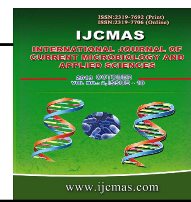

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 2 Number 10 (2013) pp. 485-495**
http://www.ijcmas.com

Original Research Article

Oxidative Stress Enzymes in *Busseola fusca*

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Keywords

Oxidative stress; reactive oxygen species; *Busseola. Fusca*; *Bt* maize; ascorbic peroxidases; glutathione S-transferases.

A B S T R A C T

Insects are exposed to endogenous oxidative stress resulting from aerobic metabolism and exogenous oxidative species that are plant defenses against them. As a result insects produce a number of antioxidant enzymes for detoxifying these reactive oxygen species (ROS). These enzymes include catalases (CAT), ascorbic peroxidases (APOX), glutathione S-transferases (GST), and glutathione reductases (GR). In the present study *B. fusca* larvae were either fed 7-day old *Bt* maize plants (MON8810) / non-transformed plants or 28-day old *Bt* maize plants/ non-transformed plants; on all four treatments 3rd instar larvae were fed for a period of 7 days. Catalase (CAT), ascorbate peroxidase (APOX), glutathione reductase (GR) and glutathione-s-transferase (GST) were demonstrated to be present in larvae from all four treatments. The levels of both CAT and APOX activity were significantly higher in larvae fed control plants, irrespective of plant age (approx 136% and 60% for 7-day old and 28-day old plants respectively, for CAT compared to *Bt* fed larvae; approx 51% and 7% for 7 day old and 28-day old plants respectively, for APOX compared to *Bt* fed larvae). GST was also significantly higher in larvae fed the young control plants (150%), but lower in the older plants compared to the *Bt*-fed larvae. GR activity, on the other hand, was higher in larvae fed 7-day old *Bt* plants, but lower in the older plants relative to the control. Although the data was significant ($p<0.01$), these differences were small.

Introduction

Reactive oxygen species (ROS) are derived from the metabolism of molecular oxygen (Halliwell, 1999). These reactive oxygen species are superoxide anion radicals (O^-_2), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl (OH^-) radical (Waris and Ahsan, 2006). Oxidative stress occurs when the balance between ROS and antioxidants is disrupted because of excess ROS, depletion of antioxidant or both (Waris and Ahsan, 2006). ROS are responsible for peroxidation of membrane lipids and micromolecules such as proteins and DNA (Ahmad et al 1990). Besides causing oxidative damage to midgut cells high concentrations of ROS also impairs

the absorption of ingested nutrients (Bi and Felton, 1995). This toxicity of oxygen resulting from aerobic metabolism is called endogenous oxidative stress (Pardini, 1995). Herbivorous insects also have to content with exogenous reactive oxygen species that are part of plant defense against them (Orozco-Cardenas and Ryan 1999).

Insects produce many enzymes for detoxifying ROS. These enzymes are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), glutathione transferase peroxidase (GSTPX) glutathione S-transferase (GST) and glutathione reductase (GR). SOD converts O^-_2 to molecular O_2 and H_2O_2 (Fridovich, 1978). H_2O_2 is scavenged by CAT, resulting in the production of water and molecular oxygen (Table 1). CAT however, is not able to scavange hydrogen peroxide at low concentrations, this is done by ascorbate peroxidate (Clavaron-Mathews *et al.,* 1997).

There are two types of glutathione – related enzymes associated with the protection against oxidative stress. These are glutathione peroxidase (GPx) and glutathione S-transferase (GST). GPx is a selenoprotein and is highly specific for glutathione (GSH) as a hydrogen donor (Sagara and Sugita 2001). Glutathione S-transferase are a major group of detoxification enzymes (Hayes and Pulford, 1995). They play a critical role in protecting tissue against oxidative damage and oxidative stress.

All eukaryotic species posses multiple cytosolic and membrane-bound GST isoenzyme (Hayes and Pulford, 1995). In mammalian organs GSTs can constitute up to 10% of cytosolic protein (Boyer, 1989). The activity of GPOX in herbivorous insects is very low (Ahmed et al 1987). Glutathione reductase (GR) enables GSTPX function by regenerating the reduced form of glutathione (Wang *et al.,* 2001). It also reduces the oxidised form of glutathione (GSSH) (Fridovich 1975). High midgut expression of antioxidant genes in herbivorous insects is hypothesized to be a protective response to ROS ingested during feeding or generated during food processing (Krishnan and Kodrik, 2006).

