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Research Article

Endosulfan induced stress responses in Cowpea (*Vigna sinensis* L.) var. *kanjikuzhy*

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Abstract

Endosulfan ($C_9H_6Cl_6O_3S$), the pesticide frequently applied in plantations, is reported as detrimental to human life and other fauna causing several growth hazards. The present study revealed the application of endosulfan which can retard plant growth affecting seed germination, growth of shoots, fruiting, etc. Seeds treated with different concentrations of endosulfan (0.01% - 2.0%) served as the experimental plants while non-treated plants as a control. A significant decrease in seed germination was observed among the experimental plants (6.6% to 0.0%) over non-treated plants (100.0%). The amylase activity is correlated with the germination percentage. Total chlorophyll content and protein content were found to be decreased by the application of endosulfan, whereas total free proline content was found to be increased from 0.66 to 1.96 mg/gm tissue. Endosulfan treatment significantly increased the levels of peroxidase and catalase activity in both root and leaf samples. This data was further confirmed using histochemical assays.

Key words: *Vigna sinensis L., endosulfan (EDS), Oxidative stress enzymes, Peroxidase, Catalase*

1. Introduction

For controlling pests, diseases, weeds and to increase the crop yield, pesticides and insecticides play an important role in agriculture. For higher yields one should apply these pesticides very carefully and at proper times. According to an estimate of USEPA [1] globally close to 5.6 million pounds of pesticides were being used annually in 1998 and 1999. These pesticides are found to be a major environmental pollutant because of their uncontrolled use, lack of degradation ability, and some other factors. Usually, pesticides are manufactured and marketed in a target specific level, but they are causing many harmful effects to the nature and other non-targeted species also. In spite of these challenges, pesticides are proved to be the unavoidable factor in agriculture and also help the farmers to increase yield of the crop. Very few studies were carried out on this specific area of pesticides and its effects on the biochemical changes happening to the crops by the application.

Ronika and Poonam [2] reported that by centuries, human beings have developed many methods to control herbs, weeds, insects, invertebrates and microorganisms that gave threats to the supply of food, fibre, public health, etc. Endosulfan ($C_9H_6Cl_6O_3S$) (EDS) is usually used as an insecticide and acaricide belongs to cyclodienes group under the name of Thiodan. IUPAC name of endosulphan is 6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro- 6, 9-

methano-2, 4, 3-benzodioxathiepine-3-oxide. The pesticide is also named as Benzoepin, Endocel, Parrysulfan, Phaser, Thiodan and Thione [3]. By the application of EDS, induction of T-cell apoptosis, mitochondrial dysfunction and oxidative stress and lipid peroxidation will happen [4, 5]. The half-life of the pesticide residue in the soil will be a few months to years, but 1-6 months in water. The half-life depends on several factors such as pH, climatic conditions, etc. The Environmental Protection Agency (EPA) has categorised EDS under toxicity class Ib chemical. Now it is no longer made in the US, but it is registered for use on 60 crops.

For controlling the insect pests, that is attacking the food and non-food crops, endosulfan is a very efficient pesticide. Lipophilic nature of endosulfan helps to get deposited in fatty tissues of the insect pests initially and finally in liver and kidney. Ultimately it causes lethality in target species by damaging the nervous system. Like other organochlorine compounds, it is also a gamma amino butyric acid (GABA) antagonist [6, 7]. Very high dose of endosulfan stimulates the Central Nervous system of the target and resulted lack of coordination, gagging, vomiting, diarrhoea, agitation, convulsions and loss of consciousness. It also causes severe damage to renal, hepatic, respiratory, reproductive and immune systems in mammals [8- 11]. The application of endosulfan is banned in more than 60 countries because of its high toxicity, higher rate of bioaccumulation and role as an endocrine disruptor [12, 13]. But in India and Brazil farmers are extensively using this pesticide.

The application of pesticides is beneficial for plants if we are applying at their lower concentration but become highly toxic if exceeding the level thereby alter the microflora of the soil also. By repeated and extensive application of pesticides, the compounds will reach the plant body and soil, and thereby alter plant growth and its biochemical activities. Keeping the above, present study was designed to monitor biochemical effects of endosulfan on *Vigna sinensis* which is an important vegetable crop in India.

2. Materials and Methods

Cow pea (*Vigna sinensis* (L.) Savi ex Hassk. var. kanjikuzhi, family Fabaceae and subfamily Papilionaceae) collected from Kerala Agricultural University, Vellanikara, Thrissur served as the material for the present study. It is an annual bush, light green medium sized pods (pod length-27cm and pod weight-7.13cm), mottled seeds, leaves long and trifoliate. Flowers shortly pedicelled borne on axillary racemes and usually 2-3 flowers on each peduncle having 3 bracts at the base, completes fruiting in 48 days.

