ijCEPr

International Journal of Chemical, Environmental and Pharmaceutical Research

Vol. 1, No.2, 74-79
September-December, 2010

Alterations in the Activity of Enzymes as a Method to Characterize Herbicide Tolerance

Santosh Kumar Singh*[1], Satish Kumar Verma[2], Md. Aslam Siddiqui[3] and Sachin Chauhan[4]

*[1]Department of Microbiology, Gayatri College of Biomedical Sciences, Dehradun(U.K.)India
[2]Department of Biotechnology, Sai Institute of Paramedical and Allied Sciences, Dehradun (U.K.) India.
[3]Department of Life Sciences, BFIT, Dehradun(U.K.) India.
[4]Department of Biotechnology, GCBMS, Dehradun(U.K.) India.
*E-mail: res_mol_bio@sify.com

Article History:
Received:19 September 2010
Accepted:6 December 2010

ABSTRACT

Exposure of *Ocimum gratissimum* seeds to Oxyfluorfen showed a varied response. At low concentration of herbicide shoot length and its fresh and dry weight mass was observed to be stimulated though it showed an remarkable decrease in root length and its mass. Chlorophyll and carotenoid contents also showed slight enhancement in values at low doses, in comparison to untreated controls. But they decreased gradually at higher concentration of herbicide. The growth in terms of total protein contents decreased progressively at high concentration (4 ppm), though at low doses (0.5 ppm), it showed an enhancement. It might be due to the increased enzymatic activities to overcome stress. Enhanced generation of active oxygen species, increased the level of total MDA contents showing high degree of lipid peroxidation, at high dose of Oxyfluorfen (% control increase = 15-40 %). Enhanced exposure of herbicide to the seedlings stimulated the antioxidant enzymes. Superoxide dismutase and catalase activity were enhanced over controls (15% - 98%) but Peroxidase activity was observed to be decreased at 4 ppm concentration (21% as compared to the untreated samples). IAA oxidase activity assays showed its greater sensitivity towards the herbicide Oxyfluorfen. A considerable oxidative damage was observed due to the treatments of herbicide in seedlings.

Keywords: Lipid peroxidation, oxidative stress, photosynthetic pigments, catalase, SOD, Peroxidase, IAA oxidase

INTRODUCTION

Since India is an agriculture based country and it is a key factor in 'Indian Economy', about 64% of the population is dependant on agriculture for their livelihood. Peoples are diverting their attention towards various applied techniques to achieve the target of fulfilling the nutritional requirements of growing population. It might help not only to increase the food productivity, but also to prevent the losses of grains and vegetables by different invader pests and herbs. The application of insecticides and herbicides, the groups of pesticides, in crop fields for selective control of pests in modern age led to serious environmental contamination resulting in greater loss of crop productivity and growth of many microorganisms [4, 23]. Applications of herbicides are favored due to their low cost, easy availability and lack of regulatory implementation. The removal of these insecticides from soil and aquatic ecosystems has become a difficult problem and as a result of this they persist in the ecosystem for longer duration of time [24] and might harm lower and higher photosynthetic non target plants. Oxyfluorfen (2-chloro-1-(3-ethoxy-4-nitrophenyl)-4-trifluoromethyl) benzene) belongs to the chemical family of diphenyl ether herbicides. This is used to control broadleaf and grassy weeds in the culture of a variety of fields, fruits and vegetables crops, ornamentals as well as non crops sites. Because Oxyfluorfen has been identified as being persistent in water and mobile in soils, there is concern for ground water contamination and harm to some non target plants.

Ocimum gratissimum, the Ban Tulsi is a part of important group of aromatic and medicinal plants that yield many essential oils and aroma chemicals and find diverse uses in the perfumery and cosmetic industries as well as in indigenous systems of medicine. It belongs to the family Labiatae (Lamiaceae). It is well known for its antioxidant potentials [10, 26]. Since activities of enzymatic antioxidants that help plants in recovering from oxidative stress such as catalases, superoxide dismutase and peroxidase are previously reported to be changed under different stresses [17, 21], authors wished to screen the effects of herbicide Oxyfluorfen on modulation of their activity, in *Ocimum gratissimum*. The authors have set forth the objective of evaluating the alterations in growth behavior and photosynthetic pigment contents, analysis of the level of lipid peroxidation and modulations in enzymatic antioxidants in plants exposed to the herbicide Oxyfluorfen.

