

Physiological and Biochemical Impairment in Bean Plants Due to Supplementary Ultraviolet Radiation and Water Stress: Possible Protective Roles of Secondary Metabolites

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Abstract: Physiological and biochemical responses of bean (*Vicia faba* L.) plants to either supplementary ultraviolet (sUV-B) radiation, and/or water (WS) stress were investigated. Both stresses reduced yield and altered net photosynthetic rates (P_N) and stomatal conductance (g_s). Such response may be due to the oxidative damage indicated by an increase in the H_2O_2 content and lipid peroxidation. Furthermore, increases in activities of stress markers indicated that sUV-B has a stronger stress effect than WS, and it caused greater membrane damage, as assessed by lipid peroxidation and osmolyte leakage. The activities of ascorbate peroxidase (APX) and superoxide dismutase (SOD) were increased under both stresses when applied alone and in combination, while catalase (CAT) activity decreased under water stress as compared to the control. The combination of drought and UV-B, were more than additive, caused more severe damage than stress factors applied separately. WS induced accumulation of UV-B absorbing secondary pigments (anthocyanin and flavonoids) which is likely to offer some protection from UV-B irradiation.

Key words: sUV-B radiation - water stress. stress markers - antioxidant enzymes – proline accumulation - secondary metabolites.

INTRODUCTION

Anthropogenic activities have resulted in the reduction of stratospheric ozone (e.g. Molina & Rowlands 1974; Alexieva et al., 2001), which led to a significant increase in ultraviolet-B (UV-B) radiation (290.320 nm) reaching the surface of the Earth (Blumthaler & Ambach 1990; Garty et al., 2007). Elevated levels of UV-B radiation can be stressful to plants (Paul & Gwynn-Jones, 2003). Plants are exposed to a multitude of natural biotic and a biotic stressors. Almost all stressors affect either directly or indirectly the photosynthetic performance of leaves (Balouchi et al. 2009). Differences in photosynthetic rates are most likely to be observed under conditions of environmental stress, like e.g. drought (Rouhi et al. 2007; Hojati et al. 2011) and UV-B (Ranjbarfordoei et al. 2011). Most of a biotic stresses are connected to anthropogenic activities which are clearly causing major changes in atmospheric chemistry (Reddy et al. 2004).

Wide inter- and intraspecific differences have been reported in response to UV-B with respect to growth and plant morphogenetic response (Kramer et al., 1991; Rozema et al., 1997; Milchunas et al., 2004) and physiological processes (Agrawal & Rathore, 2007; Tsormpatsidis et al., 2010). Some species show varied degrees of tolerance (Kolb et al., 2001) while others are sensitive to present levels of UV-B radiation (Teramura and Sullivan, 1991; Moussa & Khodary, 2004, Zu et al., 2011). UV-B induced growth inhibition is usually associated with damage to the photosynthetic apparatus and reduction of photosystem II (PSII) efficiency (Gartia et al., 2003; Tsormpatsidis et al., 2010).

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Plants have evolved a variety of biochemical adjustments as mechanisms to protect and prevent damage caused by environmental stress(s) including UV-B radiation and water stress. The most widely observed mechanisms are the accumulation of UV-absorbing compounds in the epidermal cells such as flavonoids (Treutter, 2005) and activation of antioxidant enzymes such as POD and SOD (Alexieva et al., 2001; Zu et al., 2011). These enzymes scavenge free radicals from oxygen, and offer protections to lipids, proteins and nucleic acids (e.g. Jain et al., 2004; Tsormpatsidis et al., 2010).

UV-B is species specific, as other environmental stresses, (Smith, 2000, Alexieva et al., 2001; Zu et al., 2004). The different sensitivities of plants are partially explained by their abilities to respond to UV-B through the induction of defensive pathways (Creelman and Mullet 1997, Zu et al., 2011).

Various stress factors competing with the supplemental UV radiation were shown to modify the UV radiation effects (e.g. Balouchi et al. 2009). Of these stresses, water stress is an important restricting factor that always affects agricultural productivity, particularly in arid and semi-arid regions. Feng et al. (2000; 2007) showed that co-stresses of supplementary UV radiation and drought functioned synergically and one of them could alleviate the inhibitory effects of another under conditions of arid and semi-arid soils.