In the present study an analysis was made on the effect of endosulfan (0.01%, 0.05%, 0.1%, 1.0% and 2.0% concentrations) and control without endosulfan. on seed germination, plant growth, pod development, changes in total chlorophyll content, total soluble proteins, histochemical and biochemical activities of antioxidant enzymes viz., catalase and peroxidase. For evaluation of seed germination, good and even sized seeds soaked for 24 hour in different concentration of EDS (0.01%, 0.05%, 0.1%, 1.0% and 2.0%) were sown in 10 cm petridishes layered with wet blotting paper and kept in dark for germination. The germination of the seeds was observed as emergence of radicle and the germination percentage calculated as an average of three replicates.

Equal numbers of seeds (100) of pea plants were collected and soaked in distilled water for 24 hours. After germination 6 beds were prepared and the plants were planted and watered in the morning and evening regularly. 15 days after planting, different concentrations of endosulfan (0.01%, 0.05%, 0.1%, 1.0% and 2.0%) were sprayed in the morning and evening

and grown under natural conditions of temperature, light and humidity. Plants sprayed with water only (without endosulfan) were taken as control. After 4 weeks, second and third leaves from each plant of different treatments were collected and pooled separately for the whole study. All experiments were repeated four times and average values were taken for estimation.

Percentage of seed germination was calculated based on the number of the germinated and non-germinated seeds. Height of shoot and pod was measured from randomly selected plants and average values were recorded. Total chlorophyll was calculated using Arnon's formula [14]. 100 mg of leaves were homogenized in 10.0 mL of 80.0% acetone and centrifuged at 3000 rpm for 5 minutes. The supernatant was saved and the pellet was re-extracted twice with the same solvent. Optical density (OD) was read at 645nm and 663nm against blank (80.0% acetone). Total free soluble protein content was estimated from the TCA extract of leaf and root sample following Lowry et al. [15], while total free proline was estimated following Bates et al. [16].

Peroxidase (POX) was isolated and assayed following Golbier [17] and by using guaiacol as substrate [18]. A set of samples containing reaction mixture without guaiacol was the control. Catalase (CAT) was extracted from leaf and root samples, and decomposition of H_2O_2 into H_2O and O_2 was assayed following Luck [19]. Histochemical study of POX was done with the cytochemical preparation following Alcazar's [20] method using diamino benzidine (DAB) [21, 22]. For localization of CAT, root and stem samples were used for the histochemical preparation using 3-amino triazole (AT) and DAB [23]. Photographs were taken using canon digital camera (EOS 450D, Japan).

3. Results and Discussion

A preliminary study has been carried out to study the effect of endosulfan on the physiological, biochemical and histochemical changes in *Vigna sinensis* which is a major vegetable crop in India.

The percentage of seed germination was decreased with increasing concentration of endosulfan (Fig. 1). Control seeds showed 100% germination, which slowly decreased as the concentration increased. Germination percentage showed 86.6% for 0.01% and 80.0% for 0.05% (Fig. 1). The figure 1 clearly depicts the percentage of germination was found to be completely absent in seeds treated with 2.0% of endosulfan. Increased endosulfan concentration (2.0%) causes decreased germinability (0.0%). Some previous studies also reported seed germination as a critical stage of plant growth that is sensitive to environmental pollutants and can be used as an excellent bio-indicator [24, 25]. Shekar et al. 2011 studied the effect of endosulfan individually and in combination with kitazin in *Solanum melongena* L. and observed that pesticides individually and in combination affect the seed germination, seedling growth, number of lateral roots, protein content and amylase activity [26]. Similar work on inhibition of seed germination and seedling growth is reported in Brinjal, Chilli and *Glycine max* [27], in *Pisum* [28], in *Brassica nigra* with Kitazin [29], and in *Vigna radiata* with DDT [30]. Calvelo-Pereira et al. 2010 reported the effect of pesticides on seed germination by depressing amylase activity or suppressing seed germination [31]. As concentration increases germinating seedlings would be in stress. Chemical stress could operate by inactivating hormones or enzymes and by changing membrane permeability in the seedlings leading to loss of germinability. Hence amylase activity of the seeds was also estimated. Amylase activity of geminating seeds was found to show a progressive reduction with increasing concentrations of

endosulfan (Fig. 1). Sabale and Misal [32] also studies the effect of endosulfan in Jowar and found that endosulfan treated seedlings showed a sudden increase in amylase activity in lower concentration (0.2%), whereas in higher concentrations, the activity found decreasing.