MATERIALS AND METHODS

Selection of Experimental plants and treatment of herbicide

Seeds of the wild *Ocimum* plants were surface sterilized in 5% Sodium hypochlorite solution. Seedlings (2 weeks old) were selected for the pretreatment of herbicide- Oxyfluorfen. On 10^{th} day, plantlets of each set were harvested and various parameters were analyzed with respective to the control plantlets (untreated).

Measurement of growth, photosynthetic pigment and Lipid Peroxidation levels

Length and fresh mass of 10 seedlings were recorded separately and then dried in an Oven at 60-70^{0}C for 4 days to determine dry mass. Fresh leaves (0.02 g) from different *Ocimum* species were taken and cut into small pieces and photosynthetic pigments were extracted in 80% (v/v) acetone [2]. The quantification of pigments was done by standard methods [15]. Extraction of protein from plant leaf samples were done by boiling them in 0.5 N NaOH for 4 minutes. Samples were centrifuged at 5000 rpm and supernatant was used for protein estimation using lysozyme as the standard [16]. The level of lipid peroxidation was measured in terms of total MDA contents and the reaction reagent consisted of 0.4 N TCA + 19.68 ml of distilled water + 0.4 ml of HCl + 100mg TBA [12]. Prepared leaf extract (in phosphate buffer) was added to the reaction reagent and absorbance was taken at 532 nm. MDA content was calculated as under-

Concentration of MDA = Absorbance x 6.45/ml/mg fresh wt. (1)

Estimation of Catalase (EC 1.11.10.6) and Superoxide dismutase (EC 1.15.1.1) activities

In vivo catalase activity was determined by making homogenates of leaves in fresh 50 mM of phosphate buffer (pH 7.0). In each samples catalase activity was determined by recording O_2 evolution for 1 min after the addition of 5 ml of 50 mM phosphate buffer (pH 7.0) containing 50 mM H_2O_2 [7]. Further 1 ml of cell suspension was added and O_2 evolution was monitored in darkness. For the measurement of SOD activity the reaction mixture contained 1.3 µM riboflavin, 13 mM L- methionine, 0.05 M Na_2CO_3, (pH 10.2), 63 µM p– nitroblue tetrazolium chloride (NBT) and crude plant extract [9]. Reaction was carried out under illumination (75 µmol photon m^{-2} s^{-1}) from fluorescent lamp at 25^{o}C. The initial rate of reaction as measured by the difference in increase in absorbance at 560 nm in the presence and absence of extract was proportional to the amount of enzyme.

Estimation of Peroxidase and IAA oxidase activities

Peroxidase (EC 1.11.1.7) was estimated by adding 0.1 M Phosphate buffer (pH 7.0) to homogenized leaf samples. The enzyme reaction mixture consisted of 0.1 M Phosphate buffer + 20 mM guaiacol + 12.5 mM H_2O_2 and plant extract. Optical density was measured at 436 nm [20]. IAA oxidase activity was assayed using the enzyme reaction mixture 0.071 M Phosphate buffer + 0.5 mM $MnCl_2$ + 0.05% paracoumaric acid + enzyme extract [3]. After ½ hour incubation in dark 5 M perchloric acid and 0.1 M ferric nitrate solution was added. After incubation for 60 minutes in dark, optical density was measured at 535 nm.

RESULTS AND DISCUSSIONS

It was observed that the exposure of Oxyfluorfen herbicide to *Ocimum gratissimum* for 7 days resulted decrease in root length as compared to untreated seedlings (33% decrease at 0.5 ppm and 88% at 4.0 ppm of Oxyfluorfen as compared to the control). Progressive inhibition in root fresh mass and dry mass was found out. Though the exposure of Oxyfluorfen showed varied results with shoot length (% control increase = 8% at 0.5 ppm and 17% at 1.0 ppm; % control decrease at high concentration of Oxyfluorfen was observed: % control inhibition= 53%). Shoot fresh and dry mass showed same variations as compared to the untreated cultures (Table 1). Similar findings were reported by other authors in seedlings of *Triticum aestivum* [18], Barley and maize [25] exposed to cobalt stress. It was seen that initially chlorophyll contents increased up to 78-88% while the high dose of Oxyfluorfen decreased the % control value up to 27%. Carotenoid contents showed the increase in values at 0.5 ppm to 1.0 ppm of Oxyfluorfen but high doses decreased the value up to 62% at 4 ppm concentration respectively (Table 1). Total protein contents showed initially low doses of herbicide induced the high rate of increase in protein contents that showed a decrease with time duration (days). But the high dose of Oxyfluorfen (4 ppm) was found to reduce the total protein contents speedily with increasing days (13% at 4 ppm as compared to the control) (Figure 1a). The declining trend in pigment and protein contents continued with rising concentration of the herbicide while low doses showed their recovery. The growth of photosynthesizes reflects the status of key physiological processes such as photosynthesis. Thus to understand the impact of herbicides on photosynthetic pigments and protein contents might

