounded by an autolytic membrane. The mitochondria lost their content and seemed to be devoid of matrix and cristae (Fig. 2).

Effect of pH and temperature of CAT activity.

The activity of CAT was studied at different pH

(Fig. 3). In the control sample, maximum activity was observed at pH 10. In the treated sample, there were two peaks; the minor peak was at pH 7 and major peak was at pH 10. The control had no activity at pH 3 while the treated sample showed minor activity at pH 3. Both of the samples had no activity at pH 12.

The activity of CAT in the treated and control samples was also compared at various temperatures (Fig. 4). In the control, the maximum activity was seen at 40°C and the enzyme was totally inactive at 90°C. In the treated sample, the maximum activity was observed at 50°C and the enzyme remained active at 90°C. Both of the samples were also active at 0°C.

Enzyme Kinetic analysis.

Kinetic parameters of the CAT in the control and treated samples were determined by Lineweaver–Burk plot (Fig. 5). The Km and Vmax for the control and treated samples were 1.7 mM, 0.64 mM/min.mg protein, and 0.72 mM, 0.33mM/ min. mg protein, respectively.

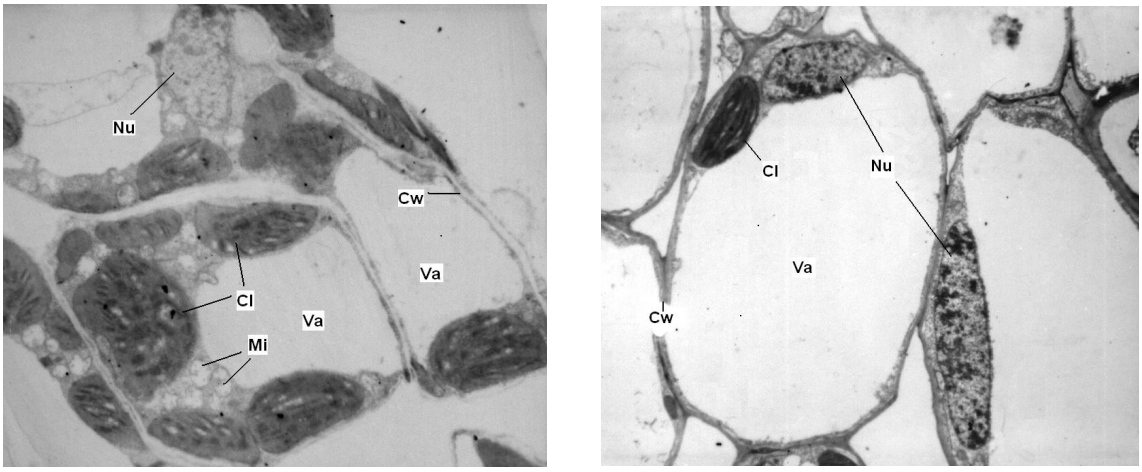

Figure 1 : TEM micrographs of normal cells (left and right) in the shoot of lentil. Nu: Nucleus, Cl: Chloroplast, Mi: Mitochondria, Va:Vacuole, Cw, Cell wall (4000 X).