During summer periods, the weather in Egypt is characterized by being anticyclonic with no rainfall and high temperature, so vegetation is often exposed to prolonged periods of drought which is reflected in lowering their relative water content and their water potential (Hassan, 2006). During such periods of drought, large depressions in photosynthetic rates are observed in many plants in the Mediterranean (Nogues and Baker, 2000) and maximum rates of CO₂ assimilation occurs either early in the morning or late afternoon (Hidema & Kumagai, 2006). This would involve heterogeneity of leaf photosynthesis (Hassan et al., 2011).

Although responses of crop physiology, growth, and yield to either water stress or UV-B radiation have been extensively studied in Northern Europe and the USA (e.g. Kakani et al., 2003; Zhao et al., 2005), knowledge of their interactive effects on crops, especially in developing countries, is extremely limited (Agrawal and Rathore, 2007). Moreover, there is paucity on the knowledge concerning the antioxidant response of plants to UV-B (e.g. Costa et al., 2002; Kakani et al., 2003; Hassan et al., 2011)

The aims of the present study were to understand the physiological and biochemical characteristics of broad bean (*Vicia faba* L.) under supplementary UV-B radiation and/or water stress, and to estimate its sensitivity and defense mechanisms under both stresses.

MATERIALS AND METHODS

Seeds of an Egyptian cultivar of bean (*Vicia faba* L.), obtained from Department of Agronomy, Faculty of Agriculture, Alexandria University, were sown 20 cm apart at the Botanical Garden (for more details of the experimental site Hassan et al., 1995; Hassan, 2006). Ten days after placing the plants half the plants were subjected to progressive drought by withholding water which provoked moderate drought stress (-0.5 MPa), while well watered plants were irrigated once a week. Well-watered and water-stressed (WS) plants were divided equally between the two sections in a split-plot design. Consequently, four treatments were distributed in each plot in a randomized Latin square design: (a) control, i.e. without UV-B radiation and well-watered (b) Plots supplied with supplementary UV (WW + sUV-B), (c) Plants subjected to water stress stress (WS) without UV-B radiation (WS . UV-B) and (d) plants were subjected to both stresses (WS + sUV-B). Twenty plants were used in each treatment (Hassan et al. 2011).

Supplemental UV-B radiation was supplied by filtered Westinghouse FS-40 sunlamps oriented perpendicular to the planted rows and suspended above the plants. Lamps were filtered with 0.13 mm thick cellulose acetate as a control (for more details Hassan et al., 2011)

No fertilisers or other fungicides were applied at either location to avoid interference with the fungicides.

Non Destructive Harvests:

Net Photosynthetic Rate (P_N), Stomatal Conductance to CO_2 (gs):

They were measured on the youngest fully expanded leaf of the main stem of all plants. Gas exchange measurements were carried out seven times at 5 d intervals to cover all growth stages (10 days after sowing) using a LI-6200 portable IRGA (LI-COR, Lincoln, USA) between 10:00 and 14:00 h (Local time).

Chlorophyll Fluorescence Analysis:

Steady-state modulated chlorophyll fluorescence of attached leaves was measured using a fluorimeter (PAM-2000, H Walz GmbH, Effeltrich, Germany) during the gas exchange measurements. Calculations were made from fluorescence parameters of the maximum quantum efficiency of PSII photochemistry (given by F_v/F_m) were measured at a PPFD of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, which was similar to the minimum mean growth PPFD (Nogues et al., 1998).

Destructive Harvests:

Plant Biomass Analysis:

At the end of the drought and / or sUV-B treatment(s) (45 days after sowing), plants were harvested destructively. Fresh weight of the above ground organs were carried out (Hassan et al., 2011).

Measurements of Hydrogen Peroxide:

At the end of the drought and / or sUV-B treatment(s) (45 days after sowing), plants were harvested destructively. The H₂O₂ assay followed the method of Wu & Tiedemann (2002). Fifteen leaf discs (10-mm diameter) were submerged in 750 μL reagent mixture containing 0.05% guaiacol and horseradish peroxidase (350 μL L21, 250 μL U mL21) in 25 mM sodium phosphate buffer (pH 7.0) and incubated for 2 h at 20°C in the dark. Then, a volume of 250 μL was transferred into 96-well microtitre plates and the absorbance was immediately measured at 4450 nm in a plate reader photometer (SLT, Spectra, Dixons Ltd, Pure Chemicals for Laboratories, Switzerland).

Commercial H₂O₂, which was used for standard curves, was calibrated by titration with KMnO₄.

Antioxidant Enzymes Assays:

Extractions of antioxidant enzymes from the leaves of the four treatments (control, WW + sUV-B, WS, sUV-B and WS + sUV-B). Leaves were cut from each treatment and immersed in liquid nitrogen and kept in a deep freezer at -80°C until the analyses were performed at Air Pollution Laboratory, Center of Excellence in Environmental Studies, King Abdulaziz University, KSA.

