Defence Mechanisms of Olive Tree (*Olea europaea*) under Heavy Metals Stress

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Abstract: Heavy metals can bioaccumulate and biotransfer from both natural and artificial sources. Given that heavy metal concentrations above normal seriously threaten both plant and animal life, their contamination of plants and water is one of the biggest problems facing the globe today that must be addressed. In this work, we attempt to evaluate the buildup of heavy metals in the soil, their penetration into plants, and their impact on the physiological and biochemical functions of olive trees. Therefore, it was interesting to research the concentrations of heavy metals in Sidi Khaled-Derna plants and soil. Soil samples' physical and chemical properties, including pH, electrical conductivity, organic matter mass, phosphorus and nitrogen content. Random soil samples were taken from the study areas, the first area as the control (Barborg) and the second as the polluted area (Sidi Khaled). The results showed that the concentrations of metals in the soil exposed to the pollution and the trees growing in it were greater than those found in the unpolluted area and were higher than the safe recommended values. While the electrical conductivity of the same sample was above the standard range set by the World Health Organization, the pH of the polluted soil was below the normal limit. In addition, the amount of phosphorus, nitrogen, and organic matter were lower than in control. Notable decline in the activity of the studied antioxidant enzymes in trees growing in the polluted area, and all of the results indicated that the stressed olive trees had higher levels of phenolic compounds and the amino acid proline, which are believed to be markers of oxidative stress in plant cells, compared to control trees.

Keywords: Anti-enzymes; Proline; Heavy metals; Phenolic compounds; Isozyme; Olive (*Olea europaea*) and Polymorphic.

1. Introduction:

The olive tree is an evergreen with a long lifespan, capable of thriving for a thousand years or more. They bear therapeutic and nourishing fruit and thrive despite harsh winters and scorching, dry summers. Olea europaea is part of the Oleaceae family, which consists of over 80 species. The olive tree has a robust, twisted, knotted trunk and a spherical crown, growing to a height of 5 to 8 meters [1]. The lower surface of the leaves is covered with fine hairs, and they are opposite, simple, leathery, oblong-ovate, sharply pointed. [2]. The leaf falls often in the spring and lasts one to three years. The tiny, yellowish-white hermaphrodite blooms are borne in clusters and emerge from the leaf axils created during the previous growth season. The olive fruit is a drupe that is usually oval or spherical. It is made up of the mesocarp (flesh), which makes up between 60 and 80 percent of the fruit's weight and endocarp (pit), which contains the seed; and the exocarp (skin), which has stomata [3]. The olive fruit becomes purplish-black when fully ripe, although some varieties can be green or turn a coppery brown. The size of the olive fruit can vary, even on the same tree, and is influenced by many factors, encompassing the specific cultivar, the amount of fruit the tree bears, soil fertility, water availability, and cultural practices [1].

Plants rarely exist under typical environmental conditions and often under extreme environmental conditions or factors causing stress, whether thermal, light, water salt, etc. [4]. The plants react to stress in various ways, including modifications to crop yields, growth rates, gene expression, and cellular metabolism [5]. Plants can suffer from several metabolic dysfunctions due to specific circumstances. Stress can induce senescence, prevent blooming and seed development, and ultimately kill agricultural plants. On the other hand, mild or short-term stresses may aid in the recovery of plants from damage, as their effects are temporary [4]. The accumulation of heavy metals (HMs) poses significant ecological, nutritional, and environmental challenges due to the disruption of natural biogeochemical cycles caused by human activities [6]. They have the ability to contaminate aquifers, the environment, soil, drinking water, and food chains. This pollution can have cytotoxic, genotoxic, and mutagenic effects on humans, animals, and plants [4].

The enemy of all aerobic species is the generation of radicals based on oxygen. These compounds, which are created as byproducts of metal-catalyzed oxidation, oxidoreductase enzymes, or the mitochondrial electron transport during aerobic respiration, have the capacity to result in a variety of harmful outcomes. At first, it was believed that the

only cells that produced ROS as part of host cell defense systems were phagocytes cells [7].

Metal toxicity levels inhibit normal plant function and metabolic processes in some ways, such as by disrupting or displacing protein structure building blocks, which results from metals and sulfhydryl groups forming bonds blocking the function of key cellular molecules [8], replacing or impairing the function of vital metals in biomolecular such as enzymes or pigments [9], and negatively affecting the integrity of the cytoplasmic membrane [10]. To counteract the negative consequences of these ROS, plant cells have evolved a defensive mechanism that includes antioxidants to protect themselves. This system includes nonenzymatic and enzymatic antioxidants; these substances act as scavengers of free radicals.

