

Interactive effects between supplemental ultraviolet-B radiation and heavy metals on the growth and biochemical characteristics of *Spinacia oleracea* L.

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Received: 04/03/2006, Accepted: 01/06/2006

The impact of supplemental UV-B (sUV-B) radiation and heavy metals: Cd (68 $\mu\text{mol.kg}^{-1}$ soil) and Ni (68 $\mu\text{mol.kg}^{-1}$ soil) singly and in combination was studied on photosynthetic and non-photosynthetic pigments, metabolites, enzyme activities, biomass accumulation and the net primary productivity of spinach (*Spinacia oleracea* L. var. All Green), a common leafy vegetable plant. Both the stresses individually and in combination resulted in the reduction of photosynthetic pigments, ascorbic acid, and catalase activity, whereas a reverse trend was observed for anthocyanin, flavonoids and proline content, lipid peroxidation and peroxidase activity. The interactive effects of both the stresses were, however, less than additive. Combined treatment of sUV-B + Cd was more deleterious as compared to sUV-B + Ni and other individual treatments.

Key words: *Spinacia oleracea*, biomass, cadmium, enzymes, nickel, pigments, ultraviolet-B.

Efeitos interativos entre radiação ultravioleta B suplementar e metais pesados no crescimento e características bioquímicas de *Spinacia oleracea* L.: Estudou-se o impacto de radiação UV-B suplementar (sUV-B) e dos metais pesados Cd (68 $\mu\text{mol.kg}^{-1}$ solo) e Ni (68 $\mu\text{mol.kg}^{-1}$ solo), isoladamente ou em combinação, sobre pigmentos fotossintéticos e não fotossintéticos, metabólitos, atividades enzimáticas, acúmulo de biomassa e produtividade primária de espinafre (*Spinacia oleracea* L. var. All Green). Ambos estresses, individualmente ou em combinação, resultaram na redução dos pigmentos fotossintéticos, ácido ascórbico, e atividade de catalase, sendo que uma tendência oposta foi observada para o conteúdo de antocianina, flavonóides e prolina, peroxidação lipídica e atividade de peroxidase. Os efeitos interativos de ambos estresses foram, no entanto, menos que se fossem aditivos. O tratamento combinado de sUV-B + Cd foi o mais deletério quando comparado com sUV-B + Ni e outros tratamentos individuais.

Palavras-chave: *Spinacia oleracea*, biomassa, cádmio, enzimas, níquel, pigmentos, ultravioleta-B.

INTRODUCTION

A decrease in the concentration of stratospheric ozone is enhancing the solar ultraviolet-B (UV-B, 280-320 nm) radiation on the earth's surface (Caldwell et al., 1998). Several studies have indicated that supplemental UV-B radiation (sUV-B) can deleteriously affect physiological processes and overall growth in a number of plant species (Tevini, 2000; Rathore et al., 2003). Recently, Agrawal et al. (2004) reported the unfavorable effects of sUV-B on several

physiological and biochemical processes in two cultivars of *Triticum aestivum* L. leading to reduction in biomass and grain yield

In recent years, besides increments in UV-B radiation, a great deal of interest has been generated on studies related to the toxic effects of heavy metals on plants. Heavy metal pollution is increasing in the environment due to mining, industrialization and other anthropogenic activities. Airborne heavy metals fall upon, react with, and are absorbed by plants

and soils near the sites of pollutant generation. A characteristic feature of toxicities due to heavy metal is chlorosis and reduction in the net photosynthetic rate leading to decreased growth and productivity (Burton et al., 1986). Among heavy metal pollutants, nickel (Ni) and cadmium (Cd) need special attention due to their widespread occurrence and potential for their toxicities. Cd in higher concentration has been shown to disturb chlorophyll synthesis (Stobart et al., 1985) and many metabolic processes like nucleic acid and protein synthesis (Bingham et al., 1976).

Synergistic action of UV-B radiation and Cd was reported by Shukla et al. (2002) on the growth of wheat seedlings. Prasad et al. (2004) reported changes due to UV-B and Cd alone and in combination on photosynthetic pigments, photosynthetic electron transport activity, enzymatic and non-enzymatic antioxidants and lipid peroxidation in a liverwort, *Riccia* sp.

Ni is considered as an essential micronutrient for plants, but is strongly phytotoxic at higher concentrations (Boominathan and Doran, 2002). Ni-induced deactivation of proteins including antioxidant enzymes, lipid peroxidation and membrane function have been reported in plants (Madhava Rao and Sresty, 2000).