Samples were weighed and ground at about 0°C in 25 mL Tris.HCl buffer containing 3 mM MgCl₂, then the homogenates were centrifuged at 20 000 for 15 min (Centrifuge 17 S/RS, Heraeus Sepatech). The supernatants were used for the enzyme assays and the results were expressed on protein basis (Bradford, 1976).

All assays were performed using a final volume of 1 mL, with at least duplicate assays undertaken on each sample. Moreover, the assays were end-point determinations (Hassan, 2006).

SOD (EC 1.15.1.1) activity was monitored according to Lee et al. (1997). The extraction mixture contained 50 mM phosphate buffer solution (pH 7.8), 13 mM L-methionine, 63 μM nitro blue tetrazolium and 2 μM riboflavin. The ability of the extract to inhibit the photochemical reduction of nitro blue tetrazolium was determined at 560 nm (Schimadzu UV-1201 spectrophotometer).

The amount of the extract resulting in 50% inhibition of nitro blue tetrazolium reaction is defined as one unit of SOD activity.

Catalase (EC, 1.11.1.6) activity was assayed in enzyme extract reaction mixture containing 50 mM phosphate buffer (pH 7.4). The reaction was started by adding 10 mM H₂O₂, and the reduction in absorbance was determined at 240 nm (Maehly & Chance, 1954).

APX (EC, 1.11.1.11) activity was determined according to Maehly & Chance (1954). The reaction mixture contained 50 mM potassium phosphate, 0.5 mM ascorbate, 0.1 M Methylene dimethyl tartaric acid (EDTA) and 0.1 mM H₂O₂, and the absorbance was determined at 290 nm.

Protein concentrations of leaf extracts were determined as described earlier (Bradford, 1976).

Pigment Analysis:

Chlorophyll was extracted in acetone from all leaves in the main stems of three plants per treatment, and determined according to Khan and Khan (1994).

Water-soluble pigments (flavonoids and anthocyanins) were extracted from leaves at the end of the experiment. Leaves were ground to a powder in liquid nitrogen before extraction in 10 cm³ of acidified methanol (HCl : methanol, 1 : 99, v/v). Absorption spectra of the extracts were determined using a Cary 210 spectrophotometer (Varian, Palo Alto, CA, USA), and the flavonoid and anthocyanin contents were estimated from absorbances at 300 and 530 nm, respectively (Nogues & Baker, 2000).

Measurements of Free Proline Concentration:

Leaves (0.2 g) were homogenized in 5 ml of 3% sulphosalicylic acid solution. After centrifugation, 2 ml supernatant, 2 ml glacial acetic acid and 2 ml 2.5% acid ninhydrin solution were added in a test tube covered with Teflon cap. The absorbance of the free proline concentration was measured at 520 nm. The proline content was expressed as $\mu\text{g g}^{-1}$ fresh weight. (Bates et al., 1973).

Measurements of Lipid Peroxidation:

Lipid Peroxidation was measured by the amount of malondialdehyde (MDA) as end product of unsaturated fatty acid peroxidation (Hassan & Twefik, 2006).

Membrane Permeability:

It was measured by Electrolyte Leakage (Alexieva et al., 2001). Five leaves from each treatment were detached and immersed in distilled water at a room temperature and the conductivity of the solution was measured after 3 hours

Statistical Analysis:

Two way ANOVA was applied to log-transformed data (Statgraphics Statistical Package 4, London, UK) to evaluate effects of WS and/or sUV-B treatments on growth and physiology of the plant. PPF_D was used as a covariate in Leaf gas exchange and fluorescence data, there was no covariate used in growth measurements. The significance of difference among treatments were compared by Fisher's least significant difference test (LSD)

Results:

Fresh weight of pod and number of seeds/pod were reduced by (27, 23%, respectively) and (-23 and -18%) and due to WS and sUV-B, respectively (Table 1). Interaction between WS and sUV-B was more than additive and they have synergistic effects on RGR. Moreover, drought stress had a higher negative effects on P_N and g_s (-54 and 41%, respectively) than s UV-B treatment (about 19% each), while exposure to both stresses caused reductions by 53 and 44% in these parameters, respectively (Table 1).

Fv/Fm showed significant responses to both stresses when applied singly and in combination (table 1).

Table 1: Effects of sUV-B and drought stress, singly and in combination on yield parameters, net photosynthetic rates (P_N), stomatal conductance (g_s) and maximum quantum efficiency of PSII photochemistry (Fv/Fm).

