The Effect of Antioxidant Proteins due to Salt Stress and Wounding in *Vicia Faba* against Bean Yellow Mosaic Virus

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Abstract: Environmental stresses like salinity and wounding are very harmful to plants and cause major economical losses especially if the plant is a major crop like bean. Exposure of plants to those types of stresses cause the production of reactive oxygen species which in turn damage the plant cellular system. To reverse this lethal effect, plants have developed a counter attack mechanism to adjust the oxygen level in the cells through anti-oxidant enzymes, a process known as oxygen scavenging. In this research, experiments have been conducted to investigate the relation between the type and magnitude of the stress, together with the timeline of bean yellow mosaic virus (BYMV) inoculation and the level of anti-oxidant enzymes if any, and the resistance to the virus infection. Salinity and mechanical wounding were used on the bean plants as types of the stress, three types of salinity concentrations and two types of mechanical wounding were used, and then the leaves of the stressed plants were inoculated with the virus immediately, after 6 hours, 1 and 3 days to study the systemic effect of the stress on signaling any antioxidant enzymes on those time intervals, and in one and two weeks as well to determine the state of the enzyme in the plant. The enzymes assayed were catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), and guaiacol-specific peroxidase (POX). Results revealed that there is a correlation between the stress and the level of the enzymes in the plant. These enzymes seem to trigger the induced resistance in the bean plants to the BYMV.

Keywords: Abiotic stress, antioxidant, ROS, oxygen scavenging, antiviral

Introduction

Due to its low cost, and being a very rich source of protein, *V.faba* or broad beans is a very important crop for humans and animals as well. It was also reported that *V.faba* has a big role in biological fixation of aerial nitrogen (Jelenic et al, 2000).

BYMV, on the other hand, is a wide host range virus that infects *V.faba* systemically, and although the virus does not really kill the plant it can spread very fast in the crop leading to a great economical loss (Checng et al, 2002).

Stresses are the negative impacts of pathogens, environment, and other species on plants and they represent major restrictions in crop agriculture. Flowers and Yeo stated that 50% of the crop land in the world is salt stressed (Flowers and Yeo, 1995). A stress can be biotic where fungi, bacteria, virus or an insect can harm a plant in some way. It can also be abiotic**,** where the harm can be caused by unavoidable factors like salinity, heat, mechanical wounding, intense sunlight, heavy rain, drought, and wind. Abiotic stresses can be even more harmful when combined together (Mittler, 2006).

Environmental stresses can however, induce plant resistance against pathogens (Babosha, 2008). Barley has been reported to have an induced resistance against pathogens when stressed abioticaly (Wiese et al., 2004). It had been reported as well that heat shock could help induce cross adaptation to many environmental challenges in maize (Ming et al., 2004).

The Prime_A_Plant Group et al., studied the state of plant priming, which is a state induced in the plant when it gets attacked by insects, pathogens or when it gets subjected to abiotic stress. The group reported that this primed state of the plant activates the defense responses of the plant more quickly and strongly than the usual (Prime-A-Plant Group et al., 2006).

To better understand how stress can induce resistance in the plant against pathogens attacks, the metabolism inside the stressed plant should be understood. Consider salinity as an example of a stress, as the salt increases in the soil the level of water decreases and consequently the ion uptake increases and reactive oxygen species increase. This abnormal generation of reactive oxygen species is the main reason of oxidative

damage to the lipids, proteins and nucleic acids of the plant cells (Noctor and Foyer, 1998, and Mittler, 2002). There are however, a series of enzymatic systems that developed in plants to revert the effect of the reactive oxygen species and conduct an oxygen scavenging process (Sairam and Tyagi, 2004). On the other hand, when a localized injury happens to a plant leaf by a herbivore, an insect or a mechanical wounding, the injury activates local and systemic responses include metabolic changes and a disorder in the damaged tissues cell structure associated with the drastic loss of water, and consequently increasing the level of the ROS in the plant, which activates the defense mechanisms systemically in the whole plant via complex wound signals (Pearce et al., 1991).

The way this oxygen scavenging works is like this: the plant gets exposed to a stress, at some point stress result in an increased level of ROS. The successive reduction of molecular oxygen to H_2O yields the intermediate radicals $O_2^{\cdot-}$, $HO^{\cdot}$ and H_2O_2 which are toxic. Now the elevation in the level of ROS triggers the production of antioxidant enzymes that naturalize the reactive bad radicals to produce more stable harmless compounds. This process is using multiple enzymes including GR, CAT, POX, and SOD.