The main goals of this research are: (i) to determine the concentrations of heavy metals in both the soil and the tissues (leaf and fruit) of olive trees, as well as to evaluate their combined effects on the morphology and physiological characteristics of the olive tree; and (ii) to investigate the mechanisms by which the olive tree (*Olea europaea* L.) withstands the detrimental effects of specific heavy metals.

2.Material and Methods:

2.1. Collection and preparation of samples

Using a stainless steel corer, samples of soil were collected from 0 to 25 cm below the surface in the research regions. The samples were then taken to the laboratory, where they were spread out on cardboard and left to air dry. Once the soil had dried, it was ground up and filtered through a 200 mm diameter sieve before being placed in dry glassware for chemical analysis. In addition, we collected control soil samples from the Barborg area, which is approximately 4.5 kilometers from Sidi Khaled, the study site. These control samples were gathered to assess soil pollution with heavy metals. We also obtained plant tissue samples (leaves and fruit) from the same locations. Before analysis, all samples were cleaned and stored in a refrigerator at -20 °C. Table (1) presents a selection of the chemical characteristics of the study's soil.

2.2. Morphological characteristics

The traits studied included tree growth [leaf area (cm^2), leaf color, and fruit shape], as well as biomass fruit size (cm^3).

2.3. Physiological analyses

2.3.1. Free proline content estimation

Bates et al. [12], used a ninhydrin reagent and a quick colorimetric approach to evaluate the proline content.

2.3.2. Total phenolics assay

The total phenolic content was quantitatively assessed using the Folin-Ciocalteu reagent, employing gallic acid as the standard reference [13].

2.3.3. Enzyme extraction

Frozen tissues (0.2 g) from each leaf and fruit was crushed to a fine powder to estimate the antioxidant activity. To extract the enzymes, the tissue powder was homogenized in 40 mM phosphate buffer pH 0.7 that contained 1.0% polyvinylpyrrolidone and 0.7 ml Ethylenediaminetetraacetic acid along with 0.1 M Tris-HCl buffer. The extraction of enzymes and activities were assessed using the supernatant fraction following centrifugation of the homogeneity for 20 minutes at 4°C at 4,000 rpm [14].

2.3.3.1. Superoxide dismutase (SOD; EC 1.15.1.1)

Its capacity to prevent the photochemical reduction of nitroblue tetrazolium chloride as detailed by Yeh and Kuo, [15], were used to measure its activity.

2.3.3.2. Catalase (CAT; EC 1.11.1.6)

According to Aebi [16], activity was assessed using mixture that contained 2.0 ml of 100 mM buffer pH 7.0, 0.5 ml of 70 mM H_2O_2, and 50 µl enzyme extract and absorbance at 240 nm.

2.3.3.3. Peroxidase (POD; EC 1.11.1.7)

The activity of peroxidase enzyme was measured using the method of Ranieri *et al*. [17].

2.3.3.4. Ascorbate Peroxidase (APX; EC 1.11.1.11)

Activity was calculated using the method of Nakano and Asada [18].

2.4. Determination of heavy elements in soil and plant samples

Metals, including copper, iron, and nickel, were examined using atomic absorption spectroscopy (Perkin Elmer 800), following the procedure developed by Lorenz *et al.* [19]. Lead and cadmium levels were estimated according to Gary *et al.* [20].

2.5. Native Protein Electrophoresis

Using catalase and polyphenol oxidase isozyme systems as molecular markers, native-polyacrylamide gel electrophoresis (Native-PAGE) was carried out following [21], to discover isozyme changes under the research conditions. 0.5 g of fresh samples were ground with 1.0 ml of extraction buffer (10% glycerol) at 4°C to extract the isozymes. After that, the extract was put into sterile Eppendorf tubes and centrifuged for ten minutes at 15,000 x g. Brior to isozyme analysis, the supernatant was moved to fresh, clean Eppendorf tubes and kept at -20°C.

2.6. Statistical Analysis

The MSTAT-C software tool was used to statistically analyse the data using the methodology outlined by Freed *et al.* (1989) [22].

