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ALTERATIONS IN ANTIOXIDATIVE POTENTIAL OF *OCIMUM* CULTIVARS AS A METHOD TO CHARACTERIZE UV-B TOLERANCE

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Abstract

The comparative alterations of UV-B tolerance and recovery on lipid peroxidation and modulation in the contents of free radicals, enzymatic and non-enzymatic antioxidants in leaves of *Ocimum sanctum*, *Ocimum basilicum* and *Ocimum gratissimum* were studied. Lipid peroxidation measured in terms of MDA level increased with UV-B doses and the ratio was higher in *Ocimum basilicum* compared to *Ocimum gratissimum*. Recovered leaves showed lower MDA content and hydroxide radicals. Ascorbate, flavonoid and proline contents increased highly in stressed and recovered leaves of *Ocimum gratissimum* compared to *Ocimum basilicum* with an increase in UV-B doses. Higher free radical scavenging capacity and more efficient antioxidant potential of *Ocimum gratissimum* cultivars were proved *in vivo*, using the thiobarbituric acid assay in liver and muscle assay systems of ovarian models. The present findings suggested that UV-B doses have modulated the antioxidative machinery of *Ocimum* cultivars, though the results proved that significant cultivars differ in responses to UV-B. Differences in responses are closely related to differences in the activities of antioxidants and overall growth responses. A significant conclusion can be drawn from the findings that improved tolerance to UV-B stress may be accomplished by increased capacity of antioxidant system of plants.

Keywords: Lipid peroxidation, Malondialdehyde, *Ocimum* species, UV-B

Introduction

Ocimum groups are reported to be widely distributed in the tropical, sub tropical and warm temperate regions of the world (Paton *et al.,* 1999). They are grown for the essential oils in leaves and stems. Essential oils from the plant have been reported to possess an interesting spectrum of antifungal properties (Dubey *et al.,* 2000; Lemos *et al.,* 2005), antinociceptive property (Rabelo *et al.,* 2003), anticonvulsant (Raj *et al.,* 2003), antioxidant (Uma Devi *et al.,* 2001; Javanmardi *et al.,* 2002 & 2003;), germicidal (Holetz *et al.,* 2003; Pessoa *et al.,* 2003), antimalarial activity (Ezekwesili *et al.,* 2003) and has found wide use in toothpastes and mouth washes as well as some topical ointments and used as a general promoter for health in herbal medicine. Crude extracts rich in natural antioxidants are increasingly of interest in food industry because they not only retard the oxidative degradation of lipids and thereby improve the quality and nutritional value of food, but also improve the resistance power against microorganism. Many medicinal plants, especially those that belong to the Lamiaceae family, contain large amounts of antioxidants other then Vitamin C, Vitamin E, flavonoids and carotenoids. The presences of many pharmacologically active compounds in *Ocimum* species provide them protection against free radical induced oxidative damage of cellular components.

Solar UV-radiations are reported to increase in the atmosphere due to several anthropogenic human activities which can damage the biological ecosystems. Numerous studies have been conducted on the effects of enhanced UV radiations on photosynthetic enzymes, pigments, proteins and antioxidant compound contents in plants (Christopher *et al*., 2010). Plants need to have special mechanisms for adjusting to the changed environment. Furthermore, many groups of stresses like heavy metals, ultraviolet radiations etc are shown to generate singlet oxygen and other active oxygen species at various sites of photosynthetic electron transport chain (Halliwell and Gutterridge, 1994). Free radical-induced lipid peroxidation of cellular metabolites may increase the level of free radicals in turn and may produce oxidative stress in cells, affecting the growth of plants.