Now, the question is, “can those detoxification enzymes used in the process of oxygen scavenging, be the reason of enhancing the plant induced resistance against pathogens?”. It was reported that doubling the glutathione in transgenic tobacco plants caused the seedlings to grow faster than the ordinary plants (Roxas et al., 1995), and that mechanical wounding of sunflower plants triggered the accumulation of the S-nitrosothiols which constitutes a signal of the detoxification process in the plant (Mounira et al., 2011). SOD was also names as the first line of antioxidant defense because it can remove the superoxide anion produced during the bio-oxidation process (Bowler et al., 1992).

In this paper, the *vicia faba* plants will be subjected to either salinity or mechanical wounding stress and then the stressed plant will be examined using the bioassay against the BYMV. For those plants showing resistance against the virus further experiments will take place to determine the level of detoxification enzymes developed in those plants and compare it with the corresponding levels in non stressed plants to determine the protective role of antioxidants expressed due to stress and the acquired resistance of the plant against the BYMV.

Materials and Methods

Plant Material

Seeds of *V.faba* (L) were surface disinfected with solution of mercuric chloride (0.1%) for 30 sec, and were washed immediately and germinated in pots containing vermiculite. Plants grown for 30 days under constant environmental conditions (23°C day : 18°C night) and were watered twice a week.

Virus inoculum

BYMV inoculum was obtained from infected *Vicia faba* leaves ground using a pestle and mortar with a little acid washed sand and distilled water (1:2 w/v). The bulk of the leaf debris and sand was removed by squeezing the pulp through three layers of muslin. The extract was centrifuged at 4,000 xg for 15 min, and the supernatant decanted and kept at room temperature over night to precipitate any proteinaceous virus inhibitor presented in the leaf sap. The supernatant was clarified by further centrifugation at 3,000 xg for 15 min. For each one of the abiotic stress treatments an exact replica of the plant was prepared for the viral bioassay after the treatment.

Abiotic stress

Different treatments according to the type of abiotic stress were applied on the bean plants. After each treatment and according to the corresponding time course the control leaves as well as the treated leaves were inoculated with BYMV according to the method below.

Salt stress

Bean plants were subjected to salinity stress by watering the base by 100, 200, and 300 mM NaCl.

Mechanical wounding

Leaves were wounded using a plastic brush having two rows. Some leaves were punctured with around 200 punctures and some were punctured with around 600 punctures.

Plant viral bioassay

Designated primary leaves of fourteen days old bean plants were inoculated with BYMV. Inoculation was done under green house conditions at 25 ± 5 °C, by dusting virus inoculum with Carborandum (600 mesh). Ten replicates were made for each virus inoculation.

The antiviral bioassay was done on the test plants with same height, and age. For each treatment, ten replicates of equal size were used. For controls, test plant leaves were treated directly with the virus inoculum without prior treatment of salinity or mechanical wounding of the adjacent leaves. After 0, 6 hours, 1 and 3 days of the stress, the designated leaves were sprinkled very lightly with 600mesh carborundum powder and inoculated gently and uniformly with virus inoculum. After inoculation, leaves were washed with distilled water. Plants were observed for the development of mosaic symptoms after 15 days. The inhibitory activity of the BYMV symptoms on bean plants due to stress was calculated according to the ratio between obviously infected plants (showing systemic symptoms) to the total inoculated plants.

SDS-polyacrylamide gel electrophoresis

Discontinuous SDS-PAGE was carried out in 12% separating gel with a 5% stacking gel according to Laemmli (1970). The proteins were visualized by staining with 0.1% Coomassie brilliant blue R-250.

Chemicals

All chemicals used in the assay of enzymes were purchased from Sigma Aldrich.

Extraction of enzymes and antioxidants

Bean leaves were homogenized with 100 mM sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, and 5 mM -mercaptoethanol. The homogenate was filtered through four layers of cheese cloth, centrifuged at 45,000Xg for 20 min. The supernatant was used as source of enzymes, antioxidants, and other components. All the steps in the preparation of the enzyme extract were carried out between 0 to 4°C.

