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# Responses of Antioxidants Enzymes to Oxidative Stress in the Floral Species Drimia Maritime

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## Abstract

In natural habitats, plants that encounter adverse growth conditions are preserved in natural reserves where rare or endangered species, such the rare plant species "*Drimia maritima*" are protected. However, abiotic stress, generated by air pollution near Bentael natural reserve - Lebanon, where '*Drimia maritima*" is widespread, is suspected to induce alteration in the biochemical status of the plant. The antioxidant potential was determined by measuring the activities variations of five different enzymes related to stress: Catalase (CAT), Superoxide Dismutase (SOD), Ascorbate peroxidase (APX), Guaiacol peroxidase (POX), and Glutathione reductase (GR). The study was conducted in Bentael natural reserve that was exposed to road works on its borders, within three various locations: location 1 directly localized on the road (P1), location 2, opposite to P1 (P2) and a control location in the middle of the reserve (Ctrl). All enzymes showed higher activities in P1 than in the other sites with proportional correlations for all the studied sites. The resulting outcome demonstrate that alteration in enzymes activities are significant element in plant responses to environmental derivatives abiotic stresses which appraise the danger of landscape activities on conserved areas' wild floral species.

## Keywords

Abiotic Stress, Antioxidant Enzymes, Antioxidant potential, Reactive Oxygen Species

## Introduction

"*Drimia maritima*"(L.) Baker, is a medicinal, ornamental plant native to especially the Mediterranean area and which belongs to the family Liliaceae. It was registered as rare species by the UNEP (United Nations Environment Program), and, in Lebanon, it is mostly found in natural reserves. Unfortunately, in a Mediterranean Reserve, specifically in Lebanon (Bentael natural reserve- Byblos), "*Drimia maritima*" is facing the danger of air pollution: a new road has been inaugurated in the south area of the reserve and it is threatening its ecosystem balance by creating an environmental stress on the fauna and flora.

Abiotic stress, major factor that affects productivity of plants, is known to stimulate the generation of "Reactive Oxygen Species" (ROS). Although these species are natural byproducts of any cell, stresses manage to disrupt the cellular homeostasis by accumulating ROS. Therefore, to identify the stress condition, the detection of ROS is necessary and hydrogen peroxide was chosen as indicator of stress effects.

Toxic hydrogen peroxide is a ROS that can act both as reductant and oxidant. This form is the most structure of ROS with a speedy capacity of diffusion across cell membranes. It instigates oxidative damage in leaf cells beginning with tissue injury and leading to senescence promotion after disruption of cell integrity and metabolic function. Its damages reach surrounding cells and initiate an antioxidative response.

One of the forms of protection against oxidative damage is enzymatic defenses. Several enzymes are known to act as antioxidants, the most important are superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6), guaiacol peroxidase (POX, E.C. 1.11.1.7), glutathione reductase (GR,

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E.C. 1.8.1.7) and ascorbate peroxidase (APX, E.C. 1.11.1.11) [1]. These enzymes, present in practically all cellular compartments, act as scavengers of ROS. The activities of these enzymes can serve as indicator of stress tolerance.

## Material and Methods

### Hydrogen peroxide determination

According to [2], 400-500mg of fresh leaves were homogenized with 5mL trichloroacetic acid (0.1%) in an ice bath. After homogenization, separation of the phase by centrifugation was done for 15 minutes at 3000g. The supernatant was then collected. 0.5mL of the supernatant was mixed with 0.5mL of potassium phosphate buffer (10mM, pH 7) and 1 mL potassium iodide (1M). The absorbance was recorded at 390nm.

### Enzymes' extraction

Fresh leaves were thoroughly grounded in an ice bath with potassium phosphate buffer (100mM, pH 7) containing poly vinylpyrrolidone (1%) and 0.2g quartz sand for until no fibrous residue can be seen. After grinding, centrifugation was realized with MPW-350R refrigerated laboratory centrifuge at 4000 rpm for 15 min and the supernatant was collected for enzyme's assays [3].