Parameter	control	UV	WS	WS+UV	LSD	d.f.
Fresh weight of pods (g)	13.84 ± 1.5	13.58 ± 1.3	10.75 ± 1.1	10.62 ± 1.3	0.27	49
No. of seed/pod	5.52 ± 0.42	5.37 ± 0.36	3.43 ± 0.21	3.58 ± 0.19	0.15	62
RGR (g g ⁻¹ day ⁻¹)	0.28 ± 0.084	0.25 ± 0.085	0.17 ± 0.081	0.22 ± 0.009	0.01	10
P_N (μmol (CO ₂) m ⁻² s ⁻¹)	18.9 ± 2.4	17.8 ± 2.2	14.6 ± 2.1	15.3 ± 2.5	0.23	35
g_s (mol m ⁻² s ⁻¹)	0.33 ± 0.02	0.31 ± 0.02	0.24 ± 0.019	0.25 ± 0.02	0.05	35
F_v/F_m	0.77 ± 0.0063	0.71 ± 0.0017	0.53 ± 0.0021	0.51 ± 0.0019	0.07	19

Values are means + SE. Least significance difference (LSD) at 5% level and d.f. are presented.

H₂O₂ and APX showed no significant response ($P > 0.05$) to water stress (Fig 1 a & d), while SOD and CAT activities were increased by 14 and 20%, respectively (Fig 1 b & c). Exposure to sUV-B caused increases in these parameters by 18, 21, 47 and 56%, respectively (Fig 1 a, b, c, d).