Assay of enzymes

CAT activity was determined by measuring the rate of disappearance of H_2O_2 following the procedure of Dhindsa et al, (1981). 0.5g of leaf sample was homogenized in 5mL of 50mM potassium phosphate buffer pH=7 and 1% PVP. Homogenized samples were centrifuged at 4°C for 10mins at 15000g. An aliquot of 1mL of the supernatant of the enzyme extract was added to the reaction mixture containing 1 mL of 1.5 M H_2O_2 and 3mL of 50 mM potassium phosphate buffer pH=7. Decrease in H_2O_2 is followed as decline 240 nm during 30 sec.

The activity of GR, on the other hand was assayed in 2 mL of 100 mM TRIS-HCl buffer (pH=7.2) containing 0.2 mM NADPH, 5mM glutathione disulphide (GSSG) and 100 µL of plant extract (Anderson, 1996). The change in absorbance at 340 nm was recorded at 25°C in a spectrophotometer. Enzyme activity was based on the oxidation rate of NADPH using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

POX activity was measured by monitoring the formation of tetra guaiacol at 470 nm, using H_2O_2 as substrate (Chance and Machly, 1955). One unit of peroxidase is defined as the amount of enzyme that caused the formation of 1 mM of tetra-guaiacol per minute.

Finally, SOD was extracted in 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1% Triton X-100 and 1% (w/v) soluble polyvinylpyrrolidone (PVP-10), and its activity was determined by the ferric cytochrome *c* method.

Statistical analysis

All data in the tables below are the means of at least ten values. Minitab statistics software was used to compare the mean values together. Analysis of variance (ANOVA) was used and the mean values were compared using lowest standard deviation test with $P<0.05$ for significant difference.

Results and Discussion

Induction of resistance as a result of salt stress

The roots of *Vicia faba* plants of about 14 days of age were watered with 100, 200, and 300 mM NaCl and the primary leaves of each of the stressed plants were inoculated with BYMV after 0, 6, 24, and 72 hours of the watering. Results in table 1 and figure 1 below, show that exposing the roots of the Bean plants to the different salt concentrations did not affect the response of the plant to the virus inoculation or induce any kind of

resistance when it is conducted immediately after salinity stress. After six hours though, the plant started to show up a peak of resistance to the virus for all concentrations. This resistance increased with the increase of concentration. This induced resistance decreased from six hours to a day and sort of stabilized afterwards till three days. These results come in accordance with those reported by Lu et al., (2003), stating that salinity treatment increased the resistance of *Suaeda salsa* against heat stress, and that this resistance increases with the salinity concentration up to 400mM NaCl.

Table 1: Percent inhibition of salt stressed *Vicia faba* plants to BYMV varying by time of inoculation after the salinity stress, and concentration of the salt stress

Time of inoculation after the stress in hours	0	6	24	72
% Inhibition at 100mM salt stress	10±0	60±1.1	50±2	50±0.1
% Inhibition at 200mM salt stress	10±0	80±0.7	60±0.7	60±1.3
% Inhibition at 300mM salt stress	10±0	80±0.9	70±0.8	60±0.9

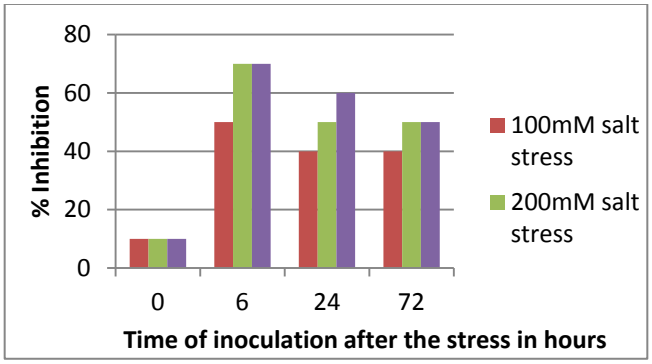

Figure 1: Percent inhibition of salt stressed *Vicia faba* plants to BYMV varying by concentration of the salt stress and timeline of virus inoculation after the stress.

Induction of resistance as a result of leaves mechanical wounding

The designated leaves of bean plants of about 14 days of age were wounded using a plastic brush having two rows. Leaves were punctured with 200, and 600 punctures. After 0, 6, 24, and 72 hours of leaves damage the adjacent leaves were inoculated with BYMV. Results in table 2 and figure 2 below, show that damaging the leaves and inoculating the adjacent ones immediately did not induce any resistance in the plant at all. Some resistance though, started to show up systemically, after 6 hours of leaves damage. This induced resistance persisted after one day of inoculation and started to decay on three days. Those results suggest that, damaging the leaves of the V*icia faba* plants did not induce the resistance until after 6 hours and showing a peak of resistance induction between 6 and 24 hours.