### Enzyme's activity assay

**Superoxide dismutase activity:** SOD activity was recorded by the diminution in absorbance of superoxide nitro blue tetrazolium complex by the enzyme. 0.1 mL of enzyme extraction was incubated with 3 mL of reaction mixture (0.5mL of 13µM methionine, 0.5mL of 63µMnitro-blue tetrazolium (NBT), 0.5 ml of 1.3µM riboflavin, 0.5 ml of 0.05M sodium carbonate and 1mL distilled water) for 10 min at 25°C under fluorescent lamp illumination. Absorbance was read at 560nm **[4]**.

**Catalase activity:** CAT activity was estimated according to [5]. 1 mL of the enzyme extract was added to 4 mL of the reaction mixture (1 mL of 0.01 M $H_2O_2$ and 3 mL of 0.1 M sodium phosphate buffer). Reaction is initiated by adding hydrogen peroxide, and stopped after adding 10mL of 2% $H_2SO_4$and incubation at 25°C for 5min. The titration of the was realized against 0.005 N potassium permanganate.

**Ascorbate peroxidase activity:** According to [6], 10µL of the enzyme extract was added to 1 mL of reaction mix (100 mM tris-acetate buffer at pH 7.0, 2 mM ascorbic acid, and 2 mM of $H_2O_2$). Every 100s, the absorbance decrease was noted at 290 nm following the oxidation of ascorbate and calculated using extinction coefficient Ɛ=2.8 $mM^{-1}.cm^{-1}$.

**Activity:** Guaiacol oxidation method was used to determine peroxidase activity. 3 mL reaction mixture (10 mM potassium phosphate buffer (pH 7.0), 8 mM guaiacol) was added to 10µl enzyme extract. The reaction was initiated by adding 0.5 mL of 1% $H_2O_2$. The absorbance's increase due to guaiacol oxidation, was recorded every 30 s at 470nm [7]. The unit of peroxidase activity was expressed using the extinction coefficient Ɛ=6.39 $mM^1cm^{-1}$.

**Glutathione reductase activity:** Glutathione reductase was assayed using the method described by [8]. The reaction was initiated by adding 0.1 mL of 2 mM oxidized glutathione (GSSG) into the mixture (1 mL of 0.2 M potassium phosphate buffer (pH=7.5), 0.1 mL of 2 mM NADPH, 0.1 mL enzyme extract and distilled water to make up a final volume of 2.9 mL). The increase in absorbance at 412 nm recorded over a period of 5 min at 25°C on Thermo/Evolution600/160908 UV/VIS Spectrophotometer.

## Results and Discussion

### Hydrogen peroxide

Hydrogen peroxide levels in "*Drimia maritima*" leaves are drawn in Figure 1. A first remark to note is that the uppermost recorded levels of hydrogen peroxide are in P1. It is shown clearly a stark difference between P1, P2 and Ctrl. Hydrogen peroxide's level decreased from P1 to P2 to Ctrl, which indicated that oxidative stress is mainly established in P1.

Hydrogen peroxide has many essential roles in plant metabolism; it's involved in variety of reactions and signaling cascades necessary for all aspect of plant growth and integration of activities [9], but, at the same time, accumulation of hydrogen peroxide related to virtually any environmental stress is potentially damaging [10].

### Superoxide dismutase

Plant is showing its highest SOD activity in P1 from both years except for May 2015 and June 2016. Ctrl is recording the lowermost activities side for all months except December 2016 year. In addition, SOD activity is increasing over the months from year to year. Airborne pollution stress had induced a gradual raise in the activity of SOD in "Drimia maritima" showing that the plant has established a strong antioxidant activity. Nonetheless, this raise in SOD activity is proven that airborne pollution stress is still affecting plants in the vicinity of road even after finishing road work activities. Superoxide dismutases constitute the first line of defense against ROS [11,12]. And because they are present in all subcellular locations, it is obvious that their activities increase with the establishment of oxidative stress.