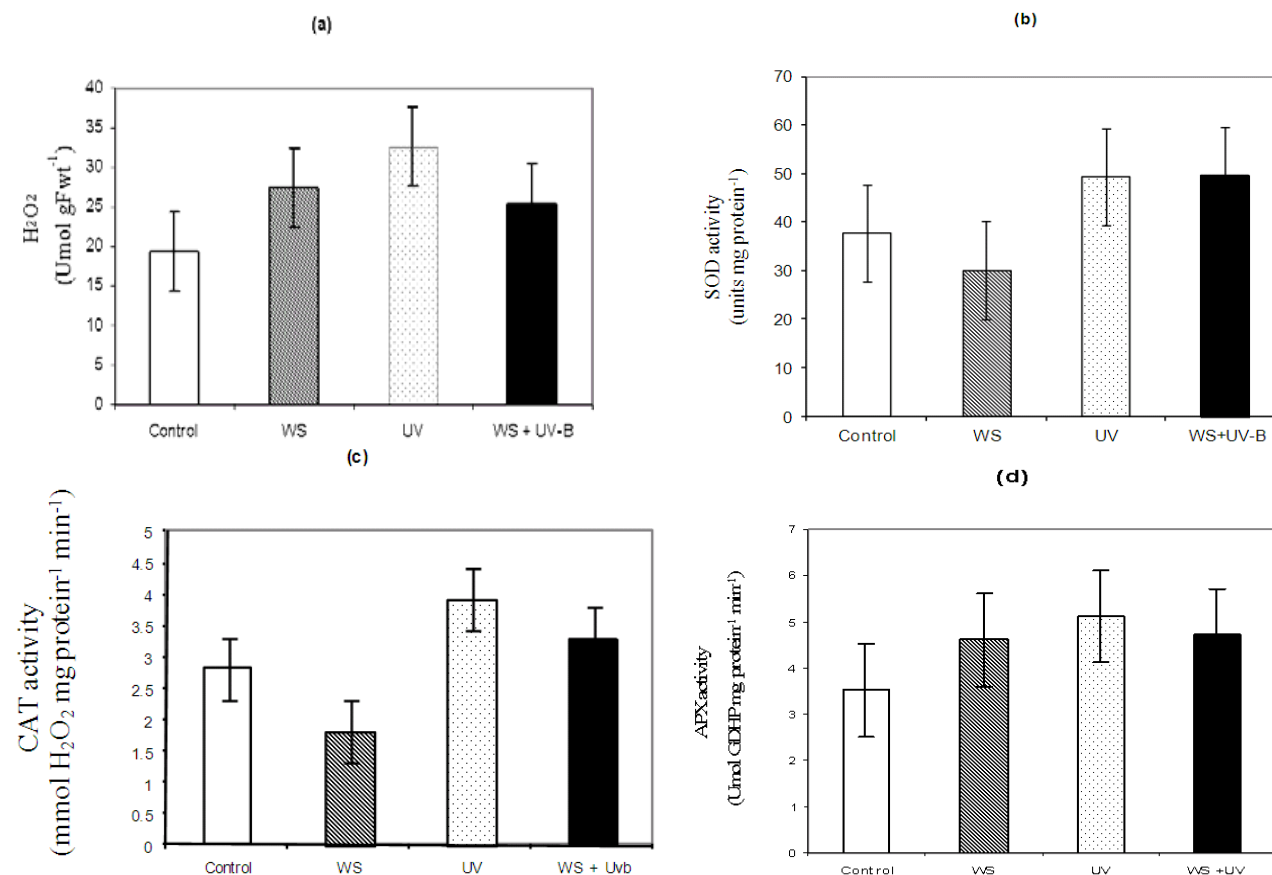


Fig. 1: Effect of water stress and sUV-B applied alone or in combination on the activities of H₂O₂ (a), SOD (b), CAT (c) and Peroxidase (d).

Each value is a mean of 8 replicates + 1 SE. Control (well watered plants without sUV-B); WS (plants subjected to waters stress only without exposure to sUV-B); UV (well watered plants exposed to sUVB) WS+ sUV-B (plants subjected to both stresses).

Exposure to both stresses was more than additive as it caused an increase by 33% in APX and H₂O₂, while it was less than additive in case of CAT and SOD, as they were decreased by 20 and 26%, respectively, (Fig 1). Furthermore, there was negative correlation between shoot fresh weight and H₂O₂ content (Data not shown)

Exposure to sUV-B caused reduction in anthocyanine by 24% (Fig 2a), while it had no significant (P>0.05) effect on total flavonoids (Fig 2b). On the other hand, WS caused increases by 75 and 46% in both pigments, respectively (Fig 2 a & b). Moreover, plants exposed to both stresses simultaneously showed increases in these pigments by 35 and 59%, respectively (Fig 2 a & b).