These results agree with those of Francia et al., (2007) who reported that wounding can induce resistance to pathogens with different lifestyles in tomato. Also, Li et al., (2009) discovered a novel Wall-associated protein kinas (WAK) gene induced in rice after mechanical wounding; this gene was proven to play an important role in plant defense. It was also reported by Ito et al., (2002), that cell death and wounding in tobacco plant induced a receptor-like protein kinase gene, which agrees with the results shown here.

Table 2: Percent inhibition of the leaves adjacent to mechanically wounded leaves of *Vicia faba* plants to BYMV varying by time of inoculation after the damage, and the magnitude of the damage

Time of inoculation after the stress in hours	0	6	24	72
% Inhibition at 200 punctures	10±0	30±0.3	25±1.1	10±0.6
% Inhibition at 600 punctures	10±0	35±0.6	30±0.3	20±1.5

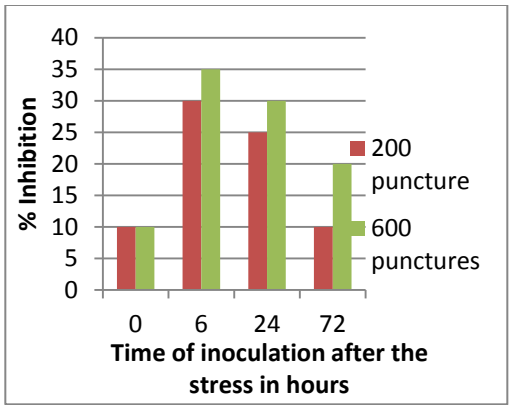

Figure 2: Percent inhibition of the leaves adjacent to mechanically wounded leaves of *Vicia faba* plants to BYMV varying by hours of inoculation after the damage, and the magnitude of the damage

Effect of stress on the total protein in the *V.faba* plants

In order to gain more understanding on what happened inside the *V.faba* plants to induce the resistance to the BYMV as noticed above, SDS-PAGE was carried over to get an insight on the proteins inside the plant and to see if there undergo any change with the different timeline and magnitude of the stress.

Results in figure 3, and figure 4 below and based on the denseometric scanning of protein patterns for the different stress magnitude and timeline (data now shown), show that there was some variation in the number of separated bands and the concentration of protein in each particular band. This variation depends on the type, magnitude of the stress and the timeline.

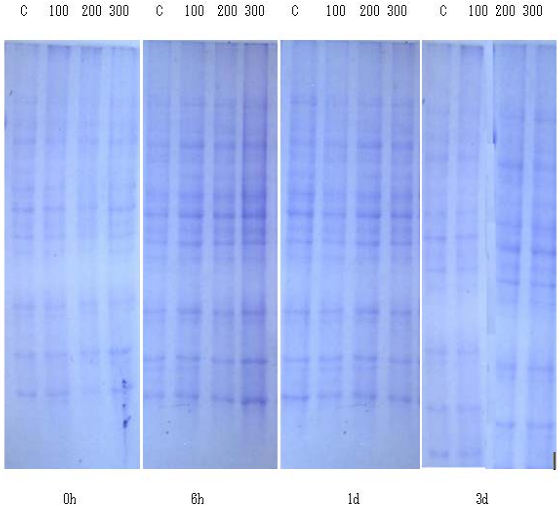

Figure 3: The SDS-PAGE of salt stressed *V.faba* plants varying by concentration of salt and time after the stress

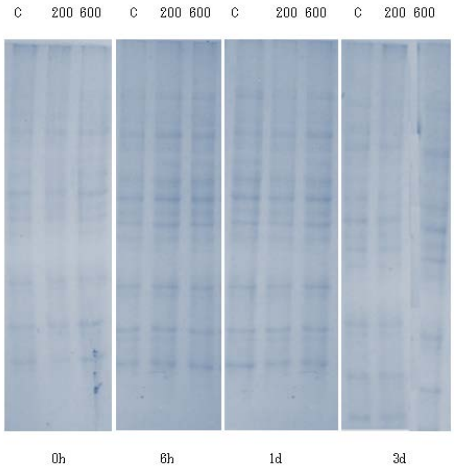

Figure 4: The SDS-PAGE of wounding stressed *V.faba* plants varying by magnitude of the wounding and time after the stress

