IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 15, Issue 8 Ser. I (August 2021), PP 63-70
www.iosrjournals.org

Estimation of heavy metals stress on oxidative enzymes, proline and chlorophyll content of *in vitro*regenerated shoots of *Tagetes patula* (L.)

Priyanka Modi*and Dr. Renu Singh#
**Department of Botany, University of Rajasthan, Jaipur-302004, India*
#Associate Professor, Seth RL Saharia Govt. PG College, Kaladera, Jaipur (Rajasthan, India)
**Author for correspondence:priyankaashna2012@gmail.com*

Abstract
This study investigated toxic impacts of heavy metals, Copper, Cadmium and Nickel over a concentration gradient of (0-25 µm) in the shoot induction medium BAP (0.5 mg/l)+ IAA (0.5 mg/l)in in vitro morphogenesis of Tagetes patula. After four weeks of treatment cellular homeostasis and detoxification to the metals were studied by analysing the growth in terms of shoot bud induction, chlorophyll content, proline accumulation and antioxidative enzyme (Superoxide dismutase (SOD), Catalase (CAT) and Peroxidase POD).In the present study the comparative toxicity of heavy metals (Nickel, Cadmium and Copper) in in vitro cultures was studied and found that cadmium was most toxic.
Key words*: Catalase, Chlorophyll, Proline, Peroxidase, Superoxide dismutase*

Abbreviations

BA	6-Benzyaminopurine
IAA	Indole-3-acetic acid
MS	Murashige and Skoog
SOD	Superoxide dismutase
CAT	Catalase
POD	Peroxidase

I. Introduction

Tissue culture is an efficient means to study the effect of abiotic stress on cell metabolism (Misra et al 2001). *In vitro* culture of plant cells in the presence of high concentrations of metals provides a useful tool to study the adaptive mechanisms of plants living in adverse environments.It offers the opportunity to develop new germplasm and it better adapted to the changing demands (Mathur et al. 2008**).**The mechanism of metal action at the physiological and biochemical levels were reviewed and studied by several workers (Saxena and Kaur 2005).Certain metal at low concentration act as an essential element for their metabolism and their high doses are toxic (Wu et al 2009). Stress induced accumulation of excessive quantities of reactive oxygen species (ROS) in plant is a typical phenomenon because ROS induced oxidative stress is an early damaging factor of stress treatment in plants

Plants have high level of physiological plasticity enabling survival of a wide variety of environmental stress to maintain cell intrigity and homeostasis. A key role in this plasticity is played by a range of protective responses. ROS are produced during normal cell metabolism and are involved in the regulation of many physiological processes. However the following exposure to unfavourable environmental conditions, ROS is formed in excess leading to a state so called oxidative stress (Apel & Hirt 2004).The level of ROS in plant tissues is controlled by an antioxidative system that consists of antioxidative enzymes and nonezymatic low molecular mass antioxidants. Superoxide dismutase (SOD), is a key antioxidative enzyme that catalyzes disproportionation of superoxide anion ($O_2^{\bullet}$**)** to H_2O_2 and O_2. Catalase (CAT,) scavenges H_2O_2 by converting it to H_2O and O_2. Peroxidase (POD) reduces H_2O_2 using several reductants, *e.g.*phenolic compounds. This enzyme has been propose to be a potential biomarker for sublethal metal toxicity in plants (Mocquot et al 1996).

Chlorophyll degradation is the routinely observed response to stress or chiefly in elevated concentration of various metals (Chen and Djuric 2001). Thus, changes in chlorophyll content and pigment ratios are important indicators of environmental stress and describes about the tolerance status of the species (Tuba et al 1996). The regeneration capability of plant grown on heavy metal was corelated to some stress related marker such as proline (Chakravarty and Shrivastava 1997, Ali at al 1998). Proline accumulation accepted as an indicator of environmental stress is also considered to have protective roles as osmoproctectant

(Hartzendorf and Rolletschek 2001), membrane stablizer (Bandurska 2001), proctective enzymes (Sharma et al 1998) and ROS scavenger.

The purpose of the present study was to contribute to a better understanding of the biochemical responses of plant in a closed system *(in vitro* explants) to copper cadmium and nickel stress. We investigated the influence of low and high metal concentration on the activities of antioxidative enzymes (SOD, CAT, and POD) and proline content in the shoot buds of *Tagetes patula*. In parallel morphogenic response and chlorophyll content were also monitored.