Table 1. Soil chemical properties

study site	PH	E.C	OM%	P%	N%
Contaminated	**5.3**	**0.89**	**3.9**	**1.2**	**0.45**
Uncontaminated	**7.1**	**0.24**	**5.4**	**1.8**	**1.3**

Where: - The required chemical analyses were performed using the analysis techniques outlined in Tan *et al.* [11].

3. Results:

3.1. Growth characteristics

Figure 1 shows that smooth and shiny fruits grow from healthy trees (control group) with dark green leaves on the upper surface and silver on the lower surface. In contrast, infected trees show red leaves, dry (wrinkled) fruits, and poor tree growth. Table 2 shows that the accumulation of heavy elements in the soil led to a significant decrease in leaf area and fruit size. We found substantial differences between the trees in the two regions regarding leaf area and fruit size: the average leaf area of trees in the contaminated area was 9.12 (cm^2), while it was 11.43 (cm^2) for unstressed trees. The size of infected fruits was recorded as 50 cm^3, compared to 62 cm^3 for healthy fruits.

Figure 1. Effects of toxicity in trees (a) and under normal conditions (b).

3.2. Proline and phenolic compounds content

The metal was the strongest inducer of proline accumulation, and uncontaminated trees showed no proportional change in proline level compared to stressed trees. The results shown in Figure 2 indicate that proline content increased in leaves compared to fruits in stressed trees (1.774 µmol g^{-1} of fresh weight) compared to those in the unstressed control group. In the similarly, the levels of phenolic compounds increased in the leaves and fruits of stressed trees, and on the contrary, the concentration of these compounds was low in the tissues of the control trees. In the same line, the leaves contained a higher concentration of phenolic compounds (0.865 mg/g dry weight) compared to the fruits (0.064 mg/g dry weight) (Table 3).

3.3. Antioxidant Enzyme Activities

The results in Figure (3 and 4) showed a significant decrease in the activity of all antioxidant enzymes in the leaves and fruits of stressed trees compared to the control group. The catalase enzyme was the least active in the leaves (290.7 mmol H_2O_2 min^{-1} g^{-1} FW) compared to the other enzymes, while the peroxidase enzyme recorded the least activity in the fruits (180.2 Δ min^{-1} g^{-1} FW).

Table 2. The impact of metals on the growth characteristics of the *Olea europaea* L. tree.

Study site	Leaf area (cm^2)	fruit size (cm^3)
Contaminated	09.12^b	50^b
Uncontaminated	11.43^a	62^a

Table 3. phenolic compounds content (mg g^{-1} dry weight) in leaves and fruit of olive trees exposed to metal stress.

Type	Area	Mean
Leaf	Contam.	0.865^a
	Uncontam.	0.162^b
Fruit	Contam.	0.064^b
	Uncontam.	0.001^c

3.4. Heavy elements concentration

The results obtained showed that the area affected by war remnants witnessed a significant accumulation of heavy elements in the soil, leaves, and fruits, and this increase exceeded the internationally declared safe limit (Table 4). The results indicated that the concentrations of these elements varied between the two parts, as their concentrations in the leaves were higher than in the fruits. Copper also recorded the highest concentration in the leaves (1.107 mg/g dry weight), while lead was the least concentrated. On the contrary, lead was recorded as the highest concentration (0.262 mg/g dry weight) in the fruits.

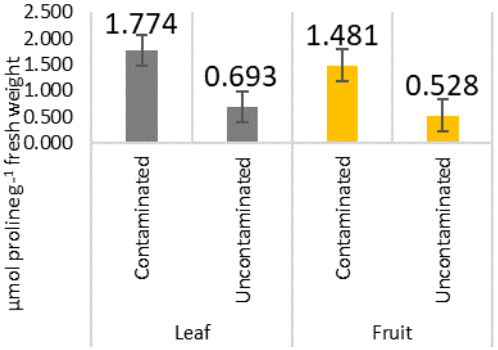

Figure 2. Proline content in leaves and fruit of olive trees exposed to metal stress.