The study was conducted to determine the presence of the following oxidative enzymes in the midgut homogenate of *B. fusca*; catalase, ascorbate peroxidase, glutathione reductase and glutathione –S-transferase.

Materials and Methods

Insect material

Third instar larvae were obtained from the International Centre for Insect Physiology and Ecology, Nairobi, Kenya. Insects were kept in controlled environmental chambers under the following conditions: 80% RH, 25°C, L12:D12. Larvae were maintained on an artificial diet as previously described (Onyango and Ochieng-Odero, 1994) before the start of the trials.

Plant material

Genetically modified maize expressing Bt Cry1Ab (Event MON810) and the non-transformed parental near isogenic line were grown under 25°C, L16:D8. Leaves were harvested when the plants were 7 and 28 days old. MON810 and the corresponding control line were supplied by Monsanto Company, St Loius.

Feeding Trials

Leaf bioassays were carried out using excised leaves from 7days old Bt maize (MON 810) plants, or the non-transformed parental line as control and 28 days old Bt maize (MON 810) plants, or the non-transformed parental line as control; 25 larvae were used per treatment. The bioassays were carried out using 3rd instar larvae (L3) but in independent experiment. The leaves (on moist filter paper to prevent desiccation) were changed every second day.

Extraction of soluble proteins for enzyme activities

Busseola fusca larvae were cold anaesthetised and whole guts dissected into chilled deionised water (1gut/50µl). Guts tissues were homogenized in 1mL of extraction buffer (100mM potassium phosphate buffer pH 7.5, 1mM DTT, 3mM EDTA, 0.4% TritonX-100 [w/v], 2% PVPP [w/v]) and centrifuged at 8000 rpm for 8 minutes at 4^oC. The protein content of the supernatant was determined by Bradford (1976) method and samples were stored at -80 ^oC.

CAT activity

The reaction mixture composed of phosphate buffer, sample extract and 3% hydrogen peroxide. Boiled samples with no CAT activity served as controls. Change in absorbance was measured at 240nm over a 3min period and decrease in absorbance was recorded. CAT activity was expressed as micromoles of hydrogen peroxide reduced per minute per milligram of protein, using an extinction coefficient of $39.4 mM^{-1}cm^{-1}$.The activity was expressed as % relative activity.

Native Gel Electrophoresis

Samples (15µg) were mixed with glycerol in the ratio of 3:1 and were loaded onto 10% native polyacrylamide gel bathed in Tris-glycine buffer (3g/L Tris + 14.4g/L glycine) according to the modified procedure of Beauchamp and Fridovich (1971). Briefly the proteins were separated at constant current (40mA) at 180V. The gels were run in the cold room at 4 ^{o} C.

Staining for CAT

CAT activity staining was performed according to Chandlee and Scandalios (1983). The gels were first pre-treated in 0.01% H_2O_2 for10 minutes and then incubated in staining solution for 15 minutes. The staining solution contained: 0.05M K-Kphosphate pH 7.5, 10mM EDTA (1ml 0.5M stock), 30mM riboflavin, 17µl Temed,10mg Nitrotetrozolium blue.

Ascorbate Peroxidase

Tissues were homogenised in buffer (phosphate 50mM) containing 0.5mM ascorbic acid. The reaction mixture contained 0.5mM ascorbic acid, 0.3% hydrogen peroxide and the supernatant of sample. Boiled samples served as controls. The rate of change in absorbance at 290nm was measured for 10 min in spectrophotometer**.** Enzyme activity was expressed as micromolar ascorbate oxidised per min per mg protein using a molar extinction coefficient of $2.8 mM^{-1}cm^{-1}$

Spectrophotometric Enzyme Assay for GST

Glutathione S-transferase activity was measured according to the methods of

Habig et al (1974). The method uses 1-chloro-2-4-dinitrobenzene (CDNB) as a substrate. Assays were performed in a reaction mixture containing 0.1M potassium phosphate buffer, 1mM GSH, 1mM CDNB and 30µg of tissue homogenate. The GST was dissolved in the potassium buffer and CDNB was dissolved in ethanol. Enzyme activity was determined by monitoring changes in absorbency at 340nm at a constant temperature of 25°C.