II. Material and Method

Dry seeds of *Tagetes patula* were procured from Namdhari Seeds Pvt Ltd, Bangalore. The seeds were washed in tap water and rinsed with 20% (v/v) extran (Merck, India) and then surface sterilized with 0.1% mercuric chloride (w/v) for 3 min followed by three rinses with sterile distilled water to remove traces of mercuric chloride. The seeds were soaked overnight in sterile distilled water and then placed on half- strength MS (Murashige and Skoog, 1962) medium to obtain the intact plantlets. The *invitro* culture of *T.patula* were established.

Culture conditions

MS medium containing 3% (w/v) sucrose and 0.9% agar (bacteriological grade Merck, India) with supplemented with BAP (0.5 mg/l)+ IAA (0.5 mg/l)was used. The pH of the medium was adjusted to 5.8 before autoclaving at 1.06 kg cm^{-2}(121ºC) pressure for 20 min. The cultures were incubated at 25 ± 1ºCwith 25 µmol $m^{-2}s^{-1}$ irradiance provided by cool fluorescent tubes (40W, Philips, India) for a photoperiod of 16 h.

***In vitro* propagation**

Leaf explants were cultured firmly with the adaxial side touching the medium in 100 ml Erlenmeyer flask containing 40 ml MS medium supplemented with plant growth regulators. The level of Nickel, Cadmium and Copper were varied from (0- 25µM).Weekly observations were recorded for 4 weeks. After 4 weeks, regenerated healthy shoot buds were used for the assay of various enzymes.

Chlorophyll estimation

Chlorophyll estimation was made following the method of Arnon (1949) using 4 week old plant material. 1g of plant material was chilled in deep freeze (-20ºC) and then homogenized in 10 ml 80 % acetone, centrifuged at 10,000 rpm for 10 min. The supernatant obtained was used for chlorophyll The absorbance of the supernatant was read at 645 nm, 665nm in a spectrophotometer(UV-2401PC Spectrophotometer, Shimadzu Corporation, Japan)against 80 % acetone solvent (Arnon, 1949). The amount of chlorophyll was calculated according to the following equation stated below

Chl. a = 12.3 (Abs 663) – 0.86 (Abs 645) V/1000xW
Chl. b = 19.3 (Abs 645) - 3.6 (Abs 663) V/1000xW
Total Chl. = Chl.a + Chl.b

Proline Estimation

Proline was estimated method given by Bates et al (1973). A measure of (1g) of plant material (4 week old) was chilled in deep freeze (-20ºC) and then homogenized in 3% aqueous sulfosalicylic acid (10ml) and the homogenate was centrifuged to 10,000 rpm for 10 min (4 ºC). The supernatant was used for the estimation of proline. The reaction mixture consisting of 2 ml supernatant, 2ml acid ninhydrin and 2 ml glacial acetic acid was boiled at 100º C for 1 hr. After termination of the reaction in ice bath for 5 min, the reaction mixture was extracted with 4 ml toluene and the absorbance was read at 520 nm. The amount of proline was calculated from the standard curve plotted with known concentration of proline.

Peroxidase enzyme assay

Peroxidase activity was determined spectrophotometrically (Shimadzu, UV-1700) according to Chanda and Singh (1997). A measure (1g)(4 week old)plant material was chilled in deep freeze (-20ºC) and then homogenized in Na-phosphate buffer (0.2 M, pH 7.0) and centrifuged at 10,000g for 15 min. The supernatant was used for estimating peroxidase activity. The assay mixture (4 ml) contained 2mlNa-phosphate buffer (0.2 M, pH 7.0), 1 ml enzyme extract, 0.5 ml (1% Guaiacol) and 0.5 ml (0.5% H_2O_2). Peroxidase (POD) activity was measured by recording the changes in absorbance at 470 nm (Δ A470) for guaiacol due to the oxidation of hydrogen donors in the presence of H_2O_2. The activity was expressed as Δ A 470 min^{-1} g fresh weight $^{-1}$.

Superoxide dismutase enzyme assay

SOD was estimated according to the method of Dhindsa et al (1981).A measure (1g)(4 week old) plant material was homogenized in 50 mM chilled/ ice cold phosphate buffer pH 7.0, 0.25 % triton x-100 (m/v) and 1 % polyvinylpyrrolidone and centrifuged at 10,000 rpm for 10 min at 4ºC and supernatant was used for enzyme assay.SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using a reaction mixture consisting of 50 mM sodium bicarbonate, 13 mM methionine, 25 mM NBT, 0.1 mM EDTA, 2 mM Riboflavin reaction was started by addition of 2 mM riboflavin and exposing to 15 W fluorescent light for 10 min. The absorbance was read at 560 nm and the total SOD activity of the samples was assayed by measuring its ability to inhibit the photochemical reduction of nitro-blue-tetrazolium (NBT). 1 unit of SOD activity was defined as the amount of enzyme, which causes 50 % inbibition of the photochemical reduction of NBT.