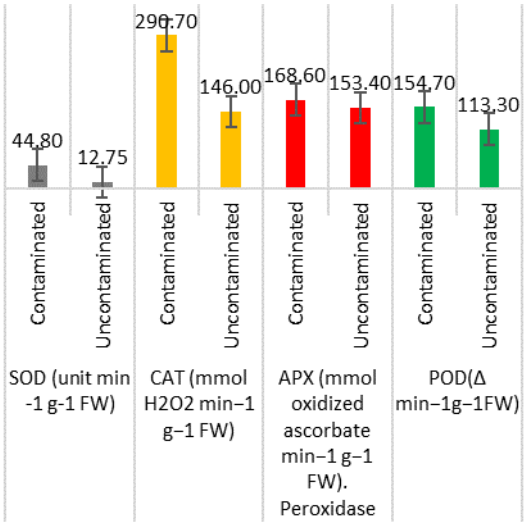

Figure 3. Effects of heavy metal on superoxide dismutase, catalase, ascorbate peroxidase, and peroxidase activity in leaves and of olive trees.

Table 4. The concentration of heavy elements in olive trees (tissues) and soil (Contaminated area).

Heavy metals (mg/g dry weight)	Type	Mean
Cu	Leaf	0.347[b]
	Fruit	0.078[c]
	Soil	1.311[a]
Fe	Leaf	0.155[b]
	Fruit	0.097[b]
	Soil	0.431[a]
Ni	Leaf	0.788[b]
	Fruit	0.086[c]
	Soil	1.089[a]
Cd	Leaf	1.294[b]
	Fruit	0.023[c]
	Soil	1.89[a]
Pb	Leaf	0.365[a]

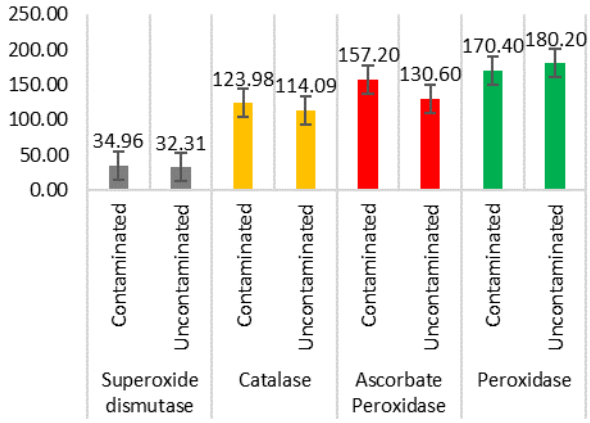

Figure 4. Effects of heavy metal on superoxide dismutase, catalase, ascorbate peroxidase, and peroxidase activity in fruit and of olive trees.

3.5. **Isozyme Overlay Pattern**

Tables 5 show the isozyme overlay patterns in olive leaves based on Native-PAGE-Electrophoresis. The symbols (+ and -) indicated the presence or absence of bands, respectively. Upon exposure to various heavy metals, peroxidase activity demonstrated varying degrees of activity. The analysis of catalase patterns based on polymorphism percentage, as well as the presence, absence, quantity, and kind of these patterns between stressed and control trees, is summarized and shown in Tables 5-6. Seven bands in all were found using CAT-isozyme analysis. Three polymorphic bands, representing 42.9% polymorphism, were found among the seven treatments, with Rf values of 0.109, 0.412, and 0.592. In contrast to the control, which displayed one absent band at Rf 0.881 of leaf, stressed leaf and fruit tissues displayed three absent bands at Rf 0.571, 0.722, and 0.820 (for leaf). control leaf tissues were compared to analyze the isozymes. Tables 6–7 show how the polymorphism was determined as a percentage. The gel was separated, then scanned and captured on camera (Fig. 5). Eight bands were identified in the PPO-isozyme by the analysis, six of which were polymorphic and accounted for 75% of the polymorphism. These polymorphic bands were identified by Rf-values of 0.222, 0.271, 0.425, 0.527, 0.552, and 0.772. Two common bands were also indicated, accounting for 25% of the total. The stressed leaf tissues exposed to heavy elements only lacked two bands, while the control sample exhibited the absence of five bands.

Table 5. The quantity and kinds of bands and the proportion of overall polymorphism produced by two isozymes from olive (*Olea europaea*).

Isozymes	Total bands	Monomorphic	polymorphic bands		Polymorphic (%)
			Unique	Non unique	
Catalase	7	3	1	3	42.9
Polyphenol oxidase	8	2	0	6	75

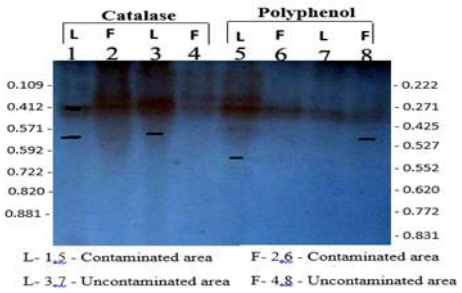

Figure 5. Electrophoretic patterns of catalase, polyphenol oxidase, and isozymes of tissue of *Olea europaea* tree.