Spectrophotometric Enzyme Assay for GR

Glutathione Reductase activity was assayed according to the method of Racker (1955). Briefly GSSG was used as a substrate which was reduced to GSH. NADPH was used a reductant. The disappearance of NADPH due to oxidation to $NADP^+$ was monitored at 340nm. The reaction mixture contained 100mM phosphate buffer, 1mM EDTA, 1mM GSSG, 0.05mM NADPH and 15µg of sample. The temperature was set at 25°C and absorbance was measured at 340nM in quartz plate.

Native gel electrophoresis for GR

Samples were run on 7.5% polyacrylamide gels. Running conditions were the same as described above for CAT.

Staining for GR

GR activity was detected on native PAGE gels in 50mL of phosphate buffer containing: 3.4 mM GSSG, 0.4 mM NADPH, 1.2mM TT(MeOH) , 0.04mM DCIP.

Statistical analysis

The mean activity for the different treatments was compared using Student-t-test to test for significant difference among the means. The relative activity (%) was determined by equating the highest activity to 100% and all the other activity was expressed as percentage relative to the highest activity.

Result and Discussion

Catalase

Native gels stained for catalase (Fig1) showed the presence of this enzyme in *B. fusca*. However, there appeared to be no difference in the intensities of the bands for the four experimental treatments. The bands indicated high molecular weight proteins. Enzyme activity assay showed significant difference ($P<0.01$) between the insects fed on control 7 days old plants and those fed on *Bt* 1 week old plants. Activity in control fed plants was approximately 136% more than in *Bt* fed insects. The same trend was also observed in insects fed on 28 days old plants. Insects fed on 28 days old control plants had approximately 60% more activity than insects fed *Bt* plants (Fig2). Insects fed transgenic maize had suppressed level of catalase in both treatments (7 days old plants and 28 days old plants). The results were not expected because catalase plays a crucial role in scavenging hydrogen peroxide to produce water and molecular oxygen. This may mean that the amount of hydrogen peroxide produced by control plants was more the one produced by *Bt* maize plants because catalase scavanges hydrogen perioxide at high concentrations. It was however; think that this scenario is highly unlikely.

Ascorbate peroxidase

Assaying for enzyme activity showed marked differences between the

treatments. There was significant difference ($P<0.01$) in activity between the insects fed 7 days old control plants and those fed 7 days old *Bt* plants. Catalase activity in control plants was significantly higher than the activity for insects fed *Bt* plants. Insects fed on control 1 week old plants had approximately 51% more activity than insects fed on 7 days old *Bt* plants. The same trend was also observed for insects fed 28 days old plants. The activity was significantly ($P<0.05$) higher for control fed insects than insect fed *Bt* plants. Insects fed 28 days old control plants had 7% more activity than insects fed on 28 days old *Bt* plants (Fig 3). Ascorbate peroxidise scavanges hydrogen peroxide at low concentrations. The results were not expected particularly considering the above results for catalase. If control plants produced higher concentrations of hydrogen peroxides than *Bt* plants the activity of catalase will be higher for control plants. However, the activity of ascorbate peroxidase will therefore be expected to be higher in insects fed on *Bt* plants because this enzyme scavanges hydrogen peroxide at low concentrations.