Catalase enzyme assay

Catalase activity was determined by the method of Teranishi et al. (1974) with some 1.0 gm plant was homogenised in 10 ml 50 mM chilled/ice cold phosphate buffer pH 7.0 and centrifuged at 10,000 rpm for 10 min at 4ºC and supernatant was treated as enzyme extract. The reaction mixture (3 ml) containing 50 mM phosphate buffer (pH 7.0) (2.7 ml) and (0.1) ml of enzyme extract. The reaction was initiated by addition of 200mM H_2O_2 (0.2 ml). The decrease in absorbance was recorded at 410 nm for 3 minutes. The catalase activity was measured using the extinction coefficient 40 mM^{-1} cm^{-1} and expressed as µMol H_2O_2 reduced/sec/g FW.

Statistical analysis

All the experiments were repeated thrice with five replicates for each treatment. The cultures were observed periodically and the percentage of responding explants and number of shoot buds developed per responding explants were recorded. The frequency of shoot regeneration was calculated as the percent of leaf explants responded out of total number of explants inoculated in a particular treatment. Data were presented as mean ± standard error (SE). Data were statistically analysed using analysis of variance (ANOVA) to test the statistical significance, and the significance of differences among means was carried out using Duncan's (1955) multiple range test at p=0.05.

III. Result

Growth effects

The *in vitro* leaf explant reared on regeneration medium supplemented with BAP (0.5 mg/l)+ IAA (0.5 mg/l)differentiated an average of 20-22 shoot buds. Addition of different concentration of heavy metals (Copper, Cadmium and Nickel) in the aforesaid regeneration medium caused differential response in terms of multiple shoot production as well as growth. $CuSO_4$ when used in lower concentration, however, favoured organogenesis, there was consistent increase in shoot buds till 4 µM but further increase in $CuSO_4$ caused decline in morphogenesis and at 25µM the shoot regeneration was severely hampered (Fig1). Cadmium chloride caused an appreciable decline in morphogenesis. Though the induction of multiple shoot buds was seen at its higher contents but the growth was very poor. At higher concentration the shoot buds showed chlorosis and necrotic symptoms followed by growth reduction and death. The average shoot number declined from 26 at 2 µM to 0 at10 µM of $CdCl_2$ (Fig1). In contrast, to $CuSO_4$ and $CdCl_2$ $NiCl_2$ proved less injurious to shoot growth compared with other metals. The treatment resulted in increase in shoot growth till 8 µM and further increase in concentration decreased shoot induction. The average shoot bud number varied from 33 shoots at 4 µM to 8 shoots at 25 µM (Fig1).

Chlorophyll content is often measured in plants to access the impact of heavy metal stress. The chlorophyll content did not change significantly in the shoot buds developed on medium supplemented with cadmium till 8 µM and on further increase chlorosis and necrosis occurred (Fig. 2). In case of nickel treatment there was increase of chlorophyll till 2 µM and further as the concentration increased the chlorophyll content decreases (Fig. 2). Similarly with shoots regenerated on copper supplemented medium there was increase in chlorophyll till 4 µM and then the decrease is observed (Fig. 2)

Proline is one of the most widespread metabolites produced in plant tissues under stress conditions. A significant increase in proline content was found in shoots exposed to heavy metal treatment. The proline content was significantly higher as compared to control. In the case of Ni the proline content decreased till 10 µM and at concentration 25 µM there was slight increase. On the contrary the level of proline increased significantly in the shoots developed on medium supplemented with Cd and Cu (Fig.3). SOD activity was considerably promoted by excess of cadmium chloride in the medium. The high activity was also seen in the shoot buds induced on the medium supplemented with copper sulphate and nickel chloride (Fig.4).

There was strong increase in the activity of catalase till 8 µM in case of shoot buds induced on the medium containing cadmium, copper and nickel and then decreased till 25 µM (Fig. 5). The catalase activity

was more pronounced with cadmium treatment than with nickel and copper. A significant increase of the peroxidase enzyme activity was observed (Fig. 6) in the shoot buds induced on medium supplemented with cadmium chloride, copper sulphate and nickel chloride. The activity was more marked with cadmium exposure as compared to copper and nickel. This enzyme has been proposed to be a potential biomarker for sublethal metal toxicity in plants.