Table 6. The activity patterns of CAT and PPO isozymes in the leaves and fruit of *Olea europaea* under normal and stress conditions.

catalase group	Rf	Contamination		Un Contamination	
		leaf	fruit	leaf	fruit
CAT 1	0.109	+	+	+	+
CAT 2	0.412	+	+	+	+
CAT 3	0.571	-	-	+	+
CAT 4	0.592	+	+	+	+
CAT 5	0.722	-	-	+	-
CAT 6	0.820	-	+	+	+
CAT 7	0.881	+	+	-	+
Total bands	7	4	4	6	5
Polyphenol oxidase group	Rf				
PPO 1	0.222	+	+	-	-
PPO 2	0.271	+	+	-	+
PPO 3	0.425	+	+	-	-
PPO 4	0.527	+	+	-	-
PPO 5	0.552	-	+	+	-
PPO 6	0.620	-	-	-	-
PPO 7	0.772	+	+	-	
PPO 8	0.831	+	+	+	+
Total bands	8	6	7	2	2

5.Desiccation:

Olive fruits develop temporary wrinkles due to root dysfunction, resulting in reduced water absorption

from physiological stress and potassium/calcium insufficiency caused by harmful soil components. Stress can impact plants by causing nutrient deficiencies, even when those nutrients are abundant in the soil. Calcium and potassium are vital nutrients that impact various physiological and biochemical processes, influencing plant growth and metabolism. These factors greatly enhance plants' ability to withstand various environmental stresses. They are involved in most cell metabolic and signal transduction activities and are essential in plant signal transduction as a second messenger. Calcium recognizes and quickly reacts to external stress signals, aiding in maintaining the cell wall and membrane's equilibrium. Furthermore, calcium promotes fruit set and fertilization, lowering flower and fruit loss following setting [19].

The color change may appear uniformly throughout the plant, starting with the older leaves. This happens when the plants are building less chlorophyll. Fruit growth will also slow down and be smaller than normal. Phosphorus deficiency results in slowed growth and decreased fruit development. The leaves will turn reddish, starting with the older leaves. A high pH may cause nutrients to take on a new form that the plant finds difficult or ineffective to absorb. Since heavy metals contribute significantly to pH decline, the effect was more noticeable in stressed olive trees than in the control group.

The results indicate that the leaf area decreased when the soil was contaminated with heavy metals, due to the impact of these elements on photosynthesis, proteins, and carbohydrates [20]. Under normal growth conditions, chlorophyll and carotenoids are synthesized in chloroplasts in a coordinated manner, both in quantity and quality.

Plants' leaf area is influenced by all genetic, environmental, and nutritional factors that affect leaf formation and size. For plants to have the largest possible leaf area, nutrients must be available; a lack of nitrogen decreases the number of growing leaves and cell division in the growing tips, which in turn reduces leaf area. Under normal growth conditions, chlorophyll and carotenoids are synthesized in chloroplasts in a coordinated manner, both in quantity and quality. However, when plants experience stress, the balance of pigment synthesis shifts towards the production of carotenoids, which alters the ultrastructure of chloroplasts. This shift leads to the degradation of chlorophyll and, ultimately, a significant reduction in biomass and plant weight [19]. The lack of accumulation of various metabolites within individual cells was the reason for this decrease in biomass. This is consistent with the study of Kouki *et al.* [21].

Plants have evolved defense mechanisms to combat the negative impacts of environmental stressors like drought, heavy metals, and other environmental conditions, including increased proline buildup [22]. Many previous studies and research have shown a close relationship between the ability of plants to resist metal stress and the concentration of proline in plant leaves (a direct relationship). In this study, we found that olive trees, when exposed to high concentrations of heavy metals, take protective measures to avoid tissue damage, and increasing the accumulation of proline molecules is one of the most important of these measures because, in addition to its effective role in the metabolism process as a protein component, proline is a soluble substance. Its distribution is widely compatible, and its accumulation is often associated with the severity of environmental stresses [23], as it accumulates in plants during negative ecological constraints. It plays an important role in stress tolerance and has been considered a factor in stabilizing proteins and molecular structures and maintaining osmotic pressure [21].

