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 seedling growth and dry weight, reduce 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 (Tavelli-Nasrabadi et al. 2011), Picea omorika (Bogdanović et al. 2007), Nicotiana tabacum (Hlavir 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) (Scandalias 1994). In the presence of a fungal toxin, cercosporin, CAT-3 levels increased in maize leaves (Williamson and Scandalias 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 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.
(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 bio-
remediation 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 homo-
geney 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 tem-
perature and relative humidity were about 30°C and 45%, respec-
tively. 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 subcel-
ular study while the other group was kept at -20°C for biochemi-
cal 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 dehy-
drated in an ethanol gradient. The specimens were embedded in epoxy resin and sectioned by an ultramicrotome (Reichert OMU3). 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 elect-
rophoresis, 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 tem-
peratures 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 opti-
mum pH was 10 in both samples. No activity was seen at pH 11.

Maximum activity was detected at 30°C in both sam-
ple (Fig. 5). Increasing the temperature above 30°C de-
creased 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.
Crude oil induces cell damage in plants. Mohammadi et al.

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. 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 ×)

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.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total activity (U)</th>
<th>Total Protein (mg/ml)</th>
<th>Specific activity (U/mg protein)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFE*</td>
<td>2</td>
<td>0.03</td>
<td>0.11</td>
<td>0.027</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>AS*</td>
<td>1.5</td>
<td>0.021</td>
<td>0.09</td>
<td>0.23</td>
<td>70</td>
<td>8.5</td>
</tr>
<tr>
<td>IEC*</td>
<td>1</td>
<td>0.013</td>
<td>0.02</td>
<td>0.65</td>
<td>61</td>
<td>24.1</td>
</tr>
</tbody>
</table>

*CFE= cell-free extract.  
*AS= Ammonium sulfate.  
*IEC= Ion exchange chromatography

Table 2: Purification details of treated samples.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total activity (U)</th>
<th>Total Protein (mg/ml)</th>
<th>Specific activity (U/mg protein)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFE</td>
<td>2</td>
<td>0.04</td>
<td>0.28</td>
<td>0.014</td>
<td>100</td>
<td>1</td>
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<tr>
<td>AS</td>
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<td>0.032</td>
<td>0.13</td>
<td>0.24</td>
<td>80</td>
<td>17.1</td>
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<tr>
<td>IEC</td>
<td>1</td>
<td>0.022</td>
<td>0.03</td>
<td>0.73</td>
<td>68</td>
<td>52</td>
</tr>
</tbody>
</table>

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 nippokensis) (Baek et al. 2004), Merkt 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 (Vetivera 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-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. arundinacea 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).

CA T is an important enzyme for removing H₂O₂, which is harmful to plant cells (Chen et al. 2012; Mhamdi et al. 2012). CA T activity, which decreases the risk of oxidative stress, changes when organisms encounter stressful conditions in the environment (Mittler 2002; Shim 2009). The amount of dicarboxylic acid increased in rice seedlings stressed by NaCl treatment, it was inversely correlated with the decrease in the CA T activity (Shim et al. 2003). Chronic exposure to high temperature (40°C) induced higher CA T activity in germinating seeds of maize (Cipriano et al. 2009). In high CO₂ (1% CO₂/21% O₂), total l-ascorbate activities in cassava leaf extract and germinating cowpea seedlings. (2007) Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. Journal of Biological Chemistry, 282, 373-384.

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