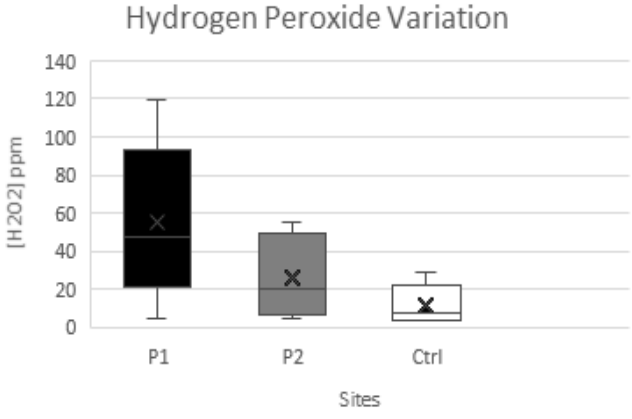


**Figure 1:** Hydrogen Peroxide variation between sites.

## Catalase

Catalase activity recorded its highest intensity in P1 sites all over the studied year except for May 2015 where P2 recorded the extreme activity. The increase of CAT activity is predicable according to the fact that SOD activity increased in the same manner. CAT has a high specificity for hydrogen peroxide [13], so its activity is related to the concentrations of the latter.

## Ascorbate peroxidase

APX activity is an ascending order in P1and P2 all over the studied years except for May 2015. In Ctrl from December 2016, a sharp rise in activity was observed. APX is known to be anefficient regulator of ROS specially for being present in a diverse number of subcellular organelles [14].

## Glutathione reductase

GR is showing its highest activity in P1 all over the months but a sharp rise in this activity was observed in December 2016. GR acts by recycling oxidized glutathione with the use of NADPH as co-factor [15].

## Guaiacol peroxidase

GPX activity is higher in P1 in all years and months except for Ctrl in December 2016, this latter result concord with the one observed in SOD an APX activities, which suggest the presence of another kind of stress that could be from biotic origin.

In the presence of oxidative stress, the studied enzymes above (SOD, CAT, GR, APX, GPX) are considered the predominant ROS scavenging system in the plant. SOD is the only known enzyme to use a free radical as a substrate [16]; nonetheless, the reaction is followed by the generation of another ROS. The effectiveness of this system is in the increased activity simultaneously of CAT and peroxidases in order to scavenge hydrogen peroxide generated by SOD. Figure 2 confirm these postulations as it is noticed synchronized fluctuations of the levels of the three enzymes [17] (Table 1).

Multiple t-test were conducted to compare the studied parameters in the months for two years of study in June 2015, the enzymes presented significant differences In December 2015, the significance was restrained to hydrogen peroxide In the second year, the only enzyme that presented significant variances was catalase along with a difference in hydrogen peroxide As shown in the table above, the differences between the antioxidants and the stress markers indicated by their significance the presence of an intense stress (Table 2).

An analysis of variance (One way ANOVA) with tukey comparisons showed a significant difference of hydrogen peroxide between sites, months and years. The significant differences were observed in enzymes when comparisons were done between sites versus month and year.

# Conclusion

In recent years, attention on the effect of anthropogenic activities on ecological communities has increased. Air quality is highly affected by human activities. Bentael nature reserve is facing a huge threat of road works on its borders [18]. The evaluation of the pollution used "*Drimia maritima*", a rare and sensitive species to pollution, to appraise the real danger of this threat, and specialized methods to estimate the oxidative stress generated from landscape activities [19]. The measurements of oxidative stress markers and scavengers in "*Drimia maritima*" demonstrated its fight against stress. The time-course evolution of Hydrogen peroxide (a Reactive Oxygen Species) contents revealed a high degree stress. Enzymatic antioxidants, presented at high values in P1,