IV. Discussion

In the present study the comparative toxicity of heavy metals (Nickel, Cadmium and Copper) in *in vitro* cultures was studied and found that cadmium was most toxic. In terms of shoot regeneration nickel and copper was found to be beneficial at lower concentration but at higher concentration reduction in growth was observed. But cadmium even at lower concentration was toxic and higher concentration was found deleterious for the growth. Similarly tissue culture of plant species such as tobacco, sunflower and soyabean have been used to understand the mechanism of metal resistance (Sobkowiak et al., 2004). Heavy metal toxicity promotes altered metabolism in plants, which is observed by changes in growth conditions. Heavy metal like Cu and Cd can modify the rate of plant development (Saxena and Kaur, 2005). The inhibitory effect of Cu may be due to Cu induced Fe deficiency leading to chlorosis and suppression of shoot growth. At higher concentration of cadmium the growth of tissue ceases and turn brown.

Browning of tissue at higher concentration may be due to the damage of phosynthetic apparatus, such as disrupted chloroplast structure and dialation of the thylakoid membrane (Quzoumidone et al 1997). Our findings corroborate with that of Ali et al 1998 where the regenerants of *Bacopa monniera* did not survive beyond 50 nm cadmium. The inhibitory effect of Cu may be due to Cu induced Fe deficiency leading to chlorosis and suppression of shoot growth subsequently all the ultrastructure changes occurred due to toxic effect of high concentration of copper.

In the present study increase in SOD activity may be due to increased level of ROS, which causes increase in the expression of gene responsible for encoding SOD (Bowler et al., 1992). Increase in the activity of SOD under conditions of cadmium toxicity is in consonance with several earlier observations (Hsu and Kao, 2004). Increase in SOD activity has been reported in adapted callus of sunflower cultivated under cadmium treatment (Costa et al., 2002). In certain plant species a decrease in level of SOD is observed at very high concentration of metal, the reason is at higher concentration the inactivation of enzyme by H_2O_2 produced in different cell compartments and from a number of nonenzymatic and enzymatic processes in cells (Luna et al., 1994).

In the present study CAT activity decrease with the increase in metal ion concentration similar results have been obtained (Sandalio et al., 2001; Shekhawat et al., 2010). CAT is sensitive to superoxide radicals, thus cadmium stress leads to inactivation of enzyme (Cakmak, 2000). The decrease may also be related with degradation caused by peroxisomal proteases or may be due to photo activation of enzymes (Sandalio et al., 2001).

V. Conclusion

Plants have high level of physiological plasticity enabling survival of a wide variety of environmental stress to maintain cell integrity and homeostasis. Exposure to unfavourable environmental conditions, ROS is formed in excess leading to a state so called oxidative stress which is controlled by an antioxidative system that consists of antioxidative enzymes and nonezymatic low molecular mass antioxidants.

References

[1]. Ali G., Srivastava P.S., Iqbal M. (1998) Effect of cadmium and copper on growth of Bacopa monniera regenerants. Biologia Plantarum 41:635-639.
[2]. Apel K., Hirt H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Plant Biology 55:373-399.
[3]. Arnon D.I., Stout P.R. (1939) The essentiality of certain elements in minute quantity for plants with special reference to copper. Plant Physiology 14:371-375.
[4]. Bandurska H. (2001) Does proline accumulated in leaves of water deficit stressed barley plants confine cell membrane injuries? II. Proline accumulation during hardening and its involvement in reducing membrane injuries in leaves subjected to severe osmotic stress. Acta Physiologiae Plantarum 23:483-490.
[5]. Bates L.S., Wadern R.P., Teare I.D. (1973) Rapid estimation of free proline for water stress determination. Plant soil 39:205-207.
[6]. Bowler C., Montagu M.V., Inze D. (1992) Superoxide dismutase and stress tolerance. Annual Review of Plant Biology 43:83-116.
[7]. Cakmak I. (2000) Role of zinc in protecting plant cells from reactive oxygen species. New Phytologist 146:185-205.
[8]. Chakravarty B., Srivastava S. (1997) Effects of Genotype and Explant duringin vitroResponse to Cadmium Stress and Variation in Protein and Proline Contents in Linseed* 1. Annals of Botany 79:487-491.
[9]. Chanda SV, Singh YD. Changes in peroxidase and IAA oxidase activities during wheat grain development. Plant Physiol Biochem 35: 245-250, 1997.
[10]. Chen G., Djuric Z. (2001) Carotenoids are degraded by free radicals but do not affect lipid peroxidation in unilamellar liposomes under different oxygen tensions. FEBS letters 505:151-154.
[11]. Costa H., Gallego S.M., Tomaro M.L. (2002) Effect of UV-B radiation on antioxidant defense system in sunflower cotyledons. Plant Science 162:939-945.