Assay of antioxidant enzymes

GR, CAT, POX, and SOD were assayed in the leaves of the stressed, non-infected bean plants, at 0h, 6h, 1d, 3d, 1w, and 2w, where h stands for hour, d stands for day, and w stands for week. The reason the assay of the enzymes was conducted on non-infected plants is that the virus itself is a biotic stress to the plant and might as well increase the level of antioxidant enzymes and in this case the assay conducted will not be objective and cannot judge for sure if the environmental stresses used generated any enzymes or not. For every assay in the stressed plant another assay has been done for the same enzyme in the non-stressed plant having exactly the same conditions to serve as a control. Results are shown in tables 3 to10 and figures 5 to 12 below. Results in tables and figures 5 to 8 show that there is almost no change in the enzymes level when measured at the time of the stress, and that there is a very noticeable elevation in the level of the four enzymes for all concentrations, comparing to the control started after six hours of the salt stress, that is in case of 100mM salt stress, 1800 for GR, 1100 for CAT, 1700 for POX, and 530 for SOD (all in IU/g tissue). This elevation was stable till after one day of stress, showing 1650 for GR, 820 for CAT, 1550 for POX, and 500 for SOD, and started to decay suddenly starting at the third day of the stress in case of GR showing 850, and POX showing 800. In case of CAT, and SOD, on the other hand, enzymes levels were 700 for CAT, and 320 for SOD, and there was a steady decay till the second week after the salt stress. Results for the 200mM, and 300mM, were similar to that of 100mM with the exception of the enzyme levels were higher in higher stress concentrations. Comparing with the result of the viral bioassay of the salt stressed plants in table 1, it can be concluded that the enzymes level elevation in the salt stressed plants implied the induced resistance inside the plant till the first day after the stress. At the third day however, the levels of the enzymes showed dramatic decay and this decay was not accompanied with a similar decay in the resistance induced in the plant against the BYMV, which implies that maybe those enzymes elevation in the plant triggered the systemic acquired resistance (SAR) of the plant against the virus and then enzymes started to decay to the normal level, but the resistance persisted in the plant. Those results come in accordance with those reported by Lu et al., (2003)

For mechanical wounding on the other hand, for 200 punctures stress, GR gave a measurement of 900 IU/g tissue after six hours of wounding, while CAT gave 930, 900 for POX, and finally 500 for SOD, showing an elevation in the level of the all enzymes but much less than their corresponding value in the salt stress. These values persisted till the first day after the stress showing 950 for GR, 750 for CAT, 950 for POX and finally 410 for SOD. Those values started to drop after he first day dramatically to almost back to normal level after two weeks of stress.

Results showed that the elevation of the enzymes in the mechanical wounding stress were much smaller than those in the case of salt stressed plants which can be perfectly correlated to the results of the virus resistance which gave better inhibition of the virus in case of salt stress than in the case of mechanical wounding. Results also showed that as the magnitude of the stress increases the enzymes levels increase and the SAR increases. Results agree with those reported by Prime-A-Plant_Group et al., (2006), and Francia et al., (2007). So, it can be concluded from these results that when the *V.faba* plants suffer salt and wounding stress and consequently suffer from an elevation in the level of ROS, the level of antioxidant enzymes increase. This increase triggers the systemic acquired resistance in the plant to fight pathogenic attacks like BYMV.

Table 3: GR enzymatic activity of the leaves of salt stressed *Vicia faba* plants, varying by time after the stress, and the concentrations of the stress

Time	0h	6h	1d	3d	1w	2w
Enzyme activity in 100mM salt stressed plant	100±0.3	1800±2.0	1650±0.4	850±1.5	350±0.3	120±0.5
Enzyme activity in 200mM salt stressed plant	110±0.3	1900±1.4	1600±0.6	870±1.1	400±0.2	100±2
Enzyme activity in 300mM salt stressed plant	100±0.2	1920±0.7	1700±1.5	900±1.2	420±0.2	150±0.5
Enzyme activity in control plant	90±0	90±0	90±0	90±0	90±0	90±0