Lipid peroxidation is a well established mechanism of cellular injury, in both plants and animals which is used as an indicator of oxidative stress in cells and

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tissues. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA), upon decomposition. Lipid peroxidation will in turn result in the elevated production of free radicals that might inhibit the antioxidant defense potential of plants (Gumieniczek, 2002). Lipid peroxidation can be affected by various conditions and substances such as pesticides, UV-radiations and other stresses. MDA, a secondary product of lipid peroxidation is used as an indicator of tissue damage (Kwon *et al*., 1964). MDA reacts with thiobarbituric acid and produces red colored products. Total antioxidant capacity can be assayed using this property of TBA. Hence the authors had been using a rapid quantifiable screening process to identify the extent of antioxidant properties of a number of volatile oils extracted from *Ocimum* species.

It is reported in many plants that various plant processes such as photosynthesis, respiration and pigment biosynthesis were affected by UV-B irradiations that stimulate the formation of free radicals and reactive oxygen species that ultimately damage living systems (Matysik *et al*., 2002). Plant metabolize these free radicals by invoking an increased antioxidant machinery that include both enzymatic antioxidants such as superoxide dismutase, catalase, peroxidase etc. (Kondo and Kawashima, 2002; Panda and Khan, 2003) and non-enzymatic antioxidants such as proline, flavonoids, ascorbate etc. Proline is an important antioxidant that helps to maintain the osmotic potential in plant cells. It is thought that its accumulation under environmental stress plays a role as osmoprotectant (Yavuz, 2000). Vitamin C or ascorbic acid acts as a co-antioxidant by regenerating α-tocopherol from α-tocopheroxyl radical and scavange free radicals. Phenolic compounds, Flavonoids, inhibit oxidation of lipids minimizing the free radical contents (Shahidi *et al*., 1992). *Ocimum* species have been attracted the attention of several workers due to the high contents of natural antioxidants. Considering the above facts, the present work was conducted with the aim to evaluate the impact of UV-B on antioxidant potentials of three *Ocimum* cultivars (*Ocimum sanctum*, *Ocimum basilicum* and *Ocimum gratissimum),* in terms of variations in the total contents of protein, ascorbate, proline, Flavonoids, enzymatic antioxidants and a comparative measurement of total antioxidant activities (in terms of antioxidant index) in liver and muscle assay systems of ovarian models (chickens).

Materials and Methods

Plant materials and UV-B Treatment

The seeds collected from naturally growing wild plants from parts of the districts Azamgarh, Chitrakoot (Uttar Pradesh) & Dehradun (Uttarakhand) India, were surface sterilized in 0.25% $HgCl_2$ On fifth day germinated seeds were transferred in a growth chamber under controlled environmental conditions with the temperature ranging from 25-27^{0}C and 78% relative humidity. They were illuminated with white fluorescent tubes (150 µM $m^{-2}s^{-1}$) for 13 hours followed by 11 hours dark period and 70-75% relative humidity. Plants were grown in soil extract liquid nutrient medium, renewed after every two days. On the 21st day, plants were exposed to ultraviolet irradiation (280-312nm) using a fluorescent tube with its main output at 312nm. The plants were exposed to different intervals of time 20 minutes, 40 minutes & 60 minutes. The desired radiation intensity was obtained by adjusting the distance between UV B (0.4 Wm^2) source and plantlets. The light temperature and humidity regimes were the same for control as well as treated *Ocimum* cultivars and after 24 hours of treatments, various parameters were analyzed.

UV-Absorbing pigment, Flavonoids: Extraction and Estimation

Flavonoids were extracted in UVB treated and untreated *Ocimum* leaves by using the method of Jordan *et al*. 1994 and were estimated as described by Mirecki and Teramura (1984). Flavonoids were extracted from leaf discs by keeping them in acidified methanol (methanol: water: HCl, 78: 20: 2, v/v) for 24h at 4^{0}C. The filtered extract was then used for measuring the absorbance at 320 nm, which is indicative of relative concentration of UVB absorbing pigments. Flavonoid contents were expressed as absorbance g^{-1} fresh mass of tissue at 320 nm.