**Table 1:** *t-test* for enzymatic stress scavengers.

| | ***Enzymatic*** | | | | |
|---|---|---|---|---|---|
| | **SOD** | **CAT** | **APX** | **GR** | **GPX** |
| | ***Sample*** | | | | |
| ***MP1-15*** | 0.884 | 0.063 | 0.649 | 0.101 | 0.435 |
| ***MP2-15*** | 0.05 | 0.023 | 0.259 | 0.32 | 0.26 |
| ***MCtrl-15*** | 0.019 | 0.938 | 0.003 | 0.16 | 0.003 |
| ***JP1-15*** | 0 | 0 | 0 | 0.209 | 0.055 |
| ***JP2-15*** | 0.001 | 0.001 | 0 | 0.002 | 0 |
| ***JCtrl-15*** | 0 | 0.205 | 0 | 0.001 | 0.001 |
| ***DP1-15*** | 0.295 | 0.012 | 0.004 | 0.135 | 0.002 |
| ***DP2-15*** | 0.135 | 0.047 | 0.004 | 0.002 | 0.004 |
| ***DCtrl-15*** | 0.044 | 0.751 | 0.007 | 0.003 | 0.006 |
| ***MP1-16*** | 0.09 | 0.021 | 0.245 | 0.563 | 0.235 |
| ***MP2-16*** | 0.149 | 0.024 | 0.985 | 0.211 | 0.947 |
| ***MCtrl-16*** | 0.124 | 0.208 | 0.154 | 0.057 | 0.161 |
| ***JP1-16*** | 0.179 | 0.03 | 0.089 | 0.713 | 0.059 |
| ***JP2-16*** | 0.839 | 0.001 | 0.091 | 0.634 | 0.074 |
| ***JCtrl-16*** | 0.503 | 0 | 0.007 | 0.319 | 0.01 |
| ***DP1-16*** | 0.038 | 0.009 | 0.008 | 0.002 | 0.003 |
| ***DP2-16*** | 0.187 | 0.002 | 0.012 | 0.038 | 0.013 |
| ***DCtrl-16*** | 0.051 | 0.018 | 0.006 | 0.249 | 0.005 |
| *p<0.05* | | | | | |

**Table 2:** One way ANOVA analysis and tukey comparisons: between sites, between months, between years, between site vs year, between site vs month vs year.

| ***F-value*** | | | | | |
|---|---|---|---|---|---|
| ***Compound*** | SITE | MONTH | YEAR | SITEX YEAR | SITEX MONTHX YEAR |
| $H_2O_2$ | 7.33** | 6.75** | 2.84 | 6.97*** | 15.43*** |
| | (a b b) | ( b a a) | ( a a) | ( a b b ) | (a b c ) |
| ***SOD*** | 3.53* | 3.26* | 7.87* | 3.34* | 8.37*** |
| | (a a b) | (a b a) | (b a) | (a a b) | (a b c) |
| ***CAT*** | 11.8*** | 4.78** | 0.0033 | 4.57** | 38.33*** |
| | (a a b) | (a b b) | (a a) | (a a b) | (a b c) |
| ***APX*** | 0.68 | 7.48** | 19.64*** | 4.3** | 49.27*** |
| | (a a a) | (b b a) | (b a) | (a a b) | (a b c) |
| ***GR*** | 5.49** | 3.48* | 0.17 | 2.42* | 29.87*** |
| | (a b b) | (a b b) | (a a) | (a a b) | (b b a) |
| ***GPX*** | 1.26 | 0.82 | 0.03 | 0.94 | 1.03 |
| | (a a a) | (a a a) | (a a) | (a a a) | (a a a) |

[a]*, ** and *** indicate significance at $p < 0.05$ with weak, moderate and highly significance

[b]Letters between parentheses show the differences among SITES, in the order P1, P2 and CTRL. Different letters denote a statistical difference with 95% confidence level (TUKEY test), with "a" being the highest concentration.