In view of the above facts, an assessment of multiple stress effects is very important in obtaining a more meaningful and realistic view of the current changes in the environment. The present investigation was aimed to assess the interactive effects of two potential stress factors i.e. sUV-B radiation and heavy metals (Cd / Ni) applied individually and in combination on the growth and biochemical characteristics of spinach (*Spinacia oleracea* L. var All Green), a common leafy vegetable plant.

MATERIAL AND METHODS

The present experiment was performed from March to April, 2003 in the field of Allahabad Agriculture Institute, Allahabad, Uttar Pradesh (24°47' N latitude and 81°19' E longitude, at an elevation of about 96 m above sea level) situated in the eastern Gangetic plains of India. Spinach (*Spinacia oleracea* var. All Green) plants were grown in a soil pot culture at the level of 68 $\mu\text{mol.kg}^{-1}$ soil Cd as CdSO₄ and 68 $\mu\text{mol.kg}^{-1}$ soil Ni as NiCl₂ in 10 pots each. The concentrations of Cd and Ni selected for experiment fall within Indian standards for heavy metals in soil (Awasthi, 2000). The pots were uniformly watered throughout the experiment in order to maintain constant soil moisture. Ten

pots without heavy metal amendment were kept as a control. Five pots of each treatment (Cd, Ni, and control) were randomly selected for sUV-B exposure.

Supplemental UV-B (sUV-B) radiation was artificially provided by Q panel UV-313 fluorescent lamps (Q-panel, Cleveland, U. S. A.) suspended above and perpendicular to the pots. Cellulose diacetate and polyester films were used to transmit sUV-B (cutoff ca. 292 nm) and exclude UV-B (cutoff ca. 318 nm). The control plants thus received only ambient levels of UV-B. Plants one week after emergence were exposed to radiation for 3 hr day⁻¹ (9:30 a.m. to 12:30 p.m.) for 45 days. The UV-B radiation at the top of the plant canopy was measured by an Ultraviolet Intensity Meter (UVP Inc., San Gabriel, CA, USA). The readings were converted to UV-B_{BE} values by comparing UV meter readings with a Spectro Power Meter (Scientech Inc, Boulder, CO, USA). Plants under polyester filter lamps received 8.6 KJ.m⁻² UV-B_{BE} on the summer solstice, measured against the generalized plant response action spectrum of Caldwell (1971). The plants beneath the cellulose diacetate film received ambient (8.6 KJ.m⁻²) + 7.1 KJ.m⁻² UV-B_{BE} that mimicked the 20 % reduction in the stratospheric ozone at Allahabad (20°47' N) during clear sky conditions during the summer solstice normalized at 300nm. The ozone column thickness was assumed to be 3.0 mm, the albedo 0 and the scatter 1.0. Three plants were sampled randomly from three replicate pots of each treatment at 15, 30 and 45 days after sowing (DAS) for determination of the various parameters.

During the study period the mean minimum and mean maximum temperatures ranged between 14.9 to 24.3°C and 30.8 to 34.6°C, respectively. The average relative humidity varied between 59.8 and 68.4 % and PAR ranged between 1,100 to 1,200 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$. There were negligible differences between temperature, relative humidity and PAR under sUV-B exposed plants and other treatments.

For all biochemical analyses leaf samples were taken at 9 a.m. and kept in a deep freezer for further analysis. There was no significant difference in the fresh/dry weight ratio of sampled tissues. Estimation of chlorophyll, carotenoids, anthocyanin and flavonoid contents was done by the methods described by Rathore et al. (2003). Leaf samples were homogenized in oxalic acid and NaEDTA extraction solution for ascorbic acid content determination as described by Keller and Schwager (1977). Determination of proline was performed by the ninhydrin assay using the method of Plummer (1979). Lipid peroxidation in the leaf tissue was measured in terms of malondialdehyde (MDA, a product of

lipid peroxidation) content by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Catalase and peroxidase activities were measured by the methods described by Agrawal et al. (2004).

For total biomass, randomly sampled plants were dried at 80°C until a constant weight was achieved.

To check the significant effects of the treatments and interactions between the treatments at different growth stages, statistical analysis was carried out using the SPSS software (SPSS Inc., version 7.0).