Estimation of total protein, ascorbate and proline contents

Protein contents were determined using Folins-Lowry method using lysozyme as the standard (Lowry *et al*., 1951). Ascorbic acid content in leaf homogenates, in UV-B treated/untreated plants were estimated using the method given by Oser (1979). Fresh leaves were ground in sulfosalicylic acid and incubated with a reaction mixture (2% sodium molybdate, 0.15 N H_2SO_4, 1.5 mM K_2HPO_4 and tissue extract) at 60^{0}C, for 30 minutes (contradictory to the time - 45 minutes, as given in standard protocol). Absorbance of the supernatant was recorded at 660 nm and the amount was calculated by using L-Ascorbate as standard. Proline contents were extracted and estimated according to the method of Bates *et al.* (1973). The amount was calculated from the standard curve in terms of µg g^{-1} FW.

Estimation of % Antioxidant index (TBARS ASSAY)

The essential oils of *Ocimum* species were isolated by hydrodistillation using an essential oil distillation apparatus (Quick Fit). The dilutions of essential oils were made in 8.1% (w/v aq.) sodium

dodecyl sulphate (SDS). All the experiments were conducted on liver and muscle organ systems excised from 21 days old chickens. Organs were stored at -20^{0}C until required. Homogenate (10%) were prepared with KCl (1.15%, w/v) using a tissue homogenizer until the tissues were completely macerated (Damien Dorman *et al.*, 1995). Three groups of experimental organisms were maintained:

Control (-): Tissue homogenate without essential oil

Control (+): Tissue homogenate + antioxidant of known property (L-Ascorbate)

Test samples: Tissue homogenate + essential oil in different concentrations.

In order for direct comparison to be made between all assays, the antioxidant index (AI) was expressed as value relative to control. It was calculated using following formula of Aeschbach *et al.* (1994) which was used to demonstrate the comparative protective properties of the essential oil in the respective assays.

% AI= (1-T/C) x 100;

Where C is the absorbance value of fully oxidized control and T is the absorbance value of the test sample.

Estimation of total Peroxide radicals and lipid peroxidation levels

Total amount of hydrogen peroxide radicals was estimated by using ferrithiocyanate method as described by Sagisaka (1976). Standardization of H_2O_2 was performed to minimize the interference of catalase. Lipid peroxidation in terms of total MDA concentration was estimated by the method of Heath and Packer (1968). The reaction reagent was prepared as: 0.4 N TCA + 19.68 ml of distilled water + 0.4 ml of HCl + 100mg TBA. Leaf extract was prepared in phosphate buffer and then added to the reaction reagent. The sample was boiled in a water bath and the absorbance was taken at 532 nm.

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

In vivo catalase activity was determined by using the method of Egashira *et al.* (1989). Plant leaves were suspended 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. To this, 1 ml of cell suspension was added and in darkness O_2 evolution was monitored. SOD activity was assayed by the method of Giannopolitis and Ries (1977). 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. 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 (Prasad and Zeeshan, 2004). IAA oxidase activity was assayed using the method of Byrant and Lane (1979). The enzyme reaction mixture contained: 0.071 M Phosphate buffer + 0.5 mM $MnCl_2$ + 0.05% paracoumaric acid + enzyme extract. 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.

Statistical analysis

All the experiments were repeated three times with variable replicates (3 to 6) per repeat and data presented are mean $\pm$ standard error (SE). The data analysis was carried out using statistical package 'SPSS 10'. Comparisons with $p<0.05$ were considered significantly different.