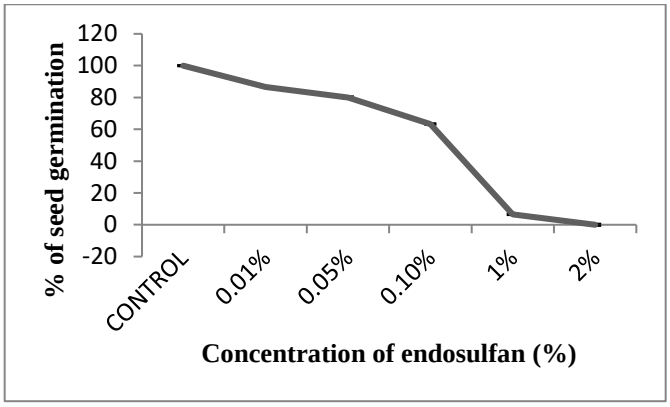

Figure 1: Graph showing the percentage of seed germination applied with endosulfan and control.

Shoot height as length is found to have a promotive effect on plants treated with 0.1 % endosulfan, and then gradually decreased from 1.0% to 2.0%. Results showed that control plants showed a maximum growth of 45 cm length. Plants treated with 0.01% and 0.05% of endosulfan showed a similar growth pattern compared to control (45 cm and 47 cm respectively). But plants treated with 0.1% concentration showed a maximum growth of 50 cm (Fig. 2). While a sudden decrease in the shoot growth was noticed in the plants treated with 1.0% and 2.0% of endosulfan (23 cm and 17 cm respectively). A promotive effect growth of pods up to plants treated with 0.1 % of endosulfan was found and then it gradually decreased. The control plants showed a pod length of 22 cm but plants treated with 0.1% showed 27 cm a little higher than control (Fig. 2). The plants treated with 1.0% and 2.0% showed a decreased pod length (11 and 8 cm). High level of endosulfan exercises a depressive effect on pod length. Perez et al. [33] reported that very low concentration of endosulfan itself reduced cell division in root meristem of *Bidens laevis* when exposed hydroponically.

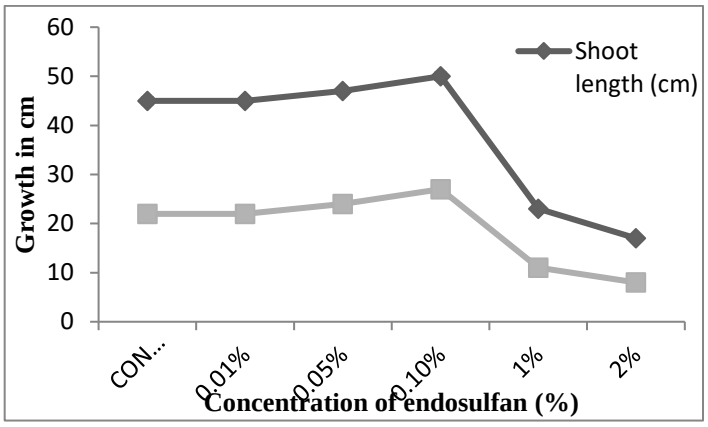

Figure 2: Graph showing the length of shoot and pods applied with endosulfan and control.

Total chlorophyll content in leaves was found to decrease with increasing concentration of endosulfan and it was 0.89 mg/gm fr.wt. in the control plants, while the higher concentrations showed decreased levels of chlorophyll content from 0.72 mg/gm fr.wt. to 0.20 mg/gm fr.wt. (Fig. 3). However, a direct depletion of chlorophyll can be observed in plants treated with 1.0%

and 2.0% endosulfan during the study period and white patches were also observed on leaf surface of these plants. Pandolfini et al. [34] suggested that loss of chlorophyll content may be due to the interference in fat metabolism and thereby inhibiting root and shoot growth, photosynthesis, nutrient uptake, leaf area, biomass, etc. or may be due to decrease in chlorophyllase.