Glutathione reductase

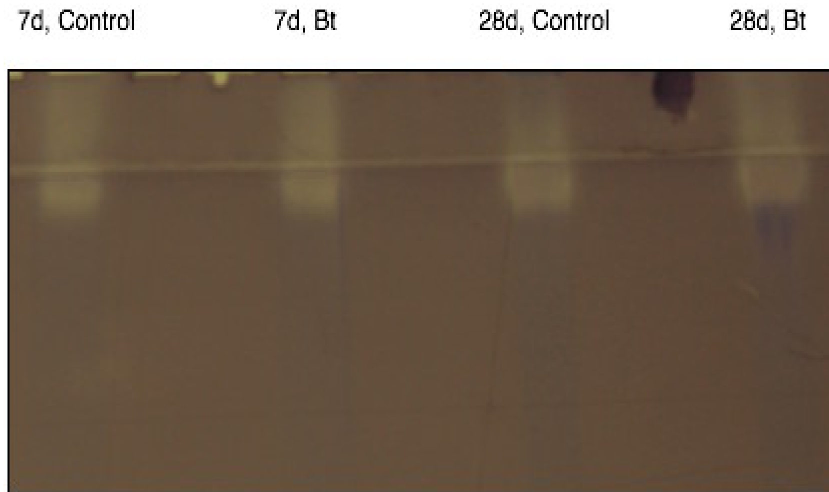
Native gels stained for glutathione reductase showed the presence of this enzyme in both treatments (Fig4). It was difficult to quantify the activity in these gels and spectrometric assays were done to determine the activity. Activity for glutathione reductase was significantly ($P<0.01$) higher for insects fed 7 days old *Bt* plants compared to those fed on 7 days control plants. Insects fed on 7 days old *Bt* had 9% more activity than those fed on 7 days old control plants. However, for 28 days plants the results were different. Activity was higher for insects fed control plants compared to those fed *Bt* plants (Fig 5). Insects fed on 4 weeks old control plants had 12% more activity than those fed on 28 days old *Bt* plants.

Glutathione S-Transferase

There was significant difference between the enzyme activity for insects fed control plants and those fed *Bt* plants. Insects fed on 7 days old control plants had higher activity (150%) than those fed 7 days *Bt* plants. However, insects fed on 28 days control plants had lower (34%) activity than those fed on 28 days *Bt* plants (Fig6). In both cases the difference was statistically highly significant at 99% confidence level. Glutathione S-transferase protects tissues against oxidative stress and oxidative damage. We expected insects fed *Bt* plants to be under more oxidative stress than those fed control plants hence more Glutathione S-transferase activity. However, the insects may not have been feeding on the *Bt* plants due to the effects of the toxin. *Bt* plants have been found to have ant-nutritive effects on insects.

The imbalance between ROS and antioxidant defenses results in oxidative stress (Ahmad *et al.,* 2006). This stress leads to peroxidation of membrane lipids (Ahmad et al 2006) and macromolecules such as DNA and proteins (Ahmad et al 1990), Oxidatively modified proteins are dysfunctional and lose catalytic or structural integrity (Krishnan and Kodrik, 2006). Protein carbonyls will tend to accumulate on the side chains of proteins as a result of oxidative stress (Krishnanand Kodrik, 2006). Oxygen toxicity resulting from aerobic metabolism is referred to as endogenous oxidative stress. Herbivorous insects are also exposed to exogenous oxidative stress resulting from plant defenses against them (Orozco-Cardenas and Ryan 1999). Insects have evolved systems to cope with both endogenous and

Figure.1 Catalase activity stain on 7.5% native-PAGE gels



Lane 1 whole gut homogenate of insects fed on 7 days non-transformed maize plants (control fed); lane 2, whole gut homogenate of insects fed on 7 days old transgenic maize (Bt fed); lane 3 whole gut homogenate of insects fed on 28 days old non-transformed maize plants; lane 4 whole gut homogenate of insects fed on 28days old transgenic maize

Figure.2 CAT relative activity of insects fed on; 7 days old non-transformed maize plants (7dys Cntrl); 7 days transformed maize plants (7dys Bt); 28 days old non-transformed maize plants (28dys Cntrl); and 28 days old transformed maize plants (28 Bt). Points and bars represent mean ± SE for triplicated independent determinations