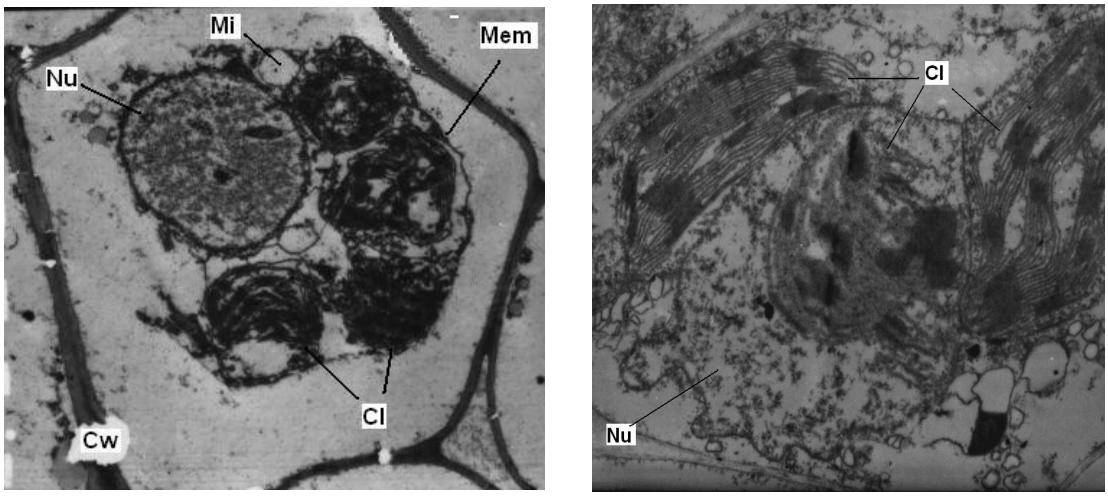

Figure 2 : Injured cells in treated samples. The chloroplasts seem to be surrounded by an autolytic membrane (left 7000 X). The nucleus has been disrupted and the chloroplasts have lost their normal shape (right 12000 X). Nu: Nucleus, Cl: Chloroplasts, Mi: Mitochondrium, Cw: Cell wall and Mem: Autolytic membrane.

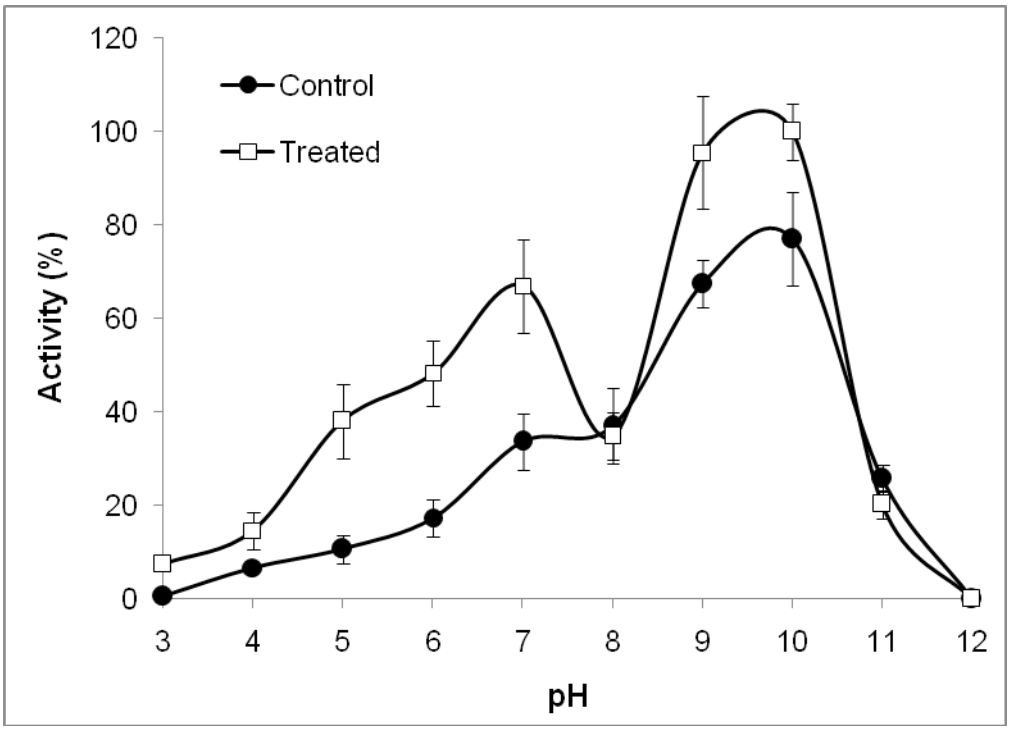

Figure 3 : The effect of pH on catalase activity in the control and treated sample.