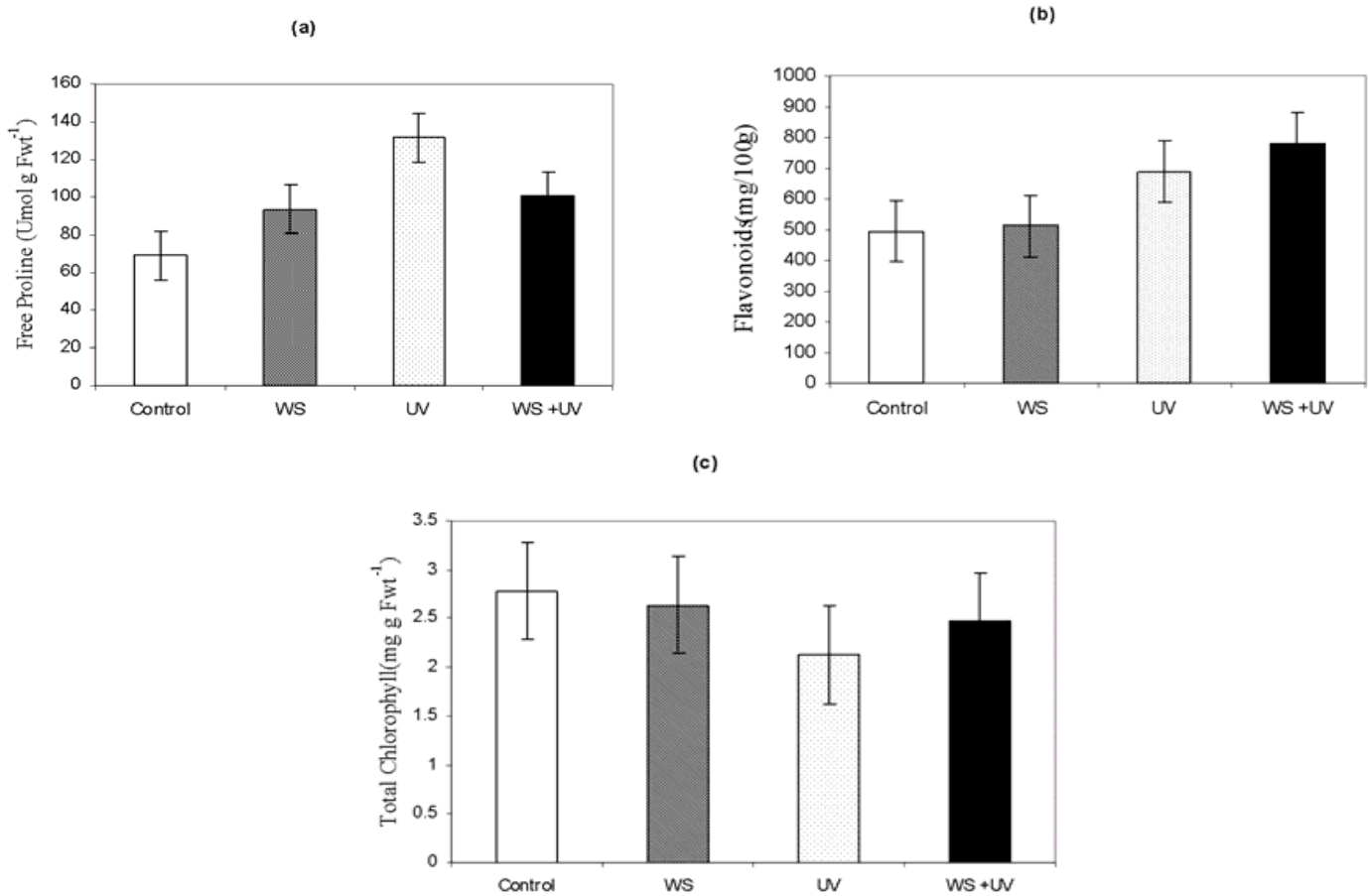


Fig. 2: Changes in total anthocyanin (a), flavonoids (b) and total Chlorophyll content (c) in leaves of bean in response to drought and UV-B stresses singly and in combination. Legends as Fig.1.

However, chlorophyll content showed the same response as growth parameters, as it was decreased by 22, 16 and 20%, due to exposure to WS, UV and both stress together, respectively (Fig 2 c). Figure 3 showed effect of waters stress and UV-B radiation applied singly or in combination on free proline, lipid peroxidation and membrane permeability. Proline was increased by 76 and 34% after exposure to WS and sUV-B, respectively (Fig 3 a), while their interaction was less than additive (19%). On the other hand, Lipid peroxidation and membrane permeability were increased by 40 and 43%, respectively (Fig 3 b & c) due to exposure to UV irradiation while water tress had no significant effect. Interaction between both stresses caused increases in these parameters by 31 and 47%, respectively (Fig 3 b & c).