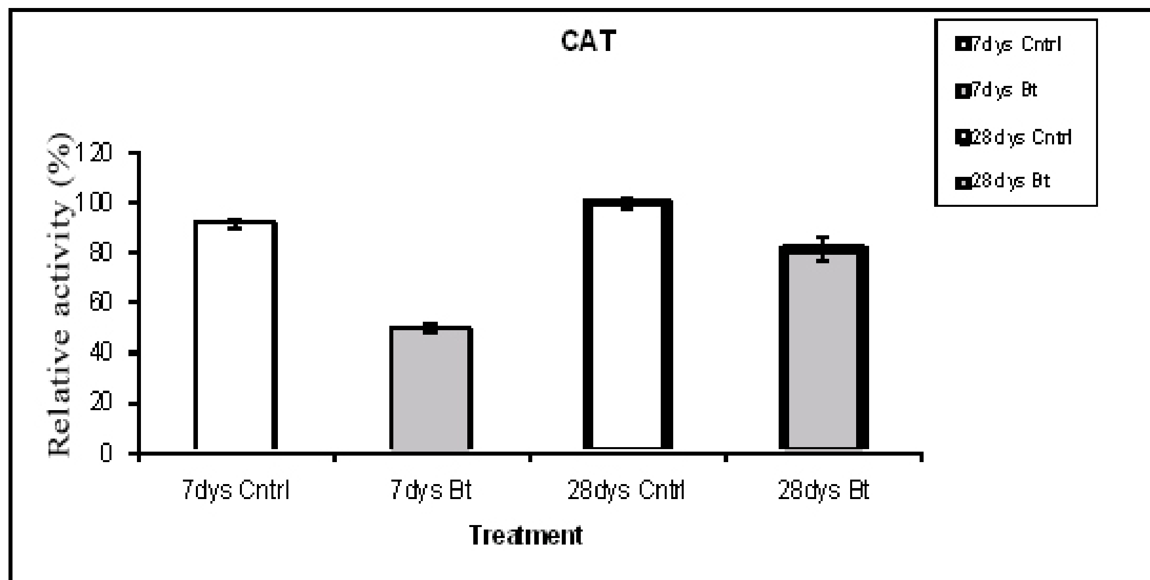


Figure.3 APOX relative activity of insects fed on; 7 days old non-transformed maize plants (7dys Cntrl); 7 days transformed maize plants (7dys Bt); 28 days old non-transformed maize plants (28dys Cntrl) ; and 28 days old transformed maize plants (28 Bt). Points and bars represent mean ± SE for triplicated independent determinations.

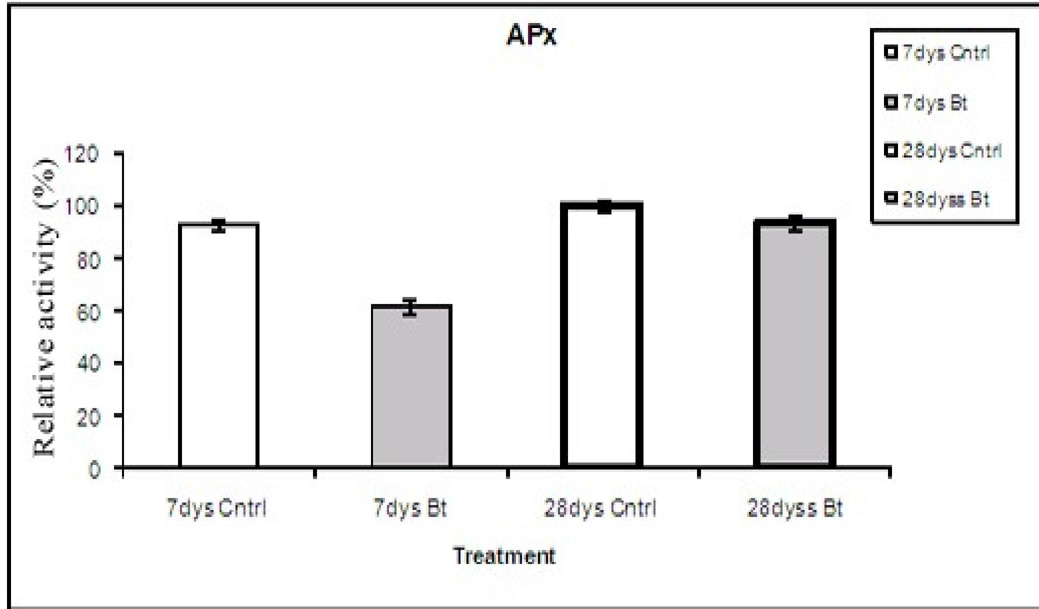


Figure.4 GR activity stains on 7.5% native-PAGE gels. Lane 1 whole gut homogenate of insects fed on 28days old transgenic maize (Bt fed); lane 2, whole gut homogenate of insects fed on 28 days old non-transformed maize plants (control fed); lane 3, whole gut homogenate of insects fed on 7 days old transgenic maize; lane 4, whole gut homogenate of insects fed on 7 days non-transformed maize plants.