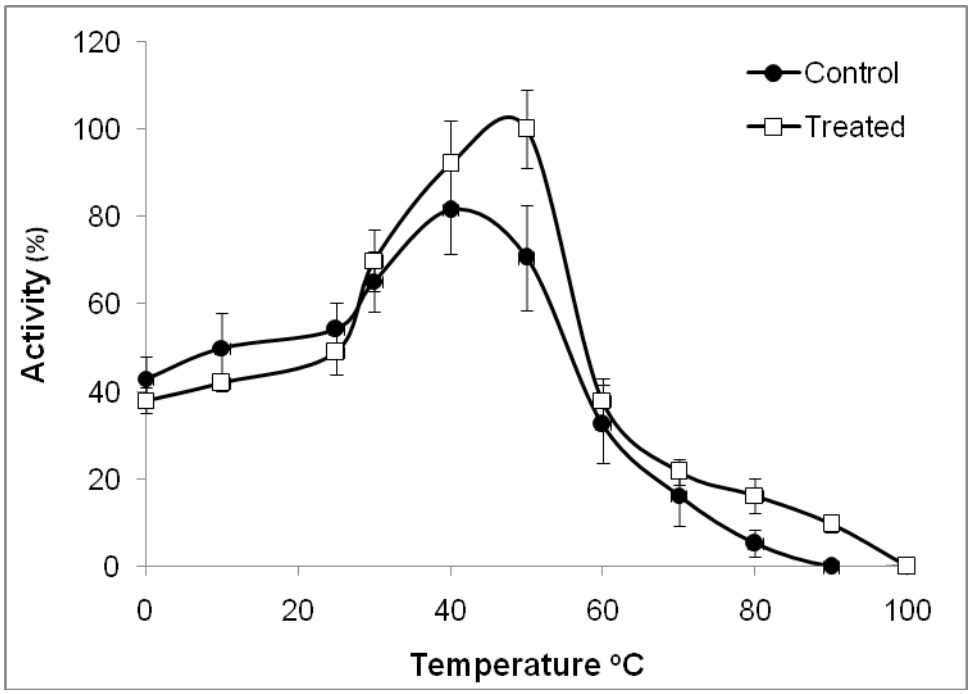

Figure 4 : Effect of temperature on the catalase in the control and treated sample.

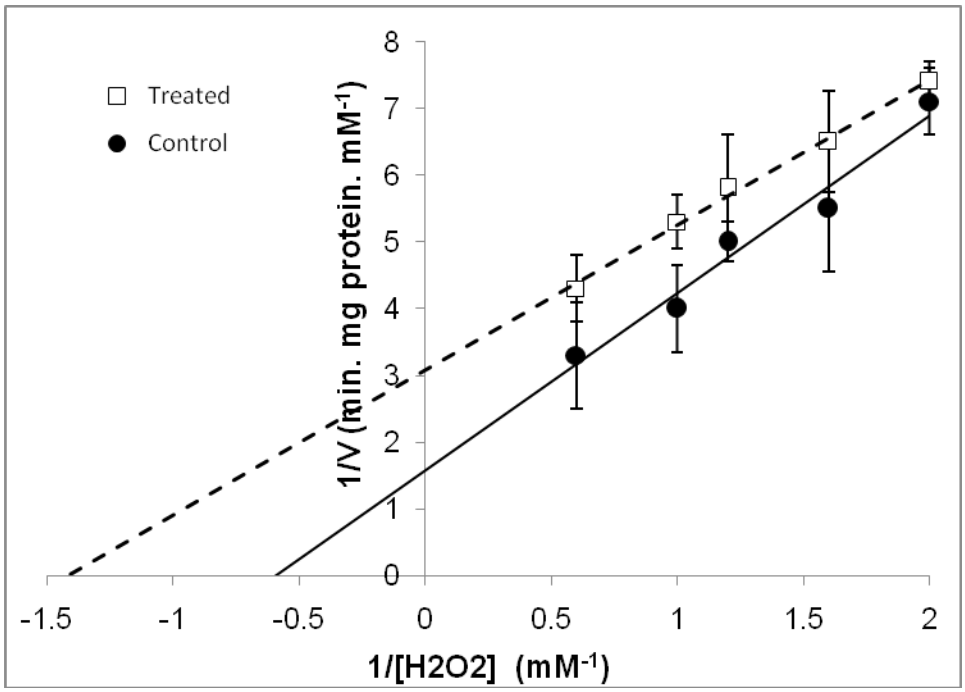

Figure 5 : Lineweaver-Burk plot of catalase. Both Km and Vmax were different in the control and treated sample.

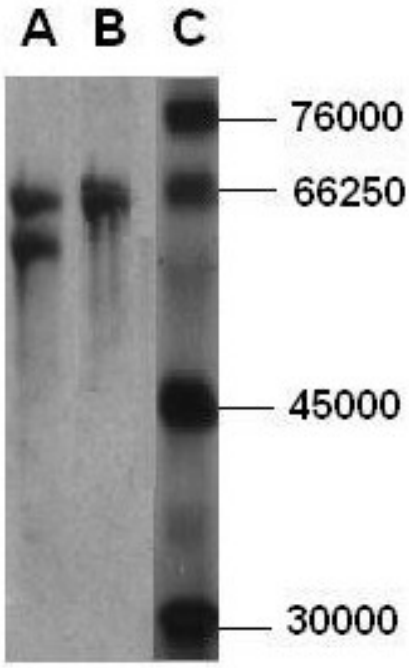

Figure 6 : Silver nitrate stained SDS-PAGE of purified catalase in the control and treated samples of lentil shoot. Lane A: Treated sample, Lane B: Control, Lane C: Protein markers.