give us clues for its impacts on total biomass yield. Initial increase in total protein contents might be due to the increase in pool of enzymatic antioxidants to overcome the stress produced by the herbicide Oxyfluorfen and other herbicides as suggested by other authors [5, 19].

Oxyfluorfen induced lipid peroxidation of the cellular components in *Ocimum gratissimum* was studied by estimating the level of MDA in treated and untreated plantlets and the related data are depicted in the figure 1b. The lipid peroxidation in non- stressed *Ocimum gratissimum* was observed as 1.60 nmol MDA (mg fresh mass)$^{-1}$. Treated plantlets showed 15-40 % increase in total Malondialdehyde contents as compared to the untreated plants. Since MDA is an intermediate compound produced due to lipid peroxidation, the measurements of its contents can be used as an index for the injury caused by free radicals produced during oxidative stress. The results obtained here are in agreement with other authors [1, 6, 11], who reported the increase in MDA content with the exposure to other stresses in *Oryza sativa*, *Cassia* sp. and *Ulva fasciata*, respectively.

It was observed that the catalase activity showed an enhancement in herbicide treated plantlets (% control induction = 15% - 97% at 0.5 ppm to 4 ppm doses). The values ranged from 0.65 to 1.11 units minute^{-1} mg protein^{-1} as compared to the control (0.562 units minute^{-1} mg protein^{-1}). Lower dose of Oxyfluorfen 0.5 ppm stimulated catalase activity a little (15%) but higher concentration increased the enzymatic activity rapidly (figure 2a). The increase in the activity of catalase might be due to the need to decompose H_2O_2 and to protect membranes. The activity of the superoxide dismutase in non-stressed plants was 5.89 Units g^{-1} minute^{-1} which indicated that plant samples appeared to be more resistant against superoxide radicals produced due to various kinds of stresses. When plantlets were treated with Oxyfluorfen, there was remarkable increase in the activity of the enzyme at high concentration of herbicide, respective to the control (figure 2b). The lower dose treatment (0.5 ppm) enhanced the SOD activity only by 11%. The enhancement in the activity of SOD may be as a consequence of increased production of $O_2^{\cdot -}$ radicals. SOD converts relatively less toxic $O_2^{\cdot -}$ radicals to more toxic H_2O_2. Thus H_2O_2 scavenging activity is increased [22].

The Peroxidase activity showed varied responses with herbicide stress. Peroxidase activity was increased with the low dose of Oxyfluorfen (0.5 ppm) by 10% and this was continued linearly with 1 and 2 ppm of the herbicide doses (6.2 and 7.1 units minute^{-1} mg protein^{-1} as compared to the control (5.15 units minute^{-1} mg protein^{-1}). but at the higher dose activity decreased by 21% as compared to the untreated samples (figure 2c). Increased activity of Peroxidase indicates more powerful mechanism of detoxification of overproduced H_2O_2. It can be depicted from the figure 2d that IAA oxidase activity increased initially showing an enhancement in the enzyme activity by 15% at 0.5 ppm Oxyfluorfen but it showed remarkable reduction in values at higher doses (10% to 73% reduction as compared to the untreated seedlings). The results were in accordance with the studies done previously in *Vigna radiata* [8].