28dys Bt 28 dys Cntrl 7 dys Bt 7 dys Cntrl

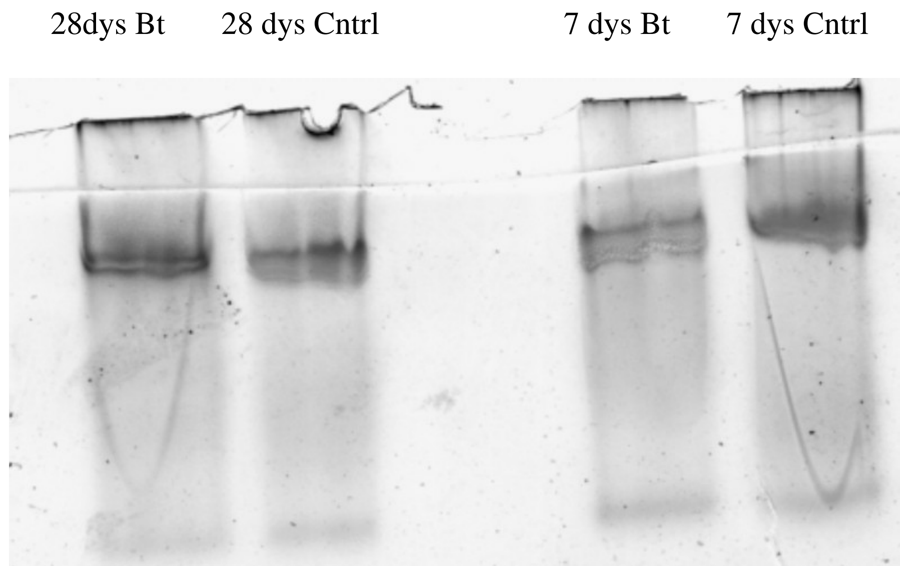


Figure.5 GR relative activity of insects fed on; 7 days old non-transformed maize plants (7dys Cntrl); 7 days transformed maize plants (7dys Bt); 28 days old non-transformed maize plants (28dys Cntrl) ; and 28 days old transformed maize plants (28 Bt). Points and bars represent mean ± SE for triplicated independent determinations.