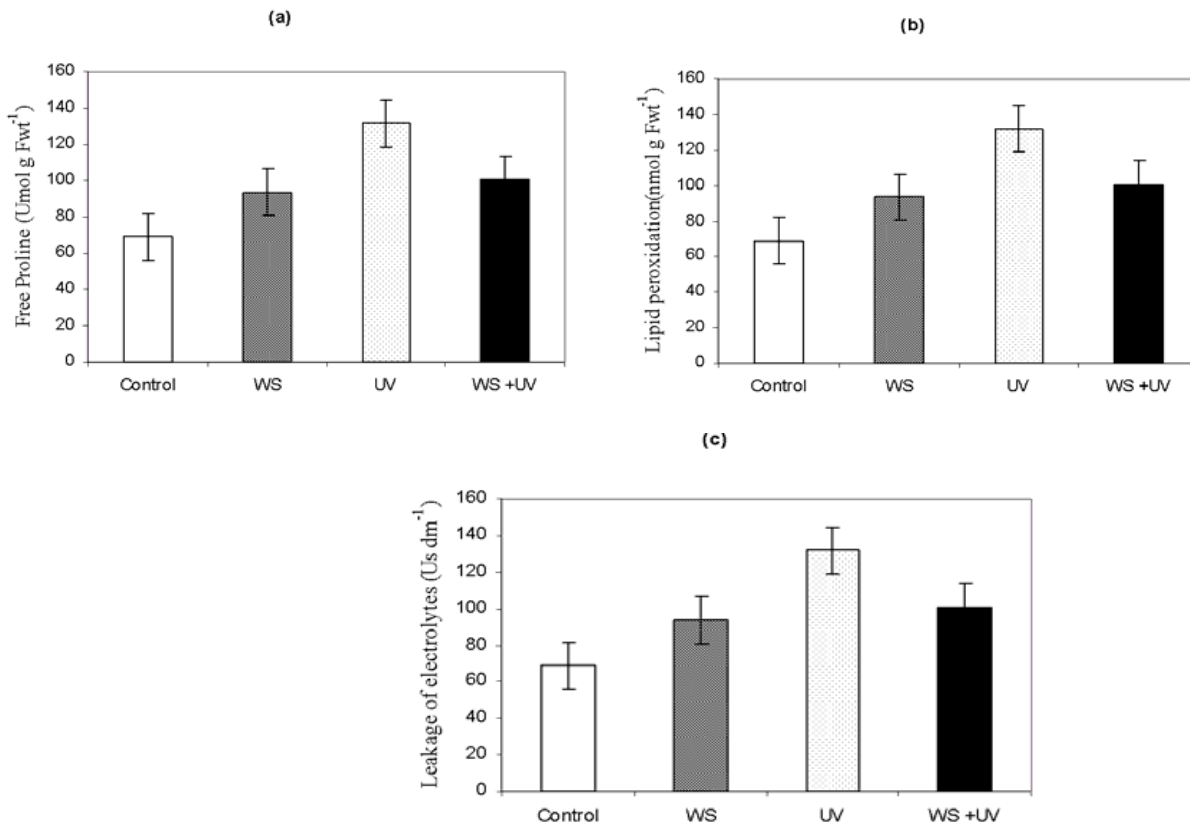


Fig. 3: Effect of waters stress and sUV-B radiation applied singly or in combination on free proline (a), lipid peroxidation (b), membrane permeability (c).

Legends as Fig.1. Lipid peroxidation is expressed as malondialdehyde (MDA) content

Discussion:

Alexieva et al. (2001) reported that there is an inter-relationship between drought and ultraviolet-B (UV-B) radiation in plant responses, in that both stresses provoke an oxidative burst. However, the mechanisms involved in the response of plants to both stresses are yet to be identified. Thus, elucidation of their interaction would help plants cope with changing environmental conditions (Tian & Lei. 2007).

UV-B radiation, causes reduction in biomass and yield and photosynthesis in various crop species suggesting that it has an inhibitory effect (e.g. Teramura & Murali, 1986; Teramura & Sullivan, 1991; Nogues and Baker, 2000; Agrwal & Rathore, 2007; Moussa & Khodary, 2008). Moreover, drought stress in the present study caused significant reductions in Fv/Fm, and yield parameters and this is in agreement with huge body of literature that stated WS causes limitation in gas exchange and growth parameters (e.g. Alexieva et al., 2001, Balouchi et al., 2009). The decline in the Fv/Fm ratio is a good indicator of photoinhibitory damage caused by environmental stress(es). However, the mechanisms involved in the response of plants to both waters stress and sUV-B are yet to be identified.

Our previous study (Hassan et al., 2011) pointed out that the exposure to drought stress resulted in slow development of water stress, with the first effect on RWC of bean leaves and there was no significant effect on *f*³PSII. This is in agreement with the results of Cornic (1994), who reported insignificant effect of mild drought stress on photosynthetic capacity of wheat leaves. Furthermore, Nogues et al (1998) found similar results on pea leaves. The photosynthetic assimilates that can be attributed to biomass is ascribed to both leaf area and net photosynthetic rates (e.g. Chisi et al., 2002; Quaggiotti et al., 2004; Hassan, 2006).

It was reported that exposure to UV-B causes a rapid loss of photosynthetic competence primarily through effects on Rubisco (Nogues & Baker, 2000; Moussa & Khodary, 2008). This further our previous findings that leaf area, growth; biomass and yield were reduced due to exposure to UV-B which suggests reducing photosynthetic supply (Hassan et al., 2011). Reduction in A could be due to reduction in Chl content, and this is supported with the results of Pal et al. (1999), who found that UV-B irradiated mung bean plants showed reduced chlorophyll content along with lower photosynthetic rates. Recently (Moussa & Khodary, 2008) found similar results in bean and barely leaves and these results were supported by results of Balouchi et al. (2009) on wheat. This was in contrast to the finding with peach grown under solar UV-B exclusion where UV-B irradiated plants showed increased A but chlorophyll content remained unaffected (Laposi et al., 2002). However, this was not the case of Nogues et al. (1998), who found no effects of UV-B on photosynthetic rates and other photosynthetic parameters in pea leaves. The differences in response may be due to species used and/or experimental design.

In their thorough review, Piri et al (2011), they showed that a decrease in photosynthetic pigments was evident during exposure to enhanced UV radiation in most crop species. It is noteworthy that a decrease of total chlorophyll occurred under reduced UV radiation, and this implied that the UV-B radiation has a negative impact on the parameters related to photosynthesis. Decrease in chlorophyll concentration due to UV-B radiation was reported previously (Piri et al. 2011). Reduced chlorophyll concentration may be due to increased chlorophyllase activity.