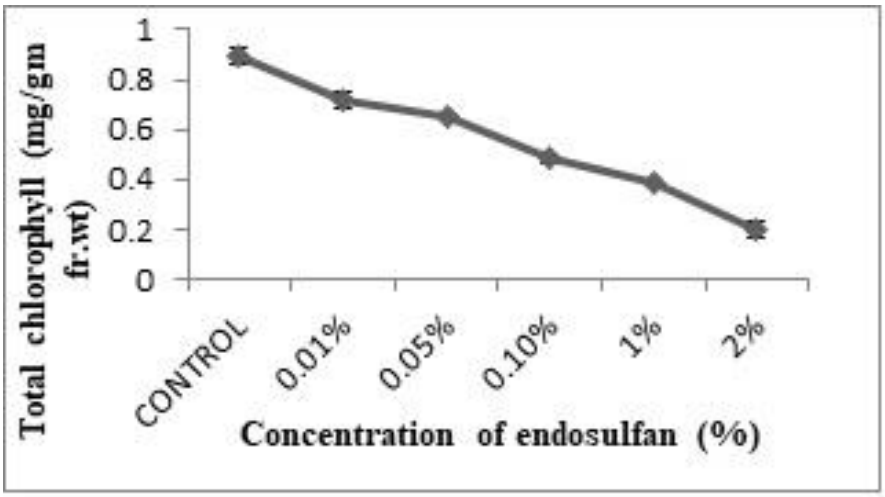

Figure 3: Graph showing total chlorophyll content in the leaves from control as well as plants treated with endosulfan.

Proteins are mainly involved in the architecture of the cell. During chronic period of stress, they are also a source of energy and need more energy to detoxify the toxic compound and to overcome stress [35]. Hence, the total soluble protein content of root and leaf samples was estimated (mg/gm fresh weight of the tissue). The decrease in protein was more pronounced among plants grown in 1.0% and 2.0% of endosulfan. Root and leaf tissues of control plants showed a total soluble protein content of 0.65 mg/gm fr.wt and 0.48 mg/gm fr.wt. respectively. But in the case of experimental plants it showed a progressive reduction from 0.58 mg/gm fr.wt. to 0.26 mg/gm fr.wt for root tissues and 0.23 mg/gm fr.wt. to 0.02 mg/gm fr.wt for leaf tissues (Fig. 4). Deterioration of protein is more pronounced in leaf tissues than in root tissues. This decreased level of protein may be due to the direct depletion of chlorophyll in plants treated with high endosulfan dose. This indicates that the application of endosulfan is directly affecting the leaf of the plants, than the roots and this reduction in protein content is due to increased hydrolysis of protein. Constantinidou and Kozlowski [36] reported the decrease in protein content is due to decrease in photosynthesis.

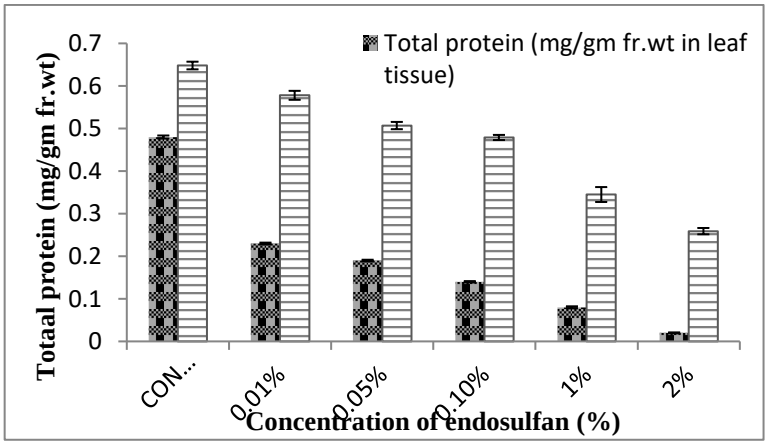

Figure 4: Graph showing total protein content leaves and root tissues taken from control and plants treated with endosulfan. In leaves the protein content decreased from 0.48 mg/gm fr. wt. (control) to 0.02 mg/gm fr. wt. (2%), while, in roots the protein content decreased from 0.65 mg/gm fr. wt. (control) to 0.233 mg/gm fr. wt. (2%).

Total free proline content was found to show a progressive increase with increasing concentrations of endosulfan. Control plants possessed free proline content of 0.66 mg/gm tissue (Fig. 5), while experimental plants showed a progressive increase in proline content from 0.75 (0.01%) to 1.96 mg/gm tissue (2.0%). Proline accumulation is a common response of plants towards stress and exists a strong relation between these two. Proline can accumulate to high levels without disturbing intercellular biochemistry and play a vital role in plant cytoplasmic adjustment during osmotic stress. Proline being a compatible osmoprotectant can accumulate in the cell sap at high concentrations reducing the water potential, thereby enhancing water uptake from the soil. Accumulation of proline is due to inhibitors of oxidation of proline oxidation and its incorporation into proteins by stress. Gzik [37] reported the accumulation of proline in plant due to drought and temperature stress. Lerudulier et al. also reported that Proline acts as a hydrophobic protectant for enzymes and sub-cellular organelles and helps the plant to tolerate or adapt to the stress condition [38]. The above studies clearly gave evidence of function of proline content and its action against stress.