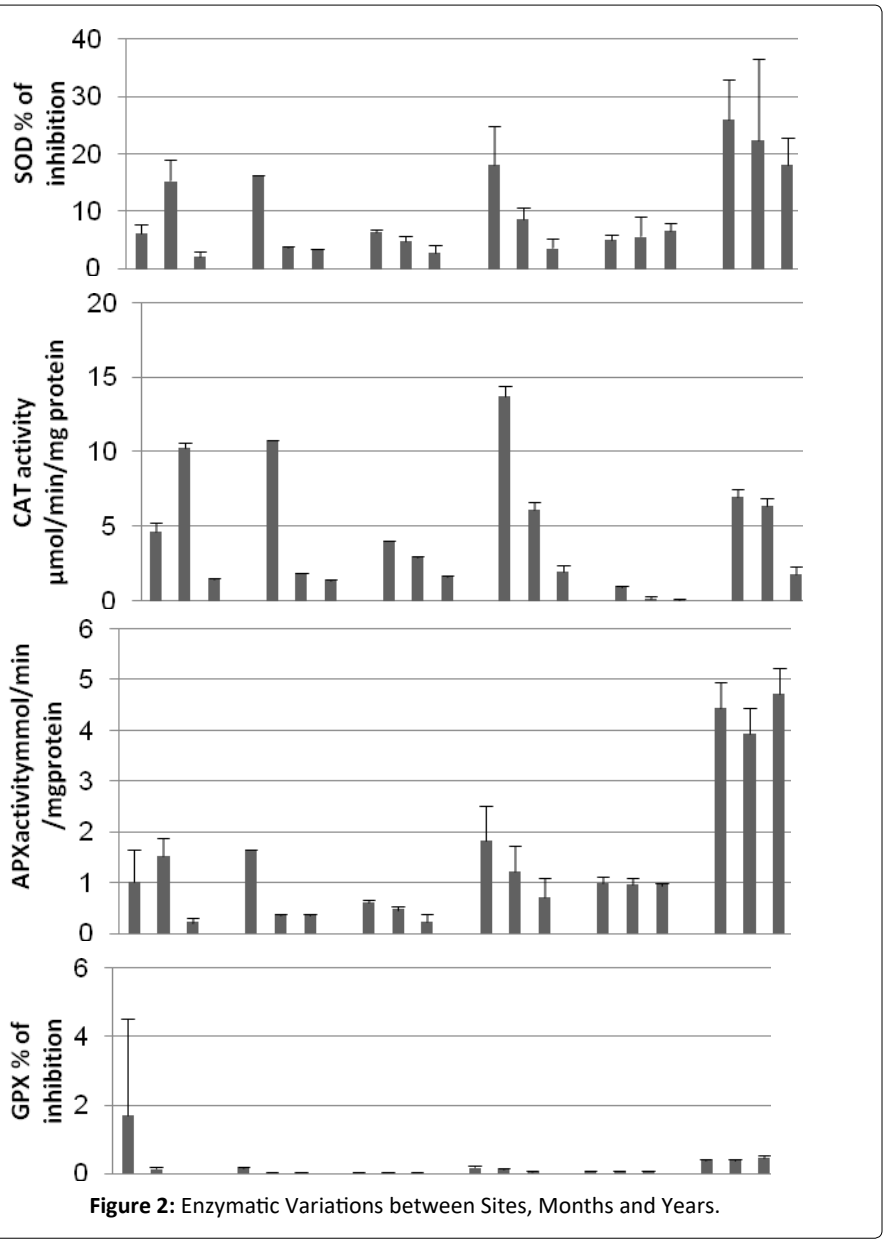


**Figure 2:** Enzymatic Variations between Sites, Months and Years.

confirmed the results of the stress marker hydrogen peroxide and pointed the danger of these anthropogenic activities. Many solutions are described such as the implementation of weather stations that include pollution detectors, the establishment of biomonitoring programs and the most important proceedings that can be executed are the organization of Green belts around cities [18].

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