Results and Discussion

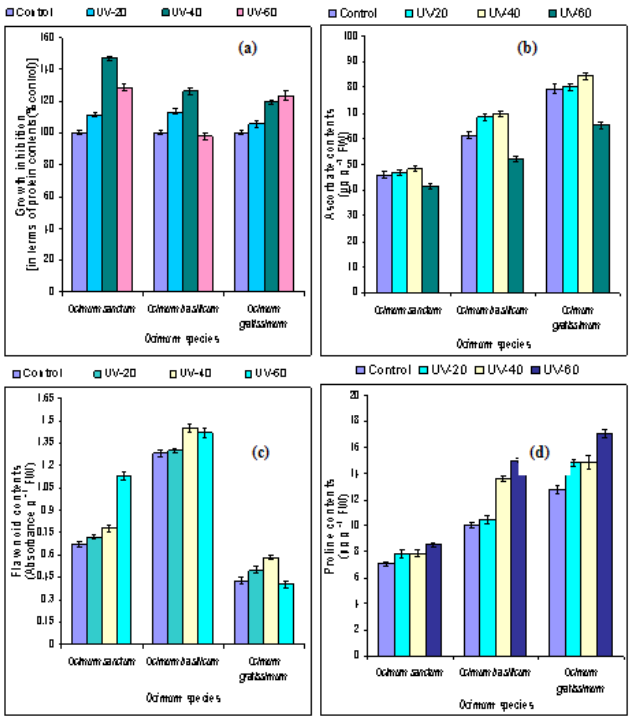
Effect of UV-B on Ascorbate, proline and Flavonoids

Besides, enzymatic antioxidants as SOD, Peroxidase and catalase, cells also contain important non-enzymatic antioxidants such as carotenoids, ascorbic acid, proline, glutathione, α-tocopherol etc., for mitigating the toxic effects of free radicals and AOS (active oxygen species) under oxidative stress. In the present work, ascorbate contents showed very little increase in the amounts (46.7 & 48.1 µg/g FW) with UV-B exposure for 20 and 40 minutes and the decrease at high doses (41.5 µg/g FW, after 60 minutes of exposure), compared to untreated samples (control = 45.76 µg/g FW), in *Ocimum sanctum* while in *Ocimum basilicum,* it was observed to increase after every 20 and 40 minutes of UV-B exposure (68.5 & 69.6 µg/g FW), followed by a reduction at 60 minutes of exposure (52.24 µg/g FW) in comparison to untreated samples (control = 61.42 µg/g FW). In *Ocimum gratissimum,* ascorbate contents showed very little increase at 20 minutes and 40 minutes of exposure (80.1 & 84.5 µg/g FW), followed by the reduction at 60 minutes of exposure (65.32 µg/g FW), when compared to control (79.47 µg/g FW) (figure 1 b). There are two possibilities regarding increase in ascorbic acid

contents; either its synthesis has increased or its regeneration rate through the Asada-Halliwell pathway has increased (as observed in *Ulva fasciata*) (Shiu and Lee, 2005). Although, the enzymes involved in regeneration of ascorbic acid has not been estimated in the present study, several reports indicate considerable increase in ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase enzymes actively involved in regeneration of ascorbic acid in response to UV-B (Ambasht and Agrawal, 2003). The chemical evolution and significance of flavonoids has been assumed to play an important role in overcoming the oxidative stress in cells (Rozema *et al.*, 2005). Evidences suggest that the presence of flavonoids in UV-B irradiated leaves could alter the perception or response of other defense mechanisms (Nijvedt *et al.*, 2001). Presently, flavonoid contents showed enhanced synthesis in UV-B treated *Ocimum sanctum* plantlets in comparison with untreated samples (control= 0.67 Absorbance/g FW). The values were found to be 0.72, 0.78 and 1.13 Absorbance/g FW (figure 1 c). In both *Ocimum basilicum* and *Ocimum gratissimum,* the contents were found to be inhibited after UV-B doses at longerduration (60 minutes). It was observed to be 1.42 and 0.4 Absorbance/g FW in *Ocimum basilicum* and *Ocimum gratissimum,* respectively. Earlier findings showed remarkable increase in the Flavonoids contents of the UV-B exposed soybean cultivars (Lizhe *et al.*, 2003). Flavonoids inhibit the enzymes responsible for superoxide anion production, such as xanthine oxidase (Hanasaki *et al.*, 1994) and protein kinase C (Ursini *et al.*, 1994). Flavonoids have also been shown to inhibit cyclo-oxygenase, lipoxygenase, glutathione s- transferase and mitochondrial succinic oxidase all involved in AOS generation (Pietta *et al.*, 2000).