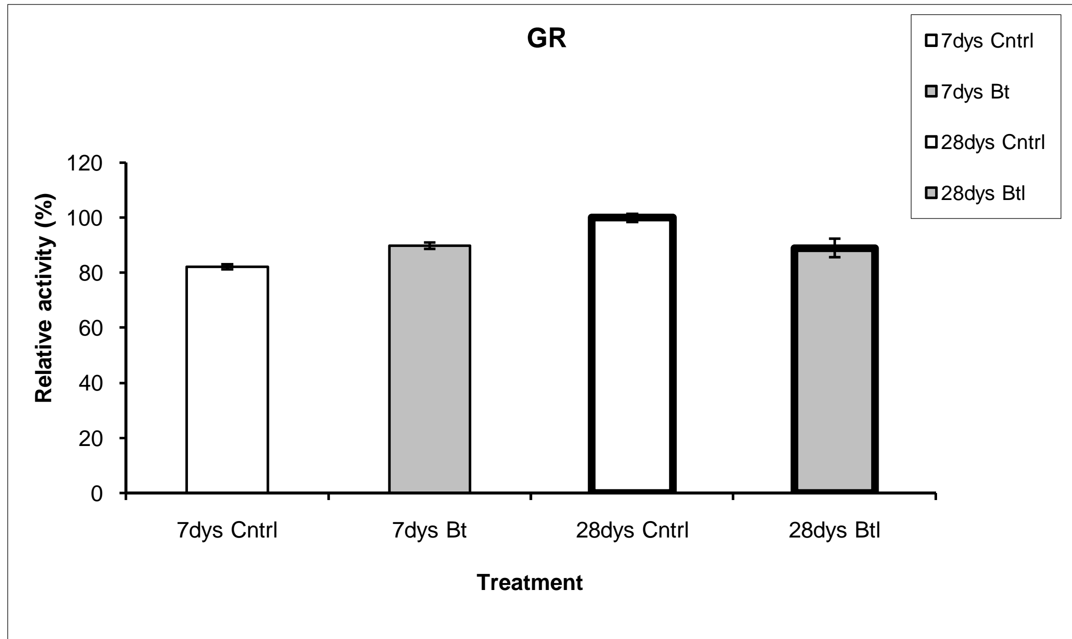
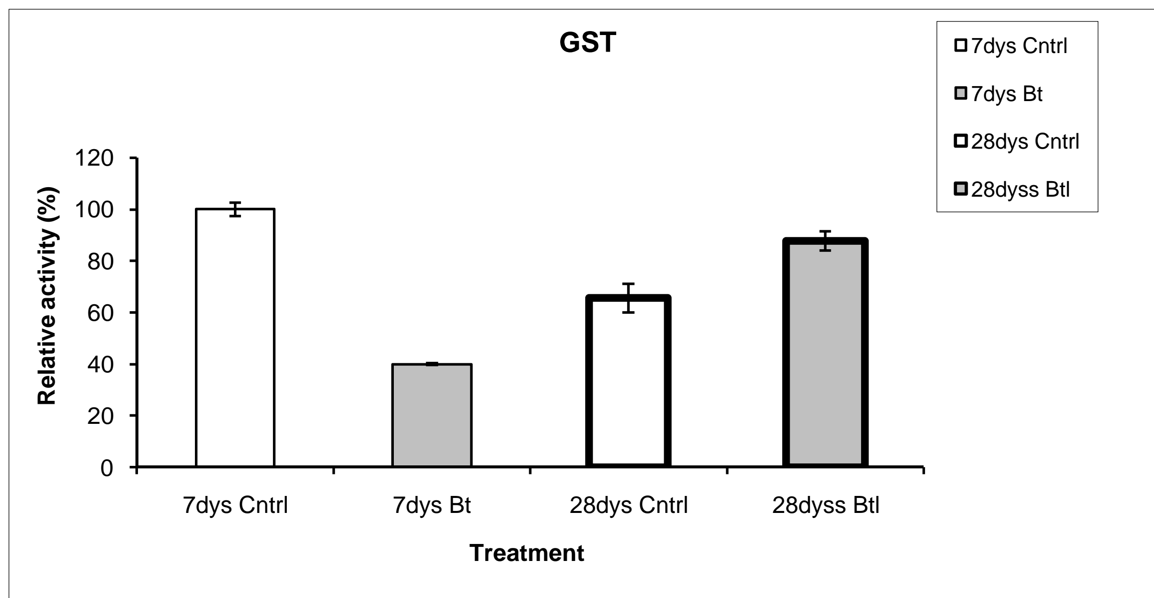


Figure.6 GST relative activity of insects fed on; 7 days old non-transformed maize plants (7dys Cntrl); 7 days transformed maize plants (7dys Bt); 28 days old non-transformed maize plants (28dys Cntrl) ; and 28 days old transformed maize plants (28 Bt). Points and bars represent mean ± SE for triplicated independent determinations.



exogenous oxidative stress. These defenses include enzymatic and non-enzymatic defenses. The following antioxidant enzymes have been found to play a crucial role in insects: catalase, superoxide dismutase, ascorbate peroxidase, glutathione reductase, and glutathione –s-transferase. We tested the presence of these enzymes (except superoxide dismutase) in the larvae of *B. fusca* and the results showed that these enzymes are all present.