Exposure of stresses like heavy metals, insecticides, pesticides, ultraviolet radiations etc are reported to induce production of active oxygen species that might trigger the responses of antioxidative defense systems [14]. The present piece of work has proved the Oxyfluorfen induced increase in the activity of enzymatic antioxidants like SOD, Catalase, Peroxidase etc. However the high doses affected the defenses adversely proving the loss and damage to recovery system. Initial increases in the enzymatic activities proved the extent of antioxidant potential of plants against free radical induced damage. It might provide suitable keys to assess the antioxidant potential of plants growing against various stresses. It can be said that the increase in the values of enzymatic antioxidants at high concentrations of herbicide might be due to their successful recovery [13]. The study also helps us to encourage the proper evaluation of the toxicity of pesticides before their uses in agricultural fields so that they might not contaminate our water and soil reservoirs and non-target organisms.

CONCLUSION

According to the results obtained, it may be concluded that the herbicide affected the enzymatic antioxidants of the non-target plants (*Ocimum* species) severely. Initial increase in the enzymatic activities might be due to the increased activities of stress relief genes and their gene products. The results also indicated that the proper estimation and evaluation of the lethal doses of pesticides must be done prior to their use in agricultural lands to avoid any damage to non target organisms.

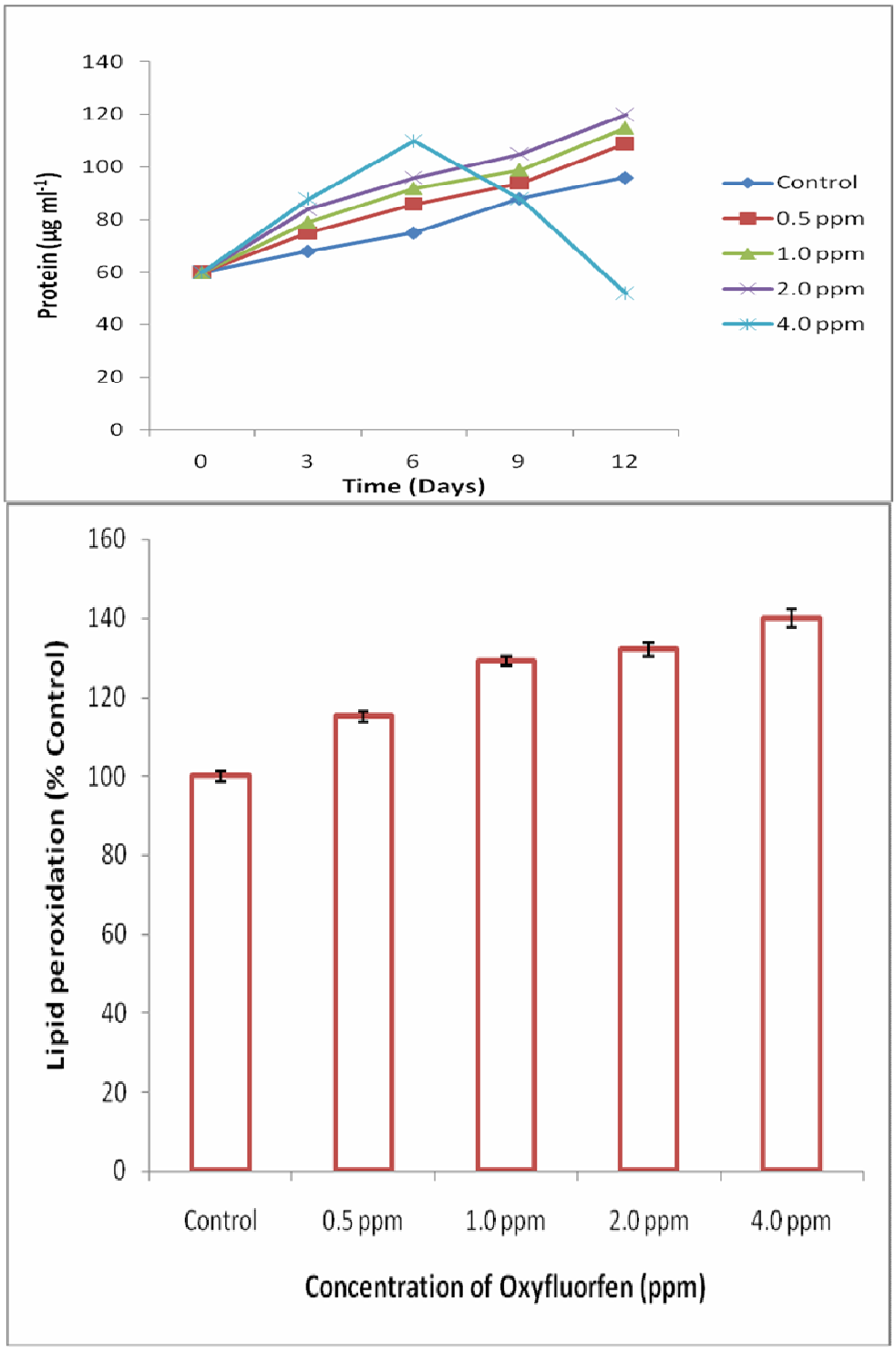

Fig.-1: Alterations in protein contents (a) and the level of lipid peroxidation (b) with enhanced exposure of Oxyfluorfen, herbicide. The values are means ±SE and significantly different from control ($p<0.05$).

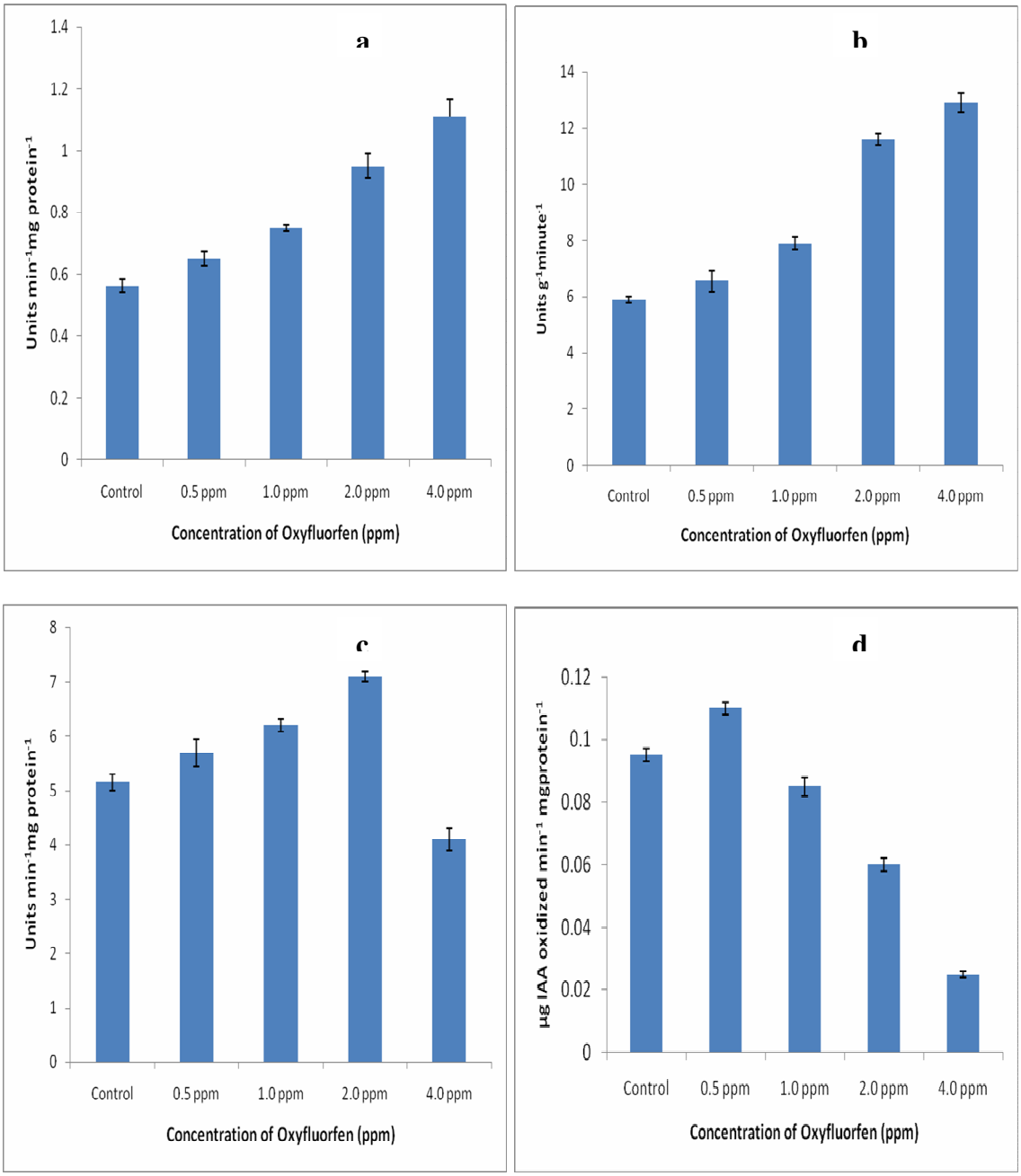

Fig.-2: Effect of increased exposure of Oxyfluorfen on Catalase (a), Superoxide dismutase (b), Peroxidase (c) and IAA oxidase activities; in *Ocimum gratissimum* plantlets. All the values were significantly different from their respective controls ($p<0.05$).