Proline contents were found to be accumulated at all the doses of UV-B in all the cultivars of *Ocimum*. At the longest duration of exposure (60 minutes), the values were observed to be 8.53, 15 & 17.1 µg g -1 FW, in comparison with control (7.07, 9.98 & 12.75 µg g -1 FW), in *Ocimum sanctum*, *Ocimum basilicum* and *Ocimum gratissimum*, respectively (figure 1 d). Similar to ascorbic acid, proline content has also been shown to be regulated in plants exposed to various stresses such as drought, salt and UV-B (Jimenez-Bremont *et al.*, 2006). The accumulation and protective effect of proline has been observed in many higher plants and bacteria as well as protozoa, algae, and marine invertebrates (Delauney and Verma, 1993). Proline is a reliable indicator of the environmental stress imposed to plants (Claussen 2005; Cechin *et al.*, 2006). It may acts as a regulatory or signaling molecule to activate multiple responses that are part of the adaptation process. Present study proved the remarkable alterations in protein (figure 1 a), ascorbate, proline and flavonoid contents in UV-B treated seedlings (%control inhibition in the ascorbate contents was observed to be 9.31%, 14.99% and 17.81% while Flavonoids showed 20.65%, 17.65% and 6.98% reduction with respect to the untreated plants in *Ocimum sanctum, Ocimum basilicum* and *Ocimum gratissimum,* respectively). Similar pattern of alterations were found in the contents of Proline also.

Figure 1 UV-B induced effect on: (a) protein contents, (b) ascorbate, (c) proline and (d) flavonoids contents, in leaves of different *Ocimum* species. The values are means ± SE and significantly different from their respective controls ($p<0.05$)



Total antioxidant activity (Antioxidant index), peroxide radicals and lipid peroxidation

The total antioxidant activity was measured in terms of antioxidant index (%) by using two bioassay systems (chicken liver and muscles) as depicted in the figures 2 (c) & (d). It was observed that both assay systems demonstrated an ability to reflect differing antioxidant capacities proportionately with dilutions of the oils. In chicken liver assay, the maximum antioxidant capacity was found in *Ocimum gratissimum* (AI= 82.94%), followed by *Ocimum basilicum* (AI= 79.47%) and *Ocimum sanctum* (AI= 74.1%), at 100 ppm concentration of essential oil (figure 2 c). Even 0.75 ppm concentration of oils extracted from *Ocimum* species were found to be more effective than the standard antioxidant (L- ascorbate) used in the present study. The results clearly indicated that the antioxidant potential of *Ocimum* species increased with the increase in oil concentrations. In chicken muscle assay, the maximum antioxidant potential was observed in *Ocimum basilicum* (80.56%), followed by *Ocimum gratissimum* (70%) and *Ocimum sanctum* (61.11%) (Figure 2 d). The differences in both, qualitative and quantitative aspects of the lipids present in the tissues within the media clearly play a vital role in the variable responses seen in the assays. The experimental organs were obtained from sources with high degree of uniform lipid contents (Noble *et al.*, 1990). Here, it is clearly evident that a distinctive inconsistency was found with various concentration of *Ocimum* oil in chicken muscle assay system, inspite of a number of repeats of the experiments. Overall dose responses for the liver and muscle systems in chickens significantly showed different responses with variations in different species. It is evident that *Ocimum basilicum* showed better protection against lipid peroxidation than *Ocimum gratissimum* and *Ocimum sanctum* in muscle assays. In the same way, *Ocimum gratissimum* was observed to show more antioxidant potential than *Ocimum basilicum* and *Ocimum sanctum* in liver assay systems. In all the cases, oil achieved similar levels of lipid protection at the 100 ppm concentration and all species of *Ocimum* showed better lipid protection, thus making it more suitable for screening for antioxidant properties. The different chemical composition of essential oils may help in explaining the observed differences in antioxidant activity. Many of the major compounds such as thymol, carvacrol, p-cymene, linalool, camphene, citronellol, eugenol, isoeugenol, α-terpenene, β- caryophylline, myrcene etc have also been demonstrated to possess *in vitro* antioxidant properties (Deans *et al.*, 1991; Aeschbach *et al*., 1994).