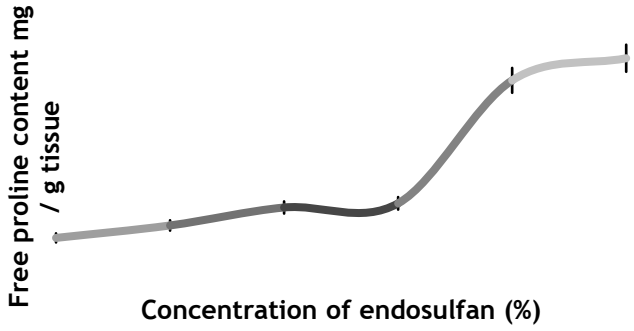

Figure 5: Graph showing total free proline content in the control leaves and plants treated with endosulfan. From the figure, it is clear that the proline accumulation is due to stress of the plant with the application of endosulfan and is very high in plants treated with 1% (1.8 mg/gm. tissue) and 2% (1.96 mg/gm. tissue) of endosulfan.

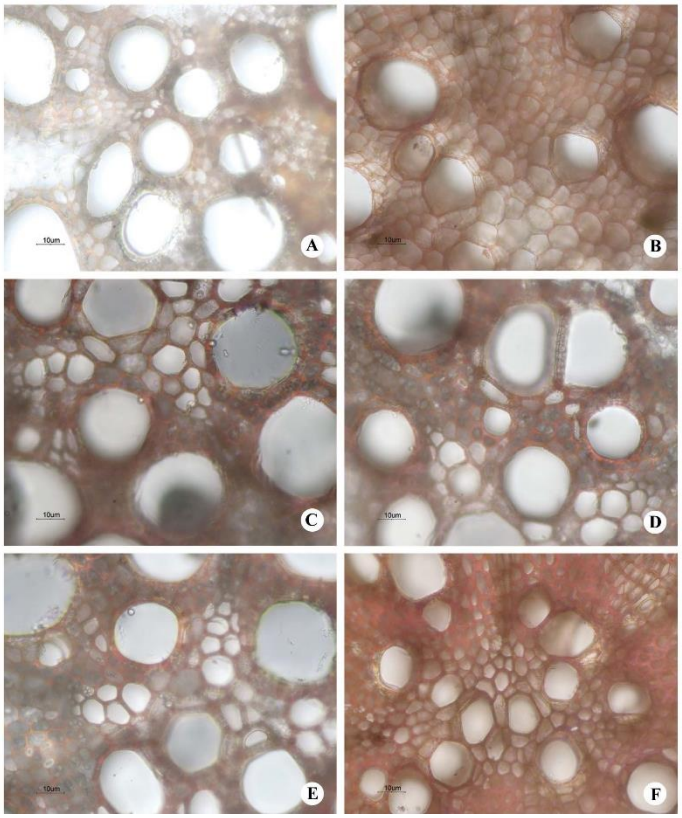

Figure 6: localization of POX in the root tissues of *Vigna sinesis* by the application of endosulfan. A. Tissues taken from the control plants showed slight brown colour deposits of DAB, B. Tissues from 0.1%treatment showed prominent brown color deposits, C. Tissues from 0.05%, D. Tissues from 0.1%, E. Tissues taken from 1.0% and F. Tissues from 2.0%. The brown color deposits of oxidized DAB are very prominent in the tissues taken from 0.1% to 2.0%.

To study the localization of POX and CAT, thin sections of roots were subjected to light microscopic analysis. DAB by the action of POX get oxidized and showed brown deposits within the cells [21]. Incubated sections showed brown color deposits of oxidized DAB indicating positive signs of POX activity. Non-treated plants showed a slight brown coloration in the stelar region with the treatment indicating the presence of POX in root tissues (Fig. 6A). Fig. 6B showed a very clear brown coloration after treatment with DAB stating that POX activity is increasing in experimental plants (0.01%) than the control. Accumulation of blackish brown color POX deposits was more prominent in root tissues of plants treated with 0.05%, 0.1%, 1.0% and 2.0% (Fig. 6C –F). This was further estimated quantitatively also.