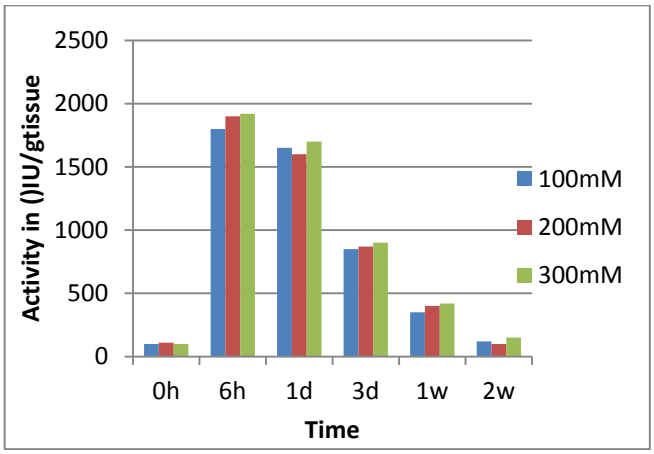

Figure 5: GR enzymatic activity of the leaves of salt stressed *Vicia faba* plants, varying by time after the stress, and the concentrations of the stress

Table 4: CAT enzymatic activity of the leaves of salt stressed *Vicia faba* plants, varying by time after the stress, and the concentrations of the salt stress

Time	0h	6h	1d	3d	1w	2w
Enzyme activity in 100mM salt stressed plant	90±0.1	1100±1.3	820±0.6	700±0.4	230±0.7	115±0.1
Enzyme activity in 200mM salt stressed plant	110±1.1	1300±0.9	900±0.7	750±0.6	300±1.5	210±0.3
Enzyme activity in 300mM salt stressed plant	100±0.3	1500±0.4	1010±0.3	830±0.4	310±0.3	115±0.2
Enzyme activity in control plant	80±0	80±0	80±0	80±0	80±0	80±0

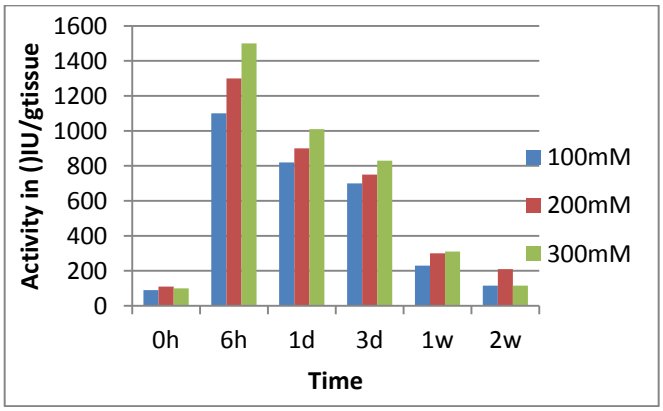

Figure 6: CAT enzymatic activity of the leaves of salt stressed *Vicia faba* plants, varying by time after the stress, and the concentrations of the salt stress

Table 5: POX enzymatic activity of the leaves of salt stressed *Vicia faba* plants, varying by time after the stress, and the concentrations of the stress

Time	0h	6h	1d	3d	1w	2w
Enzyme activity in 100mM salt stressed plant	100±0.1	1700±1.2	1550±0.2	800±0.2	420±0.1	300±0.1
Enzyme activity in 200mM salt stressed plant	110±0.5	1750±0.3	1600±0.5	760±0.4	400±0.3	310±0.3
Enzyme activity in 300mM salt stressed plant	100±0.3	1900±0.3	1600±0.3	850±0.3	515±0.4	370±1.2
Enzyme activity in control plant	95±0	95±0	95±0	95±0	95±0	95±0

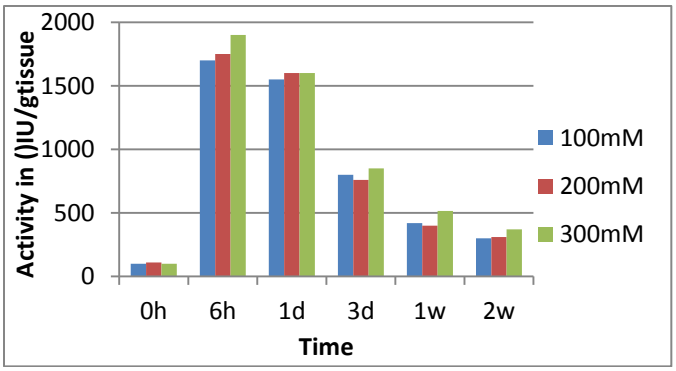

Figure 7: POX enzymatic activity of the leaves of salt stressed *Vicia faba* plants, varying by time after the stress, and the concentrations of the stress