RESULTS

Total chlorophyll and carotenoid contents declined in all the treated plants at all ages as compared to the controls (figure 1). However, per cent reductions decreased with the increase of plant age. Maximum reductions of 25.4 % in chlorophyll and 27.4 % in carotenoid contents were observed in sUV-B + Cd treated plants at 15 DAS, as compared to the controls. A three way analysis of variance showed significant variations in the above parameters due to all the factors and their interactions (table 1). Anthocyanin and flavonoid contents did not change significantly in response to individual treatments of Cd or Ni. However, they showed

a marked increase in plants treated with sUV-B alone and in combination with either metal (Cd/Ni). The maximum increments of 66.6 and 72.6 % in anthocyanin and flavonoid contents, respectively were observed at 45 DAS in sUV-B treated plants without application of any heavy metal (figure 1). Multivariate analysis of the variance test showed significant variations in anthocyanin and flavonoid contents due to sUV-B treatment, plant age, age × treatment, and sUV-B treatment × Cd/Ni interactions (table 1). Catalase activity decreased with treatment of by sUV-B, Cd or Ni and combinations of sUV-B and metals in plants at all the ages. The percentage of reductions increased with increase in age, being most severe in the combined treatments of sUV-B + Cd (67.8 %) and sUV-B + Ni (61.6 %), followed by the individual treatments sUV-B (54.8 %), Cd (32.8 %) and Ni (26.1 %) at 45 DAS (figure 2). In contrast to catalase, peroxidase activity increased in all the treatments at all ages. Peroxidase activity showed maximum increment under sUV-B + Cd (74.3 %) combinations followed by sUV-B + Ni (70.9 %), sUV-B (70.3 %), Cd (63.4 %) and Ni (50.9 %) (figure 2). A three way analysis of variance test showed significant variations in enzyme activities due to all the factors and their interactions (table 1).