[12]. Dhindsa R.S., Plumb-Dhindsa P., Thorpe T.A. (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. Journal of Experimental Botany 126:93-101.
[13]. Gallego S., Benavides M., Tomaro M. (2002) Involvement of an antioxidant defence system in the adaptive response to heavy metal ions in Helianthus annuus L. cells. Plant Growth Regulation 36:267-273.
[14]. Gallego S., Benavides M., Tomaro M. (2002) Involvement of an antioxidant defence system in the adaptive response to heavy metal ions in Helianthus annuus L. cells. Plant Growth Regulation 36:267-273.
[15]. Hartzendorf T., Rolletschek H. (2001) Effects of NaCl-salinity on amino acid and carbohydrate contents of Phragmites australis. Aquatic Botany 69:195-208.
[16]. Hsu Y.T., Kao C.H. (2004) Cadmium toxicity is reduced by nitric oxide in rice leaves. Plant Growth Regulation 42:227-238.
[17]. Luna C.M., Gonzalez C.A., Trippi V.S. (1994) Oxidative damage caused by an excess of copper in oat leaves. Plant and Cell Physiology 35:11-15.
[18]. Mathur S, Shekhawat GS, Batra A (2008) Somatic embryogenesis and plantlet regeneration from cotyledon explants of Salvadora persica L. Phytomor 58(1–2):57–63
[19]. Misra P., Datta S.K. (2001) Direct differentiation of shoot buds in leaf segments of white marigold (Tagetes erecta L.). In Vitro Cellular & Developmental Biology-Plant 37:466-470.
[20]. Mocquot, B., Vangrosveld, J., Clijsters, H., Mench, M.: Copper toxicity in young maize (Zea mays L.) plants: effects on growth, mineral and chlorophyll contents, and enzyme activities.-Plant Soil **182**: 287–300, 1996
[21]. Murashige T., Skoog F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum 15:473-497.
[22]. Ouzounidou G., Moustakas M., Eleftheriou E.P. (1997) Physiological and ultrastructural effects of cadmium on wheat (Triticum aestivum L.) leaves. Archives of environmental contamination and toxicology 32:154-160.
[23]. Sandalio L.M., Dalurzo H.C., Gomez M., Romero-Puertas M.C., Del Rio L.A. (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. Journal of Experimental Botany 52:2115-2126
[24]. Saxena D.K., Kaur H. (2005) Effect of cadmium and nickel toxicity on the peroxidase activity and carotenoids content in moss Thuidium cymbifolium. Indian Journal of Plant Physiology 10:397- 399
[25]. Sharma S.S., Schat H., Vooijs R. (1998) In vitro alleviation of heavy metal-induced enzyme inhibition by proline. Phytochemistry 49:1531-1535.
[26]. Shekhawat G.S., Verma K., Jana S., Singh K., Teotia P., Prasad A. (2010) In vitro biochemical evaluation of cadmium tolerance mechanism in callus and seedlings of Brassica juncea. Protoplasma 239:31-38.
[27]. Sobkowiak R., Rymer K., Rucinska R., Deckert J. (2004) Cadmium-induced changes in antioxidant enzymes in suspension culture of soybean cells. Acta Biochimica Polonica-English Edition-:219-222.
[28]. Teranishi Y., Tanka A., Osumi M., Fukui S. (1974) Catalase activities of hydrocarbon-utilizing Candida yeasts. Agricultural and Biological Chemistry 38:1213-1220.
[29]. Tuba Z., Lichtenthaler H.K., Csintalan Z., Nagy Z., Szente K. (1996) Loss of chlorophylls, cessation of photosynthetic CO2 assimilation and respiration in the poikilochlorophyllous plant Xerophyta scabrida during desiccation. Physiologia plantarum 96:383-388.
[30]. Wu H, Tang S, Zhang X, Guo J, Song Z, Tian S (2009) Using elevated CO_2 to increase the biomass of a Sorghum vulgare Sorghum vulgare var. sudanense hybrid and Trifolium pratense L. and to trigger hyperaccumulation of cesium. J Hazard Mater 170:861–870

Fig. 1 Effect of heavy metals on shoot bud induction from leaf explant of *Tagetes patula* cultured on MS medium supplemented with BAP (0.5 mg/l)+ IAA (0.5 mg/l)