Table 6: SOD enzymatic activity of the leaves of salt stressed *Vicia faba* plants, varying by time after the stress, and the concentrations of the salt stress

Time	0h	6h	1d	3d	1w	2w
Enzyme activity in 100mM salt stressed plant	50±0.1	530±0.1	500±1.2	320±0.1	150±0.1	75±0.1
Enzyme activity in 200mM salt stressed plant	45±0.4	600±0.2	550±0.2	400±1.9	200±0.2	100±0.3
Enzyme activity in 300mM salt stressed plant	75±1.2	730±0.1	700±0.4	600±01.3	230±0.4	80±0.1
Enzyme activity in control plant	30±0	30±0	30±0	30±0	30±0	30±0

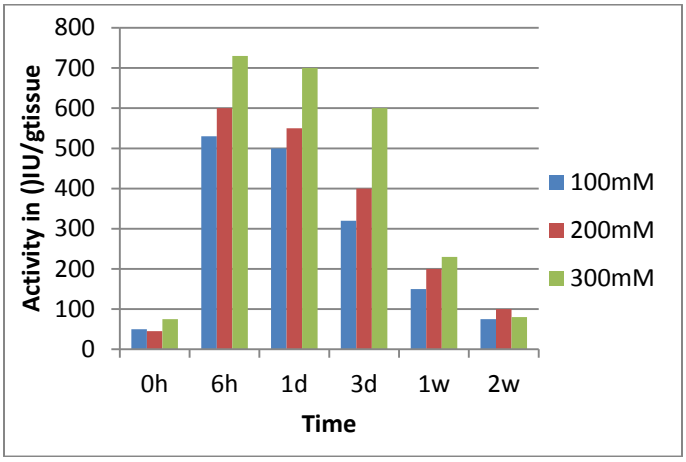

Figure 8: SOD enzymatic activity of the leaves of salt stressed *Vicia faba* plants, varying by time after the stress, and the concentrations of the salt stress

Table 7: GR enzymatic activity of the leaves adjacent to mechanically wounded leaves of *Vicia faba* plants, varying by time after the stress, and the magnitude of mechanical wounding

Time	0h	6h	1d	3d	1w	2w
Enzyme activity in 200 punctured stressed plant	120±0.1	900±0.2	950±0.3	320±0.2	200±0.1	100±0.2
Enzyme activity in 600 punctured stressed plant	100±0.2	950±0.3	970±1.2	250±0.3	260±0.6	130±0.4
Enzyme activity in control plant	90±0	90±0	90±0	90±0	90±0	90±0

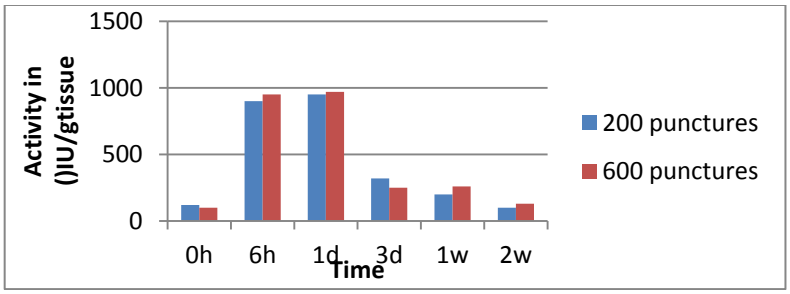

Figure 9: GR enzymatic activity of the leaves adjacent to mechanically wounded leaves of *Vicia faba* plants, varying by time after the stress, and the magnitude of mechanical wounding

Table 8: CAT enzymatic activity of the leaves adjacent to mechanically wounded leaves of *Vicia faba* plants, varying by time after the stress, and the magnitude of mechanical wounding

Time	0h	6h	1d	3d	1w	2w
Enzyme activity in 200 punctured stressed plant	90±0.1	930±0.1	750±0.3	200±0.7	150±0.7	110±0.3
Enzyme activity in 600 punctured stressed plant	75±1.2	800±0.2	710±0.5	310±0.3	170±0.5	130+1.2
Enzyme activity in control plant	80±0	80±0	80±0	80±0	80±0	80±0