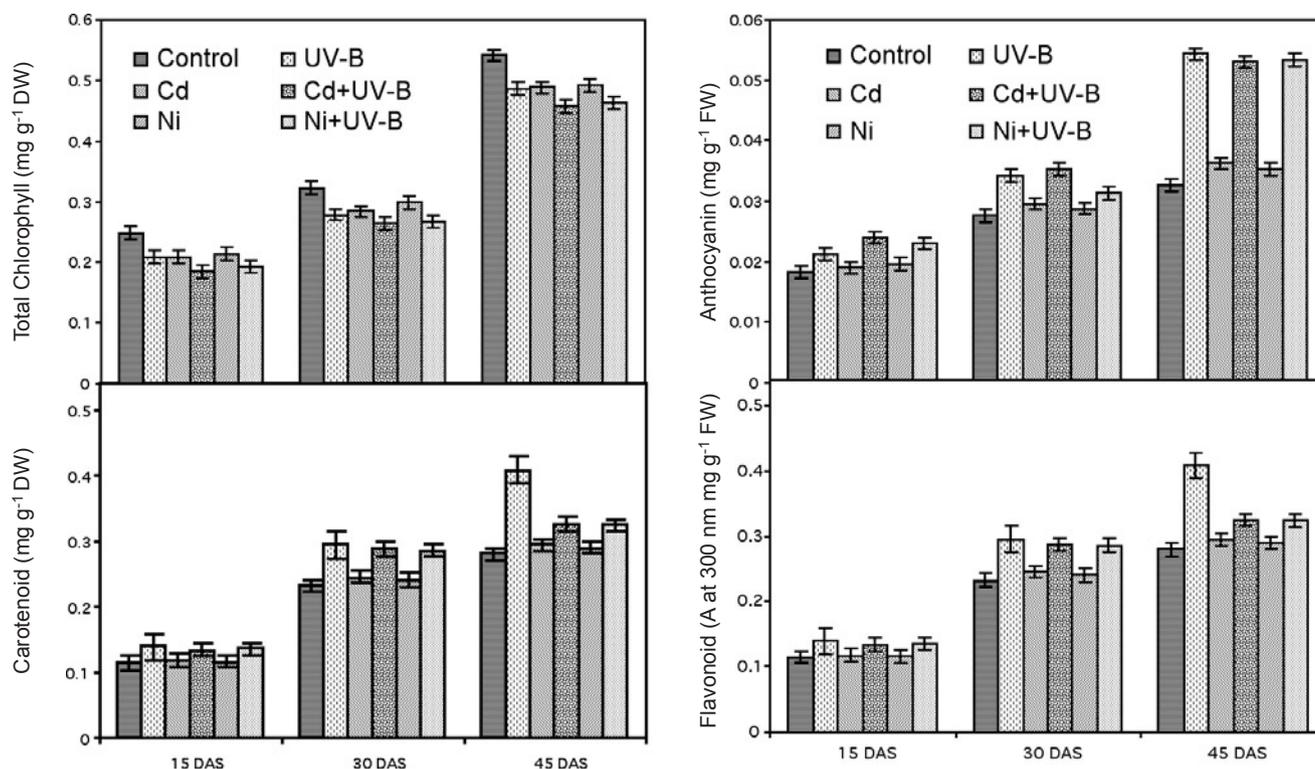


Figure 1. Effects of sUV-B radiation and heavy metal (Cd / Ni) singly and in combination on photosynthetic and non-photosynthetic pigments of *Spinacia oleracea* L. at 15, 30 and 45 DAS (mean ± S.E.).

Table 1. Variance ratio of *Spinacia oleracea* L. plants grown with and without sUV-B radiation under heavy metal treatment (Cd/Ni).

Parameters	Plant age		sUV-B treatment		Heavy metal treatment									
	(A)	(T)	(A)	(T)	Cd (H ₁)	Ni (H ₂)	A x T	A x H ₁	A x H ₂	T x H ₁	T x H ₂	A x T x H ₁	A x T x H ₂	
Total chlorophyll	***	***	***	***	***	***	***	***	***	***	***	**	**	
Carotenoid	***	***	**	**	**	**	***	**	*	**	*	*	*	
Anthocyanin	***	***	NS	NS	NS	NS	NS	NS	*	*	*	*	*	
Flavonoid	***	***	NS	NS	NS	NS	NS	NS	**	**	**	**	**	
Catalase	***	***	***	***	***	***	***	***	***	***	***	***	***	
Peroxidase	***	***	***	***	***	***	***	***	***	***	***	***	***	
Ascorbic acid	***	***	***	***	***	***	***	***	**	**	*	*	*	
Proline	***	***	***	***	***	***	***	***	***	***	***	**	*	
Lipid peroxidation	***	***	***	***	***	***	***	***	***	***	***	**	**	
Biomass	***	***	***	***	***	**	***	**	***	***	***	***	***	
NPP	**	***	***	***	***	**	***	**	***	***	***	***	***	

*** = p < 0.001, ** = p < 0.01, * = p < 0.05, NS = Non significant

An increase in the level of lipid peroxidation was observed in all the treated plants at all the ages, being maximum with the sUV-B + Cd (29.6 %) treatment followed by sUV-B + Ni (26.0 %), sUV-B (24.2 %), Ni (13.7 %) and Cd (13.1 %) at 45 DAS (figure 3). Variations in lipid peroxidation were significant due to all the factors and their interactions (table 1). Ascorbic acid content decreased due to various treatments, reduction being maximum (56.7 %) in plants treated with sUV-B + Cd at 15 DAS (figure 2). A three-way analysis of variance showed significant variations in ascorbic acid and proline contents due to plant age, sUV-B, and heavy metal treatments as well as their interactions (table 1). There was a sharp increase in proline accumulation due to different treatments at all the ages of observations (figure 2). However, combined treatments of both the stresses showed higher accumulation (47.5 and 42.9 % for sUV-B+ Cd and sUV-B+ Ni, respectively) than individual treatments of sUV-B (38.2 %), Cd (27.7 %) and Ni (26.2 %) at 15 DAS.

The total plant biomass decreased significantly in all the treated plants at all the sampling stages (figure 4). Reductions of 16.7 %, 18.6 % and 18.5 % were observed at 45 DAS with the sUV-B, Cd and Ni treatments. Maximum reductions were observed in combined treatments of sUV-B + Cd and sUV-B+ Ni, the reductions being 25.3 % and 24.1 %, respectively at 45 DAS as compared to the controls (without sUV-B/heavy metal treatment). The interactive effects of the stresses were always less than additive. Net primary productivity (NPP) showed a similar pattern as that of biomass (figure 4). A multivariate analysis of variance showed significant effects of all the factors and their interactions on biomass and NPP (table 1).