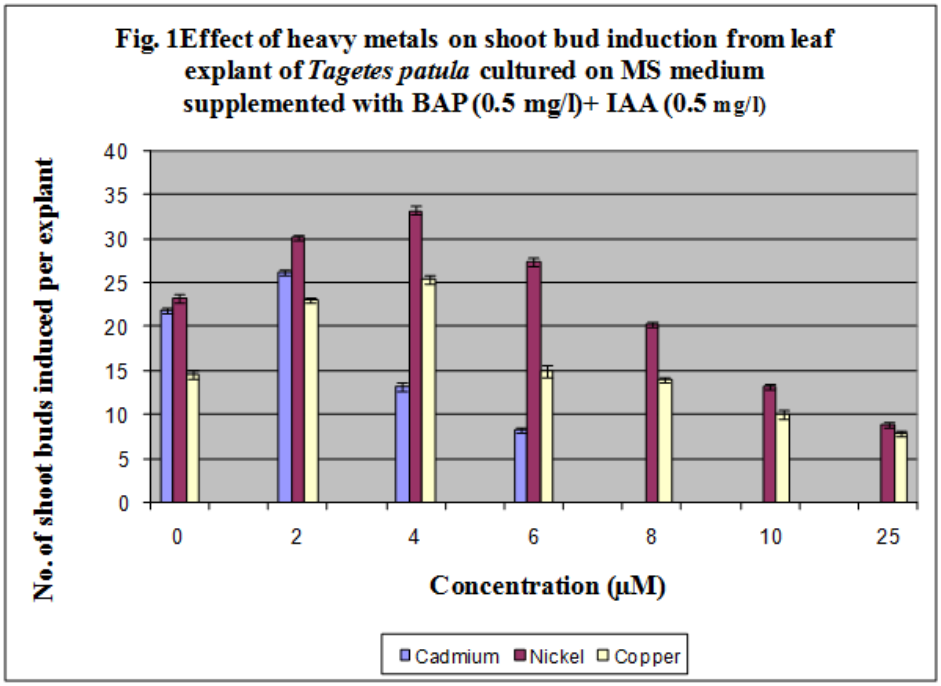

Fig. 2 Effect of heavy metals on chlorophyll content in shoot buds of *Tagetes patula* from leaf explant cultured on MS medium supplemented with BAP (0.5 mg/l) + IAA (0.5 mg/l)

Fig. 2 Effect of heavy metals on chlorophyll content in shoot buds of *Tagetes patula* from leaf explant cultured on MS medium supplemented with BAP (0.5 mg/l) + IAA (0.5 mg/l)

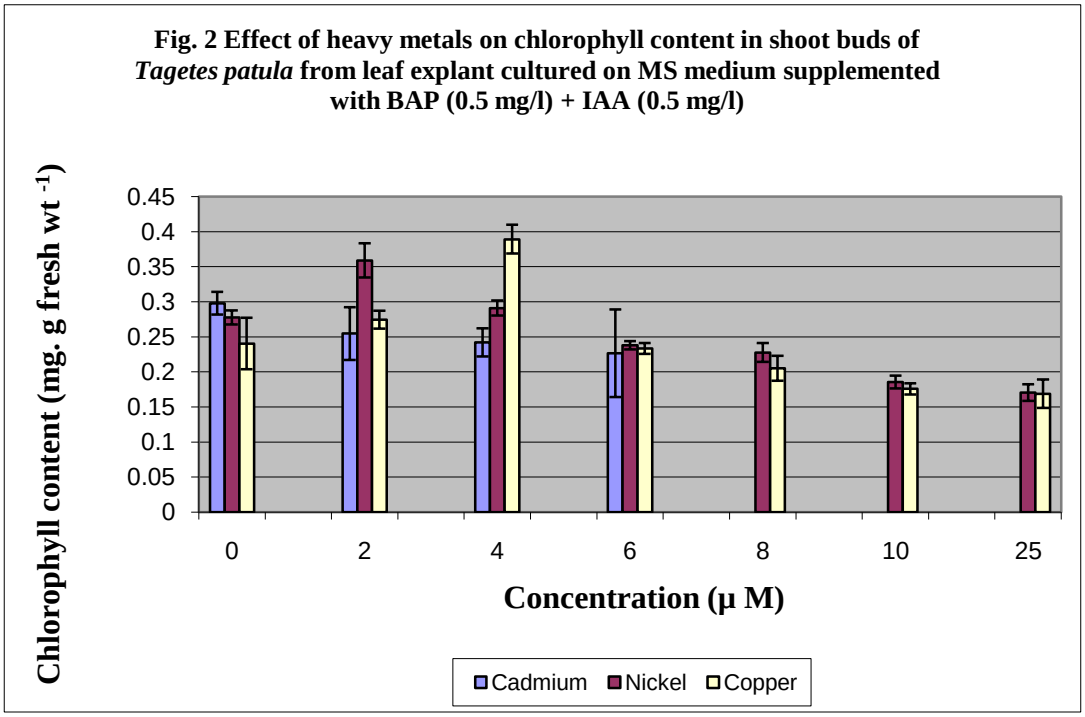

Fig. 3 Effect of heavy metals on proline content in shoot buds of *Tagetes patula* from leaf explant cultured on MS medium supplemented with BAP (0.5 mg/l) + IAA (0.5 mg/l