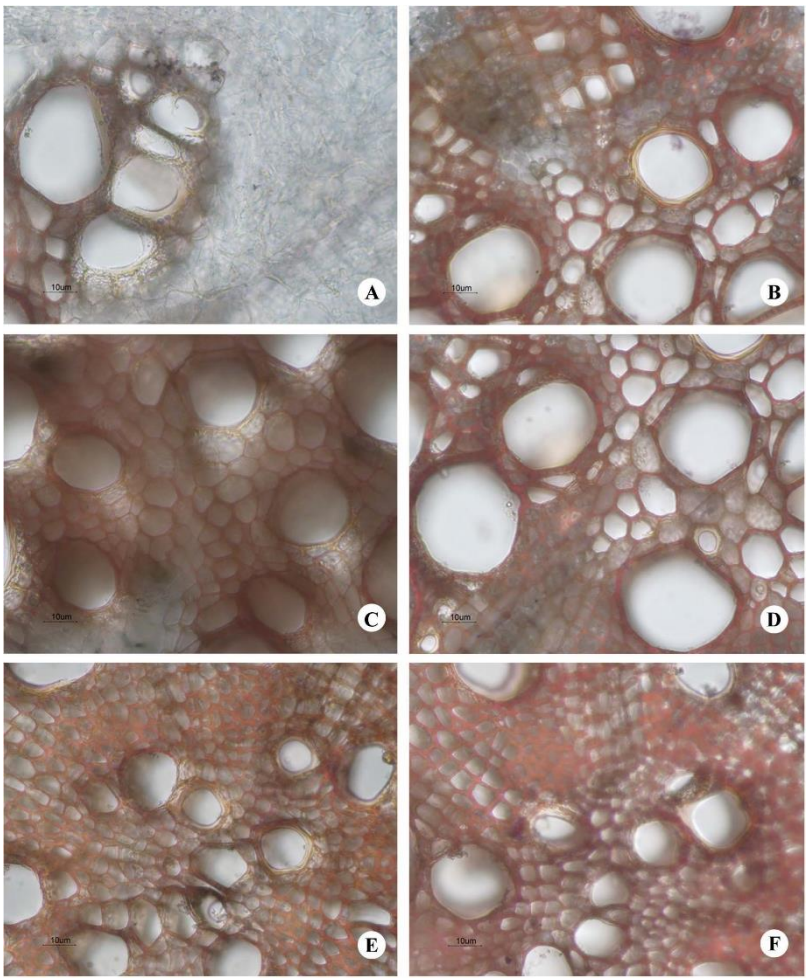

Figure 7: Localization of CAT (fully incubated in AT) in the root tissues of *Vigna sinesis* for differentiating the activity of CAT from POX. AT completely inactivated the CAT activity. The brown color deposits in the tissues indicating the presence of oxidized DAB. A. Tissues from control, B. Tissues from 0.1% , C. Tissues from 0.05%, D. Tissues from 0.1%, E. Tissues from 1.0% and F. Tissues from 2.0%.

Since the whole sections subjected to localizing POX and CAT expressed a similar color pattern, the enzyme activity was differentiated by using specific inhibitor (AT). To distinguish CAT activity from that of POX, the tissue sections were pre-incubated with AT for 1 hour (Fig. 7). The sections treated with AT showed less number of brown deposits (Fig. 7A) than the experimental sections with DAB (Fig. 7B-F). The scattered brown deposits found in the AT treated sections indicate the activity of POX. Experiments with full incubation of AT and with DAB were carried out to separate the activity of CAT from POX and the results clearly supported the presence of CAT. The root sections taken from the control plants showed the presence of brown colour deposits only in the stelar region (Fig. 8A) but from the experimental plants showed dark brown colour deposits in the stelar as well as in the cortex region indicating the presence of high levels of CAT (Fig. 8 B-F).