DISCUSSION

Chlorophyll and carotenoids are the central part of the energy manifestation of every green plant system and therefore, any significant alteration in their levels is likely to cause a marked effect on the entire metabolism of the plants. In the present experiment, both sUV-B and heavy metal (Cd/Ni) exposure decreased the chlorophyll and carotenoid contents of the test plant. Reduction in chlorophyll contents by excess Ni and Cd has been reported in the pigeon pea (Sheoran et al., 1990) and spinach (Dube et al., 2002). High Cd inhibits the formation of chlorophyll by interfering with protochlorophyllide production (Stobart et al., 1985). A 30 % reduction in the total chlorophyll of pea leaves exposed to UV-B has been reported by Day and Vogelmann (1995). A decrease of chlorophyll content by 7 % as compared to

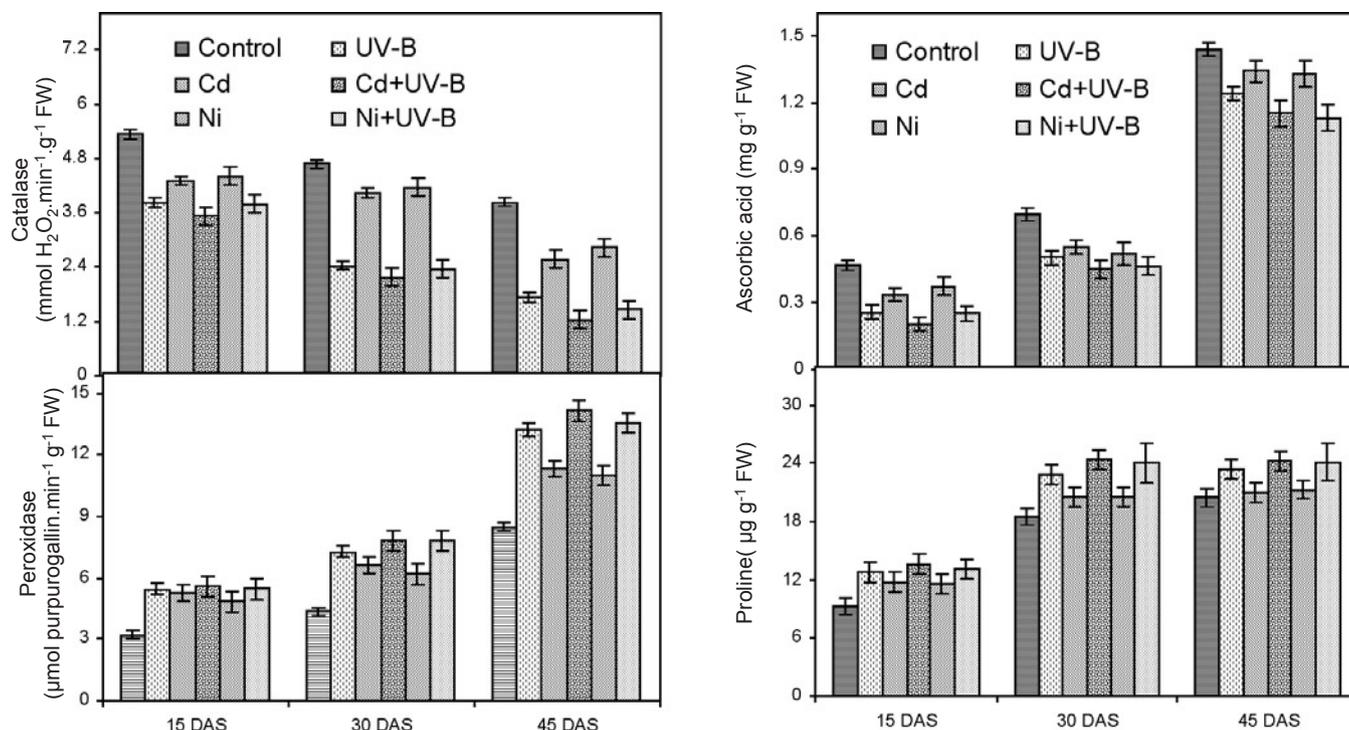


Figure 2. Effects of sUV-B radiation and heavy metal (Cd / Ni) singly and in combination on catalase and peroxidase activity, ascorbic acid and proline contents of *Spinacia oleracea* L. at 15, 30 and 45 DAS (mean \pm S.E.).

the control was reported in *Brassica campestris* L. plants after exposure to UV-B and Cd (Bornman and Dube, 1991). Several other reports have also suggested a reduction in carotenoid following sUV-B exposure (Tevini et al., 1981, Premkumar and Kulandaivelu, 1996). Carotenoids protect chlorophyll from photooxidative destruction (Middleton and Teramura, 1993) and therefore, a reduction in carotenoid could have a serious consequence on chlorophyll pigments. Prasad et al. (2004) noticed reductions of 18 %, 47 % and 58 % in chlorophyll content with UV-B, 1000 μ mol Cd and UV-B + Cd, respectively.