Fig. 3 Effect of heavy metals on proline content in shoot buds of *Tagetes patula* from leaf explant cultured on MS medium supplemented with BAP (0.5 mg/l) + IAA (0.5 mg/l)

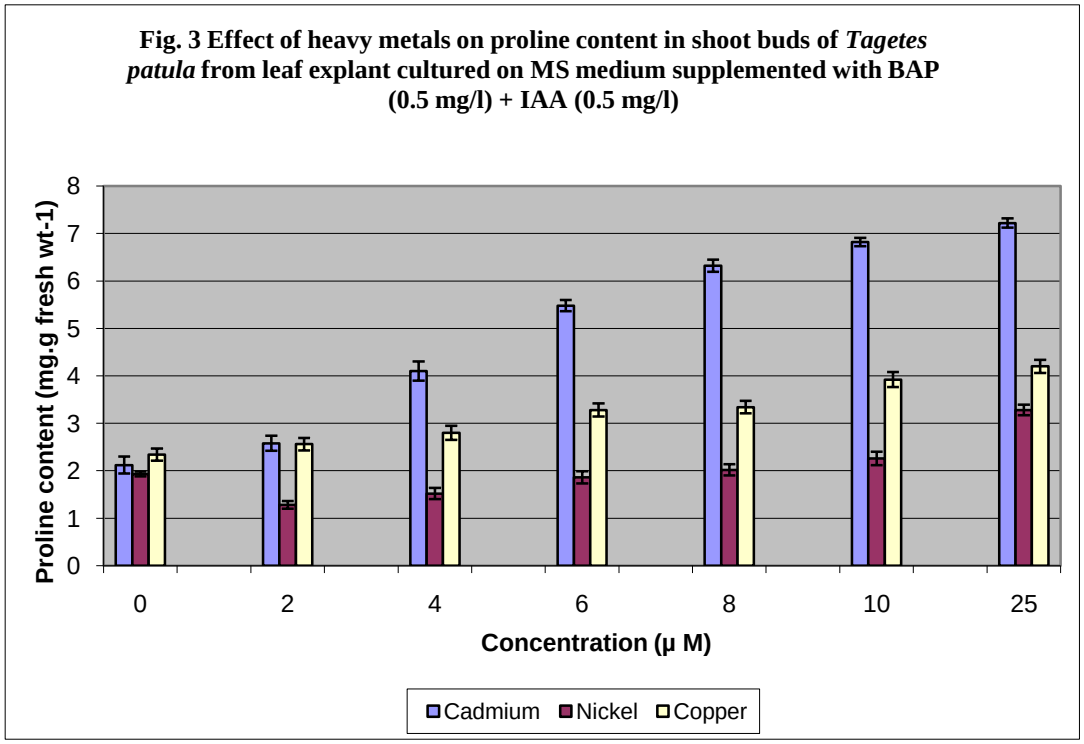

Fig. 4 Effect of heavy metals on SOD content in shoot buds of *Tagetes patula* from leaf explant cultured on MS medium supplemented with BAP (0.5 mg/l) + IAA (0.5 mg/l)