A major defense against H_2O_2 toxicity is via enzymatic removal by catalase (Ahmad *et al.,* 1988; Peas et al 2001). Activity for catalase was found to very high in samples of insects fed on control plants than those fed on *Bt* plants. This was not expected since catalase is only active at high concentrations of H_2O_2 due to its low affinity towards its substrate (Peas et al 2001) and we expected H_2O_2 concentration to be high in insect samples fed *Bt* plants.

CAT in southern armyworn has been found to be located throughout the cell (Ahmad *et al.,* 1988). However, this enzyme has been found to be absent in Malpighian tubules of *Helicoverpa zea* and *Trichoplusia ni* (Ahmad, 1992). Other studies (Krishnan and Kodrik, 2006) have found elevated levels of SOD, CAT, APOX and GSTpx when the larvae were fed potato plant diet. These antioxidants enzymes together with other low molecular weight compounds such as ascorbic acid and α-tocopherol were thought to restrict oxidative radicals from reaching metabolically active tissues (Krishnan and Kodrik, 2006). We propose that this might be the reason why the levels of catalase and ascorbate peroxidase were higher in insects fed on control plants than in insects fed on *Bt* plants. This is a more plausible if one considers that insects fed on *Bt* plants did not consume as much leaf tissues as insects fed on control plants. Phenolic compounds which represent a major line of the chemical plant defense against herbivore (Harborne, 2001) may contribute to the ROS level by undergoing spontaneous oxidation to semiquinones (Barbehenn *et al.,* 2005). This process is said to be promoted by the alkaline environment in the gut of the caterpillar. The gut of *B. fusca* has been shown to be alkaline (George *et al.,* 2008). It has been reported that plant phenolics cause a burst of ROS in the foregut and elicit antioxidant defense in the midgut (Krishnan and Kodrik, 2006).

In Colorado potato beetle ROS gradient was found along the length of the midgut, with a maximum in the lumen of its anterior and a minimum in the wall of its posterior sections (Krishnan *et al.,* 2007). In this species the adults were said to possess a more efficient control of oxidative burst (Krishnan *et al.,* 2007). Glutathione reductase catalyzes the reduction of oxidised form of glutathione (GSSG) to reduced form (GSH) (Ahmad *et al.,* 1990). This enzyme maintains a high cellular GSSG/GSH ratio. In southern armyworm GR was found in mitochondria and microsomes (Ahmad *et al.,* 1988). Insects GSTpx have been reported to be effective in targeting hydroperoxides but not the hydrogen peroxides (Felton and Duffey, 1991).

The subcellular distribution of the antioxidant enzymes in mammalian species is as follows: SOD in the cytosol and mitochondrial matrix; CAT in peroxisomes; and GPOX and GR in the cytosol and mitochondrial matrix (Chance *et al.,* 1979). It will be interested to determine the levels of all the above enzymes in the different midgut regions of *B. fusca.* As earlier noted other insects

have been shown to have different amounts of antioxidant enzymes in different regions of the gut. In our study we used the whole gut homogenate and this may not have given us the true picture of the oxidative stress in *B. fusca*. Antioxidant response in different instars of *B. fusca* could also be determined to see whether larval stage affect the levels of antioxidant enzymes in this pest. The level of oxidative stress in the plant materials used could also be determined. How this correlates with the final stress levels in the insects could provide useful insights in future control strategies. Since enzymes work best at their optimum temperature, studies could be carried out in future to determine the effect of temperature on the above enzymes.

The gut content of *Spodoptera littoralis* grown on semi-artificial diet have been shown to be alkaline and oxidizing (Krishnan and Kodrick, 2006). The foregut has been shown to be strongly oxidizing (Krishnan and Kodrick, 2006) and this facilitates the breakdown of allelochemicals (Krishnan and Sehnal, 2006). In this insect species the content of oxidative radicals dropped when the bolus passed from the foregut to the midgut, and this was attributed to the high activities of antioxidant enzymes (Krishnan and Sehnal, 2006). Likewise, the optimum pH of the gut of *B. fusca* has been found to alkaline (George, 2008). Changes (if any) in the content of oxidative radicals that occur when the bolus passes from fore gut to midgut could be the subject of future research work.

Acknowledgement

The authors would like to thank Monsanto Company, St Louis, USA, for seed material. Botswana Ministry of Agriculture provided the financial support for the study.

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