Catalase purification.

To purify CAT, ammonium sulfate precipitation and DEAE cellulose column were used. The fractions with maximum enzyme activity were used for electrophoresis. SDS-PAGE was performed to confirm the enzyme purification (Fig. 6). In the control group, only a single band was observed with the estimated Mw of 65kDa. In the treated sample, two bands were seen in the gel. One band had equal mobility to the control while another band

showed lower molecular weight with about 64 kDa.

The specific activity of purified enzyme was 0.09 and 0.11 U/mg protein for the control and treated samples respectively.

DISCUSSION

This experiment discussed the effect of 5% crude oil-contaminated soil on the cellular and biochemical changes in the shoot of lentil. 5% concentration of petroleum was chosen because previous studies have reported that this concentration could induce distinct macroscopic effect on the shoot of plants (Minai-Tehrani *et al*., 2012; Minai-Tehrani 2008). Although there are many reports on the macroscopic effects of organic pollutant on plants (Minai-Tehrani 2008; Brandt *et al*., 2006; Kirk *et al*., 2005; Merkl *et al*., 2005), few studies have focused on the microscopic level and also biochemical changes in plants (Sadunishvili *et al*., 2009; Buadze *et al*., 1998). To get deeper insights, the microscopic changes in the treated samples, the possible damage in the cell organelles such as mitochondria, chloroplasts, nucleus and the cell wall were considered and examined in detail by transmission electron microscopy (TEM). The observation with TEM micrographs showed that, in the shoots of treated samples, there were normal cells as well as injured ones. In the injured cell, nucleus and chloroplast moved to the center of the cell while, in the normal cell, they were located in the corner of the cell near the cell wall. The nucleus disrupted or changed its shape, the chloroplasts became spherical and the number of thylakoids decreased. In some cases, the chloroplasts were surrounded by a membrane which suggested autolysis of the organelle. A great vacuole was seen in normal cell but it was not distinguishable in the injured cell. All these events proposed apoptotic cell disruption in the treated samples. The presence of crude oil in the soil could enforce a stressful condition for the plant and drive some cells to apoptotic processes. Previous reports have shown the relationship between environmental stressful factors and apoptosis (Chichkova *et al*., 2010; Attar *et al*., 2009; Vartapetian *et al*., 2008). To understand the biochemical effects of crude oil on the lentil's shoot, CAT was chosen due to its role in dealing with oxidative stress. Therefore, some properties of CAT such as optimum temperature, pH and enzyme activity were considered to compare the behavior of the enzyme in the control and treated samples.

Determination of the kinetics factors of the enzyme revealed that Km of the enzyme in the treated sample was much lesser than that of the control, which suggested that affinity of CAT became much higher for its substrate. This result proposed that the plant moved to deal with oxidative stress due to the presence of oil. It has been shown that some stressful environmental factors such as heavy metals and organic pollutants could affect the CAT activity (Attar *et al*., 2009; Cheeseman 2007; Bolwell 1999).

Comparison of pH and temperature profiles of CAT in the control and treated samples showed that patterns of these profiles were different in these groups. In the control, there was only a maximum peak at pH 10 while, in the treated sample, two peaks were observed (pH 7 and 10). The difference of kinetics factors and also two distinct peaks of pH suggested that there might be other isozymes of CAT in the treated sample in addition to the usual CAT, which exists in normal cell. Purification of the enzyme and comparison of SDS-PAGE of purified CAT confirmed this suggestion. There were two bands in the treated sample while only one band

was observed in the control. These results proposed that, in the shoots of the treated sample, two isozymes of CAT were expressed to deal with a new stressful environmental condition. The diversity of CAT in plants has been revealed in previous reports (Tayefi-Nasrabadi *et al*., 2011; Bogdanovic *et al*., 2007; Mullen and Gifford 1993). In maize, three CAT isozymes have been discovered (CAT-1, CAT-2 and CAT-3), each of which was expressed under different environmental conditions (Scandalios 1994). In the presence of a fungal toxin, cercosporin, CAT-3 levels increased in maize leaves (Williamson and Scandalios 1992).

In conclusion, these results revealed that crude oil contamination could induce biochemical and subcellular changes in the shoot of lentil. At subcellular level, the cells moved to apoptotic process and disruption. At biochemical level, the cells expressed two isoforms of CAT to deal with the imposed oxidative stress.

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