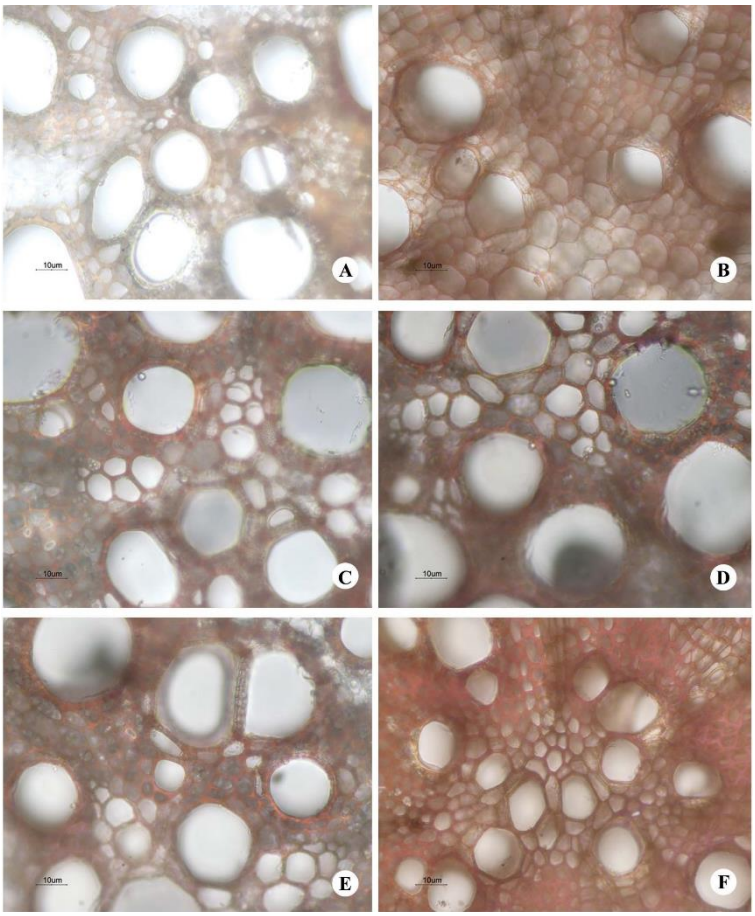

Figure 8: Localization of CAT (treated with DAB) in the root tissues of *Vigna sinesis* by the application of endosulfan. The brownish deposits indicating the presence of CAT. A. Tissues taken from control showed slight brown color deposits indicating the presence of CAT, B. Tissues taken from 0.01% showed prominent brown deposits of CAT, C. Tissues taken from 0.05%, D. Tissues taken from 0.1%, E. Tissues taken from 1.0% and F. Tissues taken from 2.0%. The presence of CAT is more prominent in tissues taken from 0.05% to 2.0%

POX activity in leaves and roots of control and experimental plants was calculated as µM of guaiacol oxidized/gm tissue/mg enzyme protein. The POX activity was found to show a progressive increase with increasing concentrations of endosulfan. The increasing POX activities were more pronounced in plants treated with 1% and 2% endosulfan. Leaves of contol plants showed a maximum enzyme activity of 6.6 µM of guaiacol oxidized/gm tissue/mg enzyme protein, while experimental plants showed an increased activity from 11.0 to 19.8 µM of guaiacol oxidized/gm tissue/mg enzyme protein (Fig. 9). The experiment was done using root tissues and the same result was observed in both control and experimental plants. 7.2 µM of guaiacol oxidized/gm tissue/mg enzyme activity was observed in control plants, while a significant increase from 8.2 to 16.2 µM of guaiacol oxidized/gm tissue/mg enzyme protein was found in experimental plants (Fig. 9). The high level of POX is a strong indication of physiological stress. Smitha et al. reported the increase in POX activity may be due to the metabolic response to environmental stress [21]. Lee [40] also reported that with Na_2SO_3 treatments, POX activity also increased.

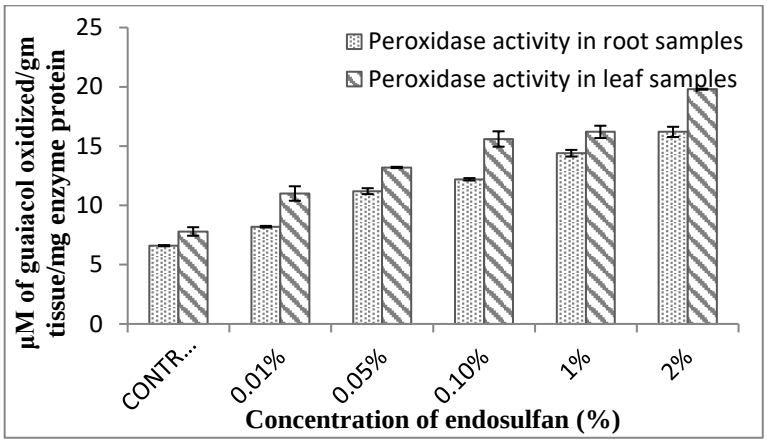

Figure 9: Graph showing POX activity in the root and leaves. The POX activity is very high in root (16.2 µM of guaiacol oxidized/gm tissue/mg enzyme protein) and leaves (19.8 µM of guaiacol oxidized/gm tissue/mg enzyme protein) of plants treated with 2% of endosulfan.