The accumulation of UV-B absorbing pigments is one of the ways by which plants alleviate the harmful effects of UV-B. In the present study, anthocyanin content increased after sUV-B treatment, but not significantly after treatment with heavy metals. An increase in anthocyanin content due to UV-B exposure has also been reported in *Vigna mungo* (Britto and DeBritto, 1995). Flavonoids showed a higher value in sUV-B and sUV-B + heavy metal (Cd/Ni) treated plants as compared to individual treatment with heavy metals or control plants. Increase in flavonoid content is in support of the results obtained by Rathore et al. (2003) in two cultivars of *Triticum aestivum* L.

Decline of catalase activity and increase in peroxidase activity was also reported by Rathore et al. (2003) in *Triticum aestivum* L. under sUV-B radiation and by Panda and Khan (2003) and Shah et al. (2001) in *Oryza sativa* plants under heavy metal toxicity. Catalase and peroxidase are important in the antioxidant defense system for scavenging H₂O₂ and free radicals (Bowler et al., 1992). Antioxidants catalase, peroxidase and carotenoids are effective quenchers of reactive oxygen species (ROS) and play an important role in adaptation and ultimate survival of plants during periods of stress. Decreased levels of catalase and carotenoids and increased levels of peroxidase induced by oxidative stress, reflected a general strategy required to overcome the stress and protect cells against damage.

Ascorbate is a major antioxidant and its reduction suggested its reaction directly with ROS generated by sUV-B, Cd, Ni and their combinations and also its utilization in reducing the oxidized form of α -tocopherol. Ascorbic acid acts as an *in vitro* biological antioxidant, and functions as a co-substrate of plant peroxidases (Halliwell, 1982). Singh (1996) also reported significant reductions in the ascorbic acid content of three leguminous plants (*Vigna radiata* L., *Phaseolus mungo* L., and *Glycine max* L.) after exposure to enhanced UV-B radiation.

Proline, an osmoprotectant, accumulated under all treatments i.e. sUV-B, Cd, Ni, and their combinations. Heavy metals and sUV-B exposure give rise to a series of reactions which generate numerous free radicals which may be reflected by altered levels of major anions and accumulation of proline

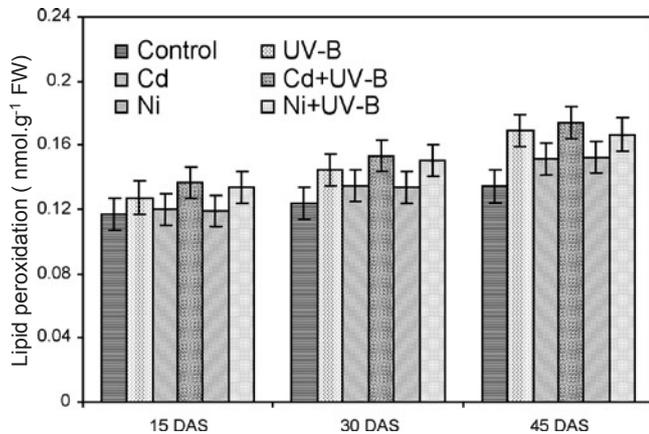


Figure 3. Effects of sUV-B radiation and heavy metal (Cd / Ni) singly and in combination on lipid peroxidation (MDA concentration) of *Spinacia oleracea* L. at 15, 30 and 45 DAS (mean \pm S.E.).