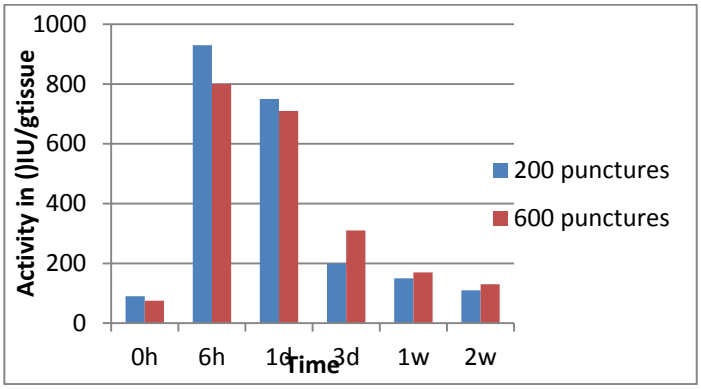

Figure 10: CAT enzymatic activity of the leaves adjacent to mechanically wounded leaves of *Vicia faba* plants, varying by time after the stress, and the magnitude of mechanical wounding

Table 9: POX enzymatic activity of the leaves adjacent to mechanically wounded leaves of *Vicia faba* plants, varying by time after the stress, and the magnitude of mechanical wounding

Time	0h	6h	1d	3d	1w	2w
Enzyme activity in 200 punctured stressed plant	120±0.1	900±0.3	950±0.6	320±0.3	200±0.3	110±0.3
Enzyme activity in 600 punctured stressed plant	100±0.3	950±0.2	970±0.5	250±0.7	220±1.1	120±0.2
Enzyme activity in control plant	95±0	95±0	95±0	95±0	95±0	95±0

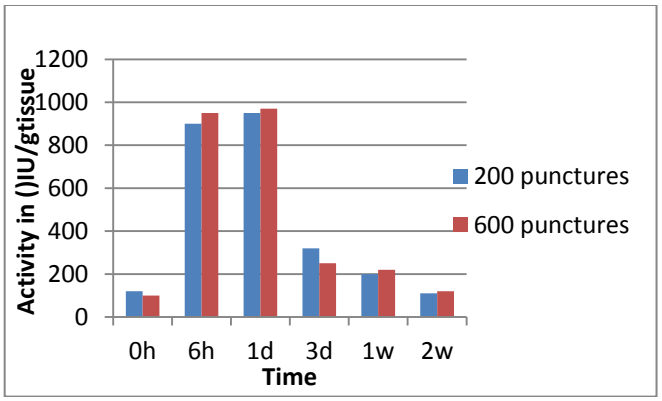

Figure 11: POX enzymatic activity of the leaves adjacent to mechanically wounded leaves of *Vicia faba* plants, varying by time after the stress, and the magnitude of mechanical wounding

Table 10: SOD enzymatic activity of the leaves adjacent to mechanically wounded leaves of *Vicia faba* plants, varying by time after the stress, and the magnitude of mechanical wounding

Time	0h	6h	1d	3d	1w	2w
Enzyme activity in 200 punctured stressed plant	35±0.1	500±0.4	410±0.2	320±03	120±0.5	75±0.6
Enzyme activity in 600 punctured stressed plant	55±0.2	620±0.3	500±1.5	350±0.9	130±0.3	60±0.3
Enzyme activity in control plant	30±0	30±0	30±0	30±0	30±0	30±0

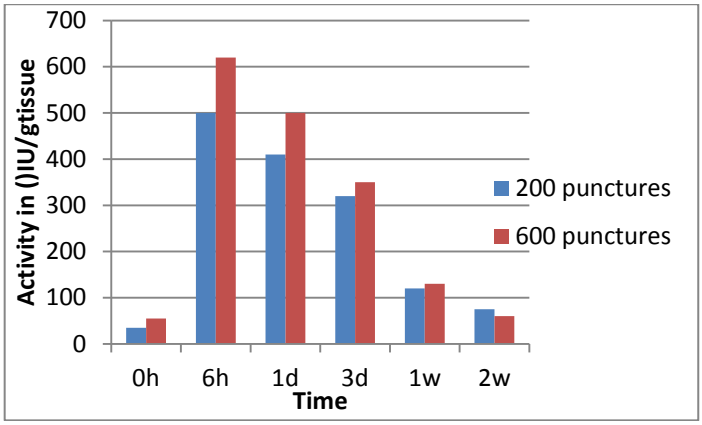

Figure 12: SOD enzymatic activity of the leaves adjacent to mechanically wounded leaves of *Vicia faba* plants, varying by time after the stress, and the magnitude of mechanical wounding

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