CAT activity in leaves and roots were also calculated as µM of H_2O_2 destroyed/minute/mg enzyme protein in both control and experimental plants. Like that of POX, the CAT activity was found to show a progressive increase with increasing concentrations of endosulfan. Increased activities were more pronounced in plants treated with 1% and 2% endosulfan. Leaves from control plants showed a maximum enzyme activity of 13.6 µM of H_2O_2 destroyed/minute/mg enzyme protein, while the experimental plants showed an increased activity from 15.0 to 27.2 µM of H_2O_2 destroyed/minute/mg enzyme protein (Fig. 10). In the case of root tissues, 8.6 µM of H_2O_2 destroyed/minute/mg enzyme protein was observed in control, and a significant increase from 11.2 to 34.6 µM of H_2O_2 destroyed/minute/mg enzyme protein was found among experimental plants (Fig. 10). These results are also supporting the histochemical and quantitative data of CAT (Fig. 6 & 9). The data confirms the detoxifying nature of CAT for removing the ROS. Apart from the peroxidatic action of POX on H_2O_2 for the formation of water, CAT can perform a catalytic by way of producing water and oxygen. In other words, the scavenging property of CAT by detoxifying H_2O_2 tends to produce more oxygen in the cell system. Besides the catalytic action of CAT under *in vivo* conditions the enzyme is capable of reacting a peroxidatic way.

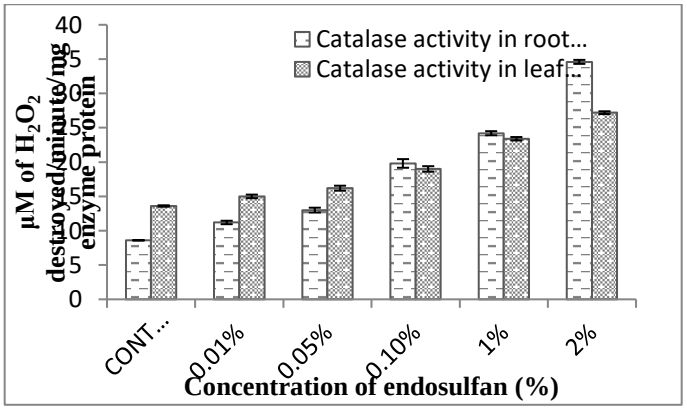

Figure 10: Graph showing the CAT activity in the root and leaves. The CAT activity is very high in root (34.6 µM of H_2O_2 destroyed/minute/mg enzyme protein) and leaves (27.2 µM of H_2O_2 destroyed/minute/mg enzyme protein) of plants treated with 2% of endosulfan.

Amylase activity in germinating seeds of control and experimental plants was calculated as U/mL and showed a progressive decrease with increasing concentrations of endosulfan. The seeds collected from control showed maximum activity of 43.87 U/ml, while seeds of experimental plants showed a significant reduction from 41.21 to 18.6 U/ml (Fig. 11). The amylase activity is correlated with the data obtained from the percentage of seed germination (Fig.1).

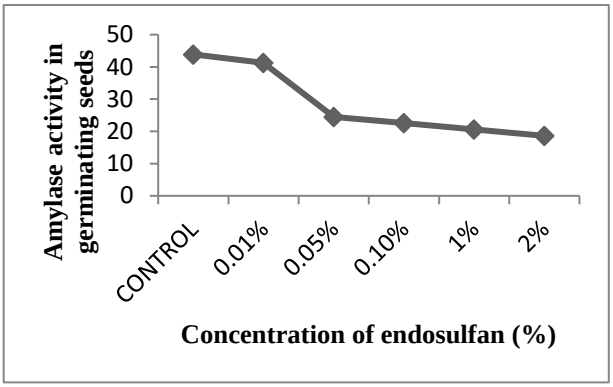

Figure 11: Graph showing the amylase activity in the germinating seeds. The amylase activity is decreasing from the control (41.21 U/mL) to 2.0% (18.6 U/mL).

Mathur et al., studies were carried out in *Vigna mungo* and treatment of pesticides may impose an osmotic stress and thereby causing damage to membrane structure [41]. Vidyasagar et al. also reported under stress conditions, the lysosomes are broken down and resulting in an increased level of several hydrolytic enzymes [5]. In the present study, seed germination, growth of shoot and pod, total chlorophyll, protein, proline contents, biochemical and histochemical data of peroxidase and catalase enzymes. These parameters showed considerable changes with the increase in endosulfan concentration.

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