The level of hydrogen peroxide radicals were enhanced in all the UV-B treated plantlets of *Ocimum* species, in comparison with untreated controls. The values of peroxide radicals in *Ocimum sanctum* were observed to be 39.1, 49.3 and 59.5 µmol/g fresh weight at 20 minutes, 40 minutes and 60 minutes of UV-B exposure, respectively (figure 2 a), in comparison with control (28.9 µmol/g fresh weight). It was found to be 32.3, 42.5 and 54.4 µmol/g fresh weight in comparison with control (23.8 µmol/g fresh weight) in *Ocimum basilicum* and 47.6, 59.5 & 62.0 µmol/g fresh weight (control= 34.0 µmol/g fresh weight), in *Ocimum gratissimum,* at 20 minutes, 40 minutes and 60 minutes of UV-B exposure. Hydrogen peroxide radicals may be very damaging, since they can attack lipids in cell membrane, proteins in tissues or enzymes and DNA to induce oxidations, which cause membrane damage, protein modification and DNA damage (Levine *et al.,* 1994; Halliwell, 1997). UV-B induced lipid peroxidation of the cellular components in *Ocimum* species was studied by estimating the level of MDA and the related data are depicted in the figure 2 (b). Total MDA contents in untreated controls was 1.56, 1.53 and 1.60 nmol MDA (mg fresh mass)$^{-1}$ in *Ocimum sanctum, Ocimum basilicum* and *Ocimum gratissimum,* respectively. It is clear from the figure 2 (b) that the level of lipid peroxidation was increased in all the treated samples. UV-B exposure upto 60 minutes caused lipid peroxidation to increase by 15.8%, 55.2% and 24.8% in *Ocimum sanctum, Ocimum basilicum* and *Ocimum gratissimum,* respectively. The results obtained here are in agreement with the works previously done (Kramer *et al.,* 1991; Agrawal and Pandey 2003).

Enzymatic antioxidants

It was observed that the catalase activity showed enhancement in plantlets exposed to 60 minutes of UV-B irradiation (% control induction was 49.36%, 46.4% & 45.9% in *O. sanctum*, *O. basilicum* and *O. gratissimum*, respectively). Treatments of shorter durations stimulated catalase activity a little but high duration exposures increased the enzymatic activity rapidly (figure 3 a). 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 5.79, 5.84 & 5.96 Units min^{-1}mg protein^{-1} in *O. sanctum*, *O. basilicum* and *O. gratissimum*, respectively (figure 3 b). It 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 duration exposure of UV-B radiations, respective to the controls. 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. The Peroxidase activity showed varied responses with

UV-B treatments. Peroxidase activity was increased with low duration treatments and this was continued linearly but at the higher intensities, their values were inhibited in all *Ocimum* species as compared to the untreated samples (figure 3 c). Increased activity of Peroxidase indicated more powerful mechanism of detoxification of overproduced H_2O_2. IAA oxidase activity increased initially showing an enhancement in the enzyme activity but it showed remarkable reduction in values at higher doses (43%, 48% & 38.8% reduction as compared to the untreated *O. sanctum*, *O. basilicum* and *O. gratissimum*, respectively) (figure 3 d). The results were in accordance with the studies done in *Vigna radiata* (Garg *et al*., 1988).