Table-1: Effect of Oxyfluorfen on different growth parameters of *Ocimum gratissimum*.

Parameters	Concentration of Oxyfluorfen				
	Control (untreated)	0.5 ppm	1.0 ppm	2.0 ppm	4.0 ppm
Root length (cm)	3.89±0.80	2.60±0.34	2.45±0.14	2.13±0.13	0.45±0.06

Shoot length (cm)	12.35±0.45	13.5±0.30	14.56±0.26	11.20±0.19	5.80±0.16
Root fresh mass (g/root)	0.190±0.05	0.125±0.08	0.10±0.006	0.078±0.001	0.035±0.004
Root dry mass (g/root)	0.03±0.04	0.020±0.001	0.016±0.004	0.010±0.002	0.008±0.002
Shoot fresh mass (g/shoot)	0.98±0.36	1.01±0.036	1.060±0.04	0.85±0.04	0.450±0.05
Shoot dry mass (g/shoot)	0.098±0.28	0.110±0.040	0.125±0.024	0.080±0.02	0.045±0.004
Chlorophyll contents	0.118±0.056	0.210±0.045	0.280±0.14	0.10±0.003	0.085±0.002
Carotenoid contents	0.054±0.008	0.068±0.04	0.090±	0.044±0.03	0.020±0.006

*Values are means± SE of triplicate samples

REFERENCES

1. Agarwal S., Pandey V., Indian Journal of Plant Physiology **8:3** (2003) 264.
2. Arnon D.I., Plant Physiology **24** (1949) 1–13.
3. Byrant S.D., Lane F.E., Plant Physiology **63** (1979) 696.
4. Cedergreen N., Streibig J.C., Pest Management Science **61** (2005) 1152.
5. Christopher D.N. *et al*., International Journal of Environmental Research and Public Health **7** (2010) 3298.
6. Dai Q. *et al*., Physiol Plant **101** (1997) 301.
7. Egashira T. *et al*., Plant Cell Physiol **30** (1989) 1171.
8. Garg N. *et al*., Res Bull Punjab University Sci **39** (1988) 196.
9. Giannopolitis C.N., Ries S.K., Plant Physiology **59**(1977) 309.
10. Gupta S.K. *et al*., Indian Journal of Experimental Biology **40** (2002) 765.
11. Hasanuzzaman M. *et al*., American Journal of Plant Physiology **5** (2010) 295.
12. Heath R.L., Packer L., Arch Biochem Biophysics **125** (1968) 189.
13. Holt J.S., Plant Physiol Plant Mol Biol **44** (1980): 203.
14. Kondo N., Kawashima M., Journal of Plant Research **113** (2000) 311.
15. Lichtenthaler H.K., Welburn W.R., Biochem Soc Trans **11** (1983) 591.
16. Lowry O.H. *et al*., J Biol Chem **193** (1951) 265.
17. Meriles J.M. *et al*., Journal of Phytopathology **154** (2006) 309.
18. Prasad S.M. *et al*., Biochem Cell Arch **2** (2002) 29.
19. Prasad S.M. *et al.*, Photosynthetica **43:2** (2005) 177.
20. Prasad S.M., Zeeshan M., Environment and Experimental Botany **52** (2004) 175.
21. Rao M.V., Paliyath G., Osmrod D.P., Plant Physiology **110** (1996) 125.
22. Ratcliff A.W., Busse M.D., Shestak C.J., Applied Soil Ecology **34** (2006) 114.
23. Shetty P.K. *et al*., Current Science **79** (2000) 1381.
24. Singh PK., Arch Microbiology **89** (1973) 317.
25. Vergaro O., Hunter J.G., Annals of Botany **17** (1952) 317.
26. Young I.S., Woodside J.V., Journal of Clinical Pathology **54** (2001) 174.

[ijCEPr-122/2010]