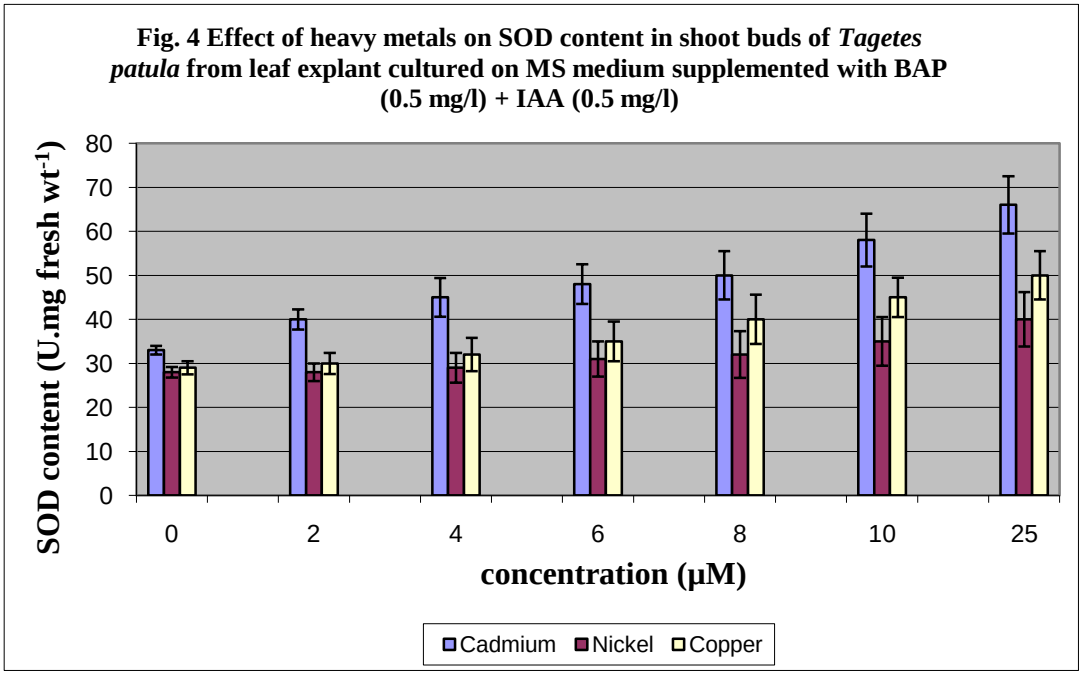

Fig. 5 Effect of heavy metals on catalase enzyme activity in shoot buds of *Tagetes patula* from leaf explant cultured on MS medium supplemented with BAP (0.5 mg/l) + IAA (0.5 mg/l)

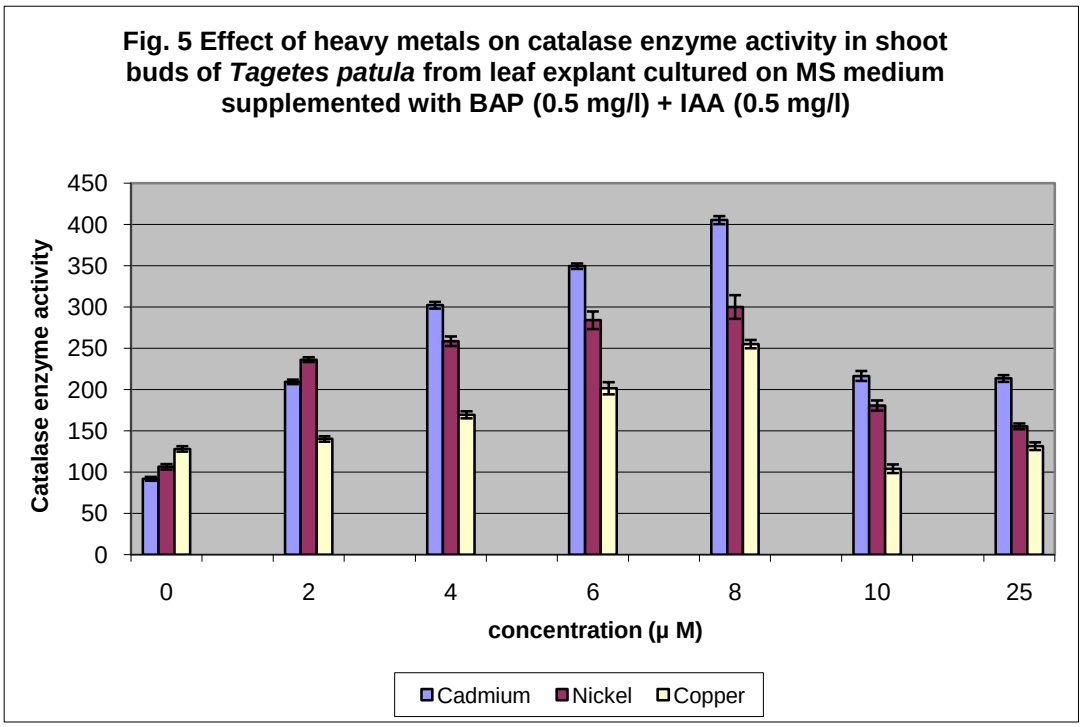

Fig. 6 Effect of heavy metals on peroxidase enzyme activity in shoot buds of *Tagetes patula* from leaf explant cultured on MS medium supplemented with BAP (0.5 mg/l) + IAA (0.5 mg/l)