Figure 2: UV-B induced formation of hydrogen peroxide radicals (a), total MDA contents (after 3 days of treatments) & differential responses in Antioxidant Index to various dilutions of *Ocimum* essential oils using the chicken liver (c) and chicken muscle (d) assay systems. The values represented are mean ± SE and significantly different from their respective controls (p<0.05)

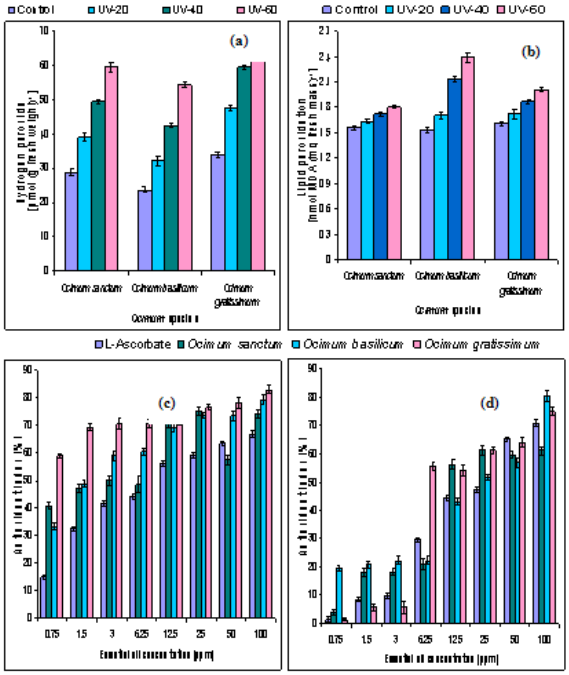
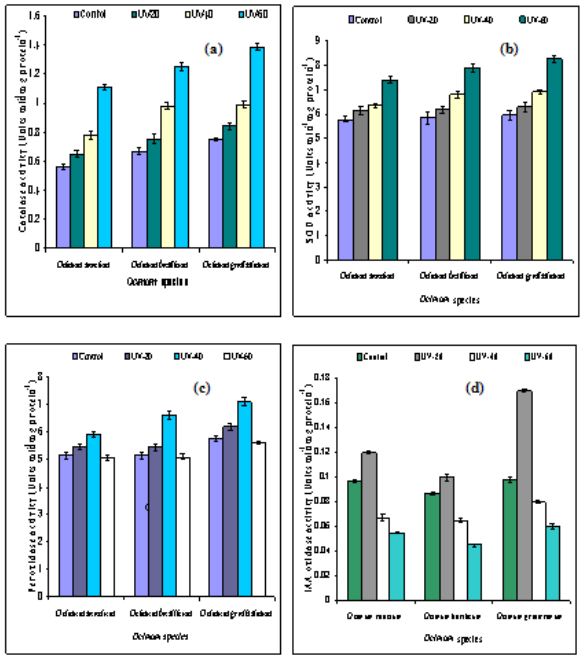


Figure 3: UV-B induced alterations in Catalase (a), Superoxide dismutase (b), Peroxidase (c) and IAA oxidase activities; in *Ocimum* plantlets. All the values were means ± SE and significantly different from their respective controls (p<0.05)



The current work provides preliminary information and methodologies for rapid quantifiable screening of antioxidant potentials of *Ocimum* cultivars. *Ocimum* species are widely used in many traditional medicines prescribed under different systems of medicine. It is, therefore, important to study the comparative antioxidant properties of different *Ocimum* species. Overall growth of the *Ocimum* species was found to be retarded by longer UV-B exposures. Slight enhancements in the contents of enzymatic and non-enzymatic antioxidants due to the low duration exposures may be the sign of recovery from oxidative stress condition. Antioxidants as Flavonoids, proline and ascorbate contents showed usually enhancements proving their potential for scavenging reactive oxygen species. *In vitro* screening of antioxidant activities in three *Ocimum* species, showed varied responses. Increasing potential with the increase in oil concentrations proved the varied antioxidant activities in *Ocimum* species. *Ocimum gratissimum* was proved to be better antioxidant for liver and *Ocimum basilicum* for muscles, in oxidative stresses. It was observed that there was an intimate relationship among the contents of natural antioxidant and recovery potential of plants from oxidative stress conditions in terms of antioxidant index.

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