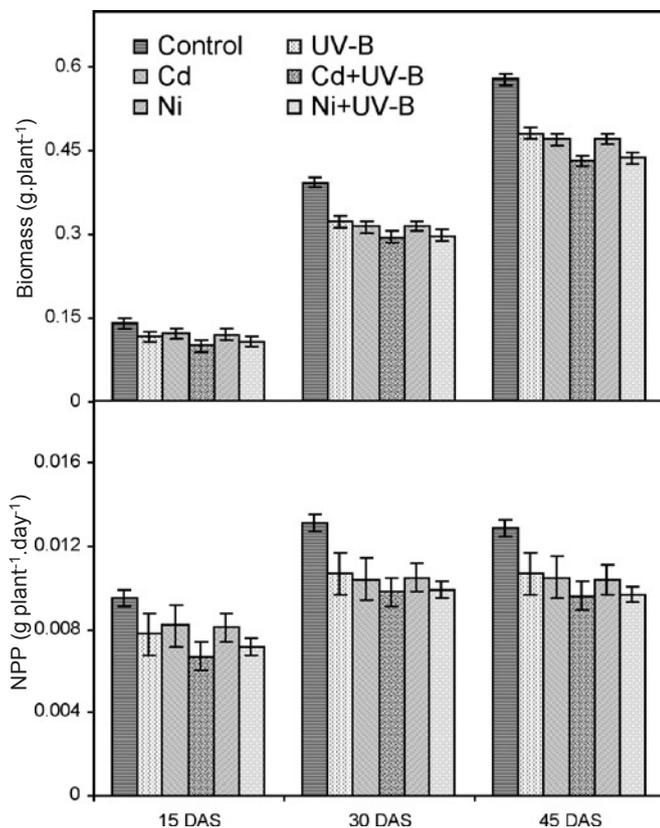


Figure 4. Effects of sUV-B radiation and heavy metal (Cd / Ni) singly and in combination on biomass and net primary productivity of *Spinacia oleracea* L. at 15, 30 and 45 DAS (mean \pm S.E.).

(Alia and Pardha Saradhi, 1991). Proline is supposed to participate in the reconstruction of chlorophyll, activates the Krebs cycle and constitutes an energy source (Saxe, 1991). It is also an important part of structural proteins and enzymes and participates in repair processes. Recently, Chris et al. (2006) have shown that simultaneous exposure of sUV-B and NaCl produced an additive effect on MDA and proline accumulation in a cyanobacterium *Cylindrospermum* sp.

In the present study, LPO increased due to various treatments at all ages. A combination of sUV-B and heavy metal (Cd/Ni) produced more LPO as compared to individual treatments of either stress. The effect of UV-B on the membrane is related to the oxidation of the membrane components by free radicals (Predieri et al., 1995). Similar responses were observed in crop plants for various heavy metals (Somashekaraiah et al., 1992). Ni produced a constant increase in MDA concentration (about 47 %) in *Nicotiana tabacum* (Boominathan and Doran, 2002). Studies of Shah et al. (2001) suggested that rice plants freely absorbed Cd and its accumulation in tissues was paralleled with enhanced lipid peroxidation and marked elevation in the levels of antioxidant enzymes. Lipid peroxidation also increased in *Riccia* sp. thalli with rising concentration of Cd and UV-B exposure alone and also in combined treatments of Cd and UV-B.

Reduction in biomass accumulation is often a reliable indication of the plant's sensitivity to various stresses, as it represents the cumulative effects of damaged or inhibited physiological functions. The exposure to sUV-B and heavy metal stress caused significant reductions in biomass accumulation and NPP. Similar reductions in the biomass yield were observed by Shukla et al. (2002) in *Triticum aestivum* (wheat) seedlings due to the combined stress of UV-B and Cd. Haghiri (1973) also reported a sharp decline in the yield of dry matter in soybean and wheat at the lowest level of applied Cd (2-5 ppm). Dube et al. (2002) noticed reductions of 10.8, 20, 38.4 and 72.4 % in spinach biomass with the application of 5, 10, 20 and 40 mg Cd.kg⁻¹ soil, respectively.

Concluding, the present investigation showed that sUV-B and heavy metal treatments caused oxidative stress in plants leading to reductions in photosynthetic pigments and consequently the biomass of spinach plants. Proline accumulation appears to be an additional defense against UV-B and metal-induced oxidative stress. The combined effects of sUV-B and Cd caused the strongest reduction in biomass at final harvest followed by the treatments sUV-B + Ni, Cd, Ni and sUV-B. Interactive effects of sUV-B + Cd/ sUV-B+ Ni, however, were always less than additive. This

study further concludes that an increasing availability of UV-B in a natural system would intensify metal toxicity in the plants inhabiting metal rich environments.

Acknowledgements: The authors are thankful to C.S.I.R. (New Delhi) for financial assistance and to Prof. R.B. Lal, Vice Chancellor and Dr. G. Abraham, Head, Botany Department, Allahabad Agricultural Institute-DU, Allahabad for the necessary facilities and encouragements.

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