The significant effect of sUV-B on the concentrations of H₂O₂ is in agreement with results of other researchers (Rao and Ormrod 1995, Hideg and Vass 1996; Prasad et al. 2005). increase in lipid production caused by stress may have occurred because of the accelerated formation of reactive oxygen species (ROS) [i.e., singlet oxygen (¹O₂) and \cdot EOH]; ROS attack lipids, particularly unsaturated fatty acids, and the accelerated formation of ROS results in the formation of peroxidation products, the main one of which is MDA (Forman and Fisher 1981, Zu et al. 2011). The reaction of such radicals with macromolecules, particularly lipoproteins, can cause faster peroxidative damages as observed from the destruction of membrane lipids (Asada 1992, Alexieva et al. 2001).

On the other hand, anthocyanins and flavonoids are affected differently by UV radiation. These pigments play an important role against UV damage in higher plants (Balouchi et. 2009). We found the the highest levels of anthocyanin and flavonoids were obtained in UV-B radiation while the lowest content were bserved in plants exposed to water stress (Figure 3). An increase of UV absorbing compounds caused by UV was well documented in previous studies (Alexieva et al., 2001; Rozema et al. 2002; Balouchi et al. 2009). Flavonoids compounds have effective radical scavenging capabilities and can directly contribute to enhanced photoprotection against UV-B radiation. The increases in UV-B absorbing compounds, mainly in flavonoids, are recognized as a general response to UV-B stress (Flint et al. 2004). These results suggest that the UV-B absorbing compounds are mainly synthesized in leaves and they are used to protect leaf tissue under exposure to UV. However, it seems to be produced through similar mechanisms as in the case of UV induction. Flavonoids and related compounds absorb strongly in the UV-region but not in the photosynthetically active regions of the spectrum (Cen and Bornman 1993), allowing photosynthesis to continue while UV wavelengths are attenuated at the epidermis.

Water stress and UV radiation lead to the increase of the contents of proline in leaves of bean in this experiment, which indicates that some wilting-induced proline accumulation occurred (Balouhci et al. 2009). It was reported that plants exposed to UV radiation accumulate proline that could protect plant cells against UV radiation-induced peroxidative processes (Saradhi et al. 1995). A marked increase in proline accumulation under UV-B in the present study is in agreement with the results of Balouchi et al. (2009) and this could represent adaptive responses to oxidative damage induced by UV radiation. Proline is known to be involved in alleviating cytosolic acidic associated with several stresses (Kurkdjian and Guern 1989). The removal of excess H⁺ occurring as a result of proline synthesis may have a positive effect on reduction of the UV-B induced damage. It suggests that UV radiation- induced proline accumulation protects plants against UV radiation promoted peroxidation processes.

CAT, APX, and SOD are key enzymes of the antioxidant defence system. The SOD, POD, APX, and CAT activities are also associated with UV-B exposure and other stresses such as water stress, as these enzymes act as antioxidant compounds to help reduce photooxidative damage in plant leaves. SOD accelerates the conversion of superoxide to H₂O₂, whereas CAT and APX catalyze H₂O₂ breakdown (Alexivea et al. 2011, Zancan et al. 2008, Wang et al. 2010). The results of the present study indicate that the CAT, APX, and SOD activities were positively affected by supplementary UV-B radiation. Therefore, a preferential synthesis/activation of this enzyme by bean leaves in the present study counteracts oxidative stress. The increase in the CAT, APX, and SOD activities are frequently observed under stressful conditions (Yazici et al. 2007, Mishra et al. 2009, zu et al. 2011).

The severe reduction in yield and photosynthetic efficiency were confirmed the synergistic effects of both drought stress and sUV-B.

Conclusion:

In conclusion, we found that UV-B radiation and water stress increased UV screen pigments, MDA and antioxidant enzymes although water stress decreased yield and pigment production. Moreover, the present investigation showed that supplemental UV-B radiation and water stress caused adverse effects on activity of photosystem II in bean plants leading to reductions in photosynthetic gas exchange and Chl pigments. Parameters such as Fv/Fm, Chl content and A were useful indicators of the plant's response to UV-B. Our

understanding of the relationships between crop growth and the atmospheric environment was developed substantially in the past few decades. Still, the factor of climate change and its impact on crops and food production will be further explored in future studies because global change climate might be critical event in future centuries; available data may not adequately characterize the potential effect of future, such as simultaneous changes in climate change and UV-B radiation

There was an interaction between sUV-B and WS, where the first delayed and reduced the severity of the latter through a reduction in plant water loss rates and through reductions in leaf area and g_s .

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