The accumulation of proline was also marked in olive trees under different stresses, such as heavy metals [24], and salt stress [22]. Our results are consistent with the findings of Mousavi *et al.* [25], who suggested that Cu stress increased the level of proline in leaves of olive trees in comparison to the control. Similar results were obtained ~~from~~ olive tree leaves under copper stress by Rana *et al*. [26].

Proline accumulated results from the disruption of amino acid breakdown caused by mineral stress. Also, the accumulation of glutamate and ammonia in plants exposed to stress leads to the induction of proline formation by direct and indirect effects on the ratio of building materials and resulting materials. Carbohydrates also play a role in inducing proline by inhibiting the breakdown enzymes and reducing their activity (proline dehydrogenase and proline oxidase).

Radical and non-radical forms of ROS are created as byproducts of metabolic processes in various locations within the cell. Plants undergo oxidative stress due to the synthesis and production of ROS in higher quantities in cellular compartments when transition heavy metals and other environmental stressors are present. Cell proteins, lipids, and nucleic acids are all damaged.

Plant cells have developed various enzymatic and non-enzymatic defense mechanisms to combat the threat of reactive oxygen species (ROS). The primary antioxidant enzymes include superoxide dismutase, catalase, glutathione peroxidase, and ascorbat~~e~~ peroxidase.

Peroxisomes include glutathione peroxidase and catalase, while mitochondria, apoplasts, cytosol, chloroplasts, and ascorbate peroxidase are also found in peroxisomes [27]. The ascorbate-glutathione cycle is present in most cellular compartments studied so far. Because APX has a high affinity for hydrogen peroxide (H_2O_2), it is likely that this cycle plays a crucial role in regulating reactive oxygen species levels in these compartments [27]. The production of toxic active oxygen species, which results in oxidative stress, is one of their negative impact on plants. We found through this study that the increase in the

concentration of phenolic compounds was clear in the leaves and fruits of olive trees growing in soil contaminated with heavy elements due to their defensive role against oxidation processes. This agrees with Lee *et al.* [28], on the study of the mango plant. Increased activity of antioxidant enzymes and elevated levels of non-enzymatic components are essential for plants to endure stressors such as metal toxicity. Initially, it was thought that these components primarily acted as osmotic buffers. However, besides aiding in osmotic adjustment, they also play a critical role in maintaining the native state of macromolecules, likely by scavenging reactive oxygen species (ROS). Strong evidence suggests that an effective antioxidative system is linked to reduced oxidative damage and enhanced tolerance to environmental stressors.

Research indicates that higher antioxidant levels and improved activity of radical-scavenging enzymes are associated with plants' tolerance to oxidative stress. When plants face environmental stressors, the balance between the production of reactive oxygen species (ROS) and the detoxification capabilities of the antioxidant system can be disrupted, leading to oxidative damage. An enhanced ability to scavenge or detoxify activated oxygen species is linked to increased tolerance to harmful environmental conditions. Additionally, ascorbate peroxidase and superoxide dismutase (SOD) were somewhat inhibited under high Pb and Cd stress, and excessive ROS also led to a noticeably increased degree of oxidative damage [24].

Electrophoretic patterns of peroxidase isozymes revealed eight bands in the control group and seven in the catalase group. The bands and the activity of the esterase isozyme are decreased, and those of the peroxidase isozyme are increased. According to Yahia *et al.* [29], peroxidase isozymes can mark salinity stress tolerance in plants. They discovered that the peroxidase isozyme profile changed under stress conditions, potentially leading to shifts in gene expression within the cells. In general, isozyme pattern variations can be identified on zymographs. Either non-expression or induction of isozymes is of immense significance, especially under stress, which severely hampers the normal metabolism of plants and their physiological state during development. The number of isozymes obtained varied markedly with the specific enzyme.

6.Recommendation:

The environment and the health of people, animals, and plants are directly harmed by pollution. Since soil is a non-renewable natural resource that can take thousands of years to build and renew, soil pollution is a serious issue and challenge for residents and government organizations. At the same time, soil can respond quickly to environmental deterioration, sometimes occurring within a few years or decades. Therefore, we recommend reducing human activities that are the main cause of soil, water, and plant pollution and trying to get rid of pollutants using many well-known preventive or therapeutic procedures.

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