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Research Article

The Effects of Ultraviolet Radiation on the Contents of Chlorophyll, Flavonoid, Anthocyanin and Proline in *Capsicum annuum* L.

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Abstract: Although ultraviolet radiation is potentially harmful, it is an important component of terrestrial radiation to which plants have been exposed since invading land. Since then, plants have evolved mechanisms to avoid and repair UV radiation damage. Therefore, it is not surprising that photomorphogenic responses to UV-B and UV-C are often assumed to be adaptations to harmful radiation. Most of the compounds accumulated are directly involved in UV-B and UV-C protection: they are either efficient in filtering excess radiation or in scavenging radicals. In this study plants were grown for 5 weeks in controlled environment room. The plants were grown in vermiculite medium using pots. Before UV treatments, plants were irrigated with nutrient solution (Hoagland solution) for 5 weeks. Then plants were exposed to UV-A (320-390 nm), UV-B (312 nm) and UV-C (254 nm) irradiation with a density of 6.1 (Wm^{-2}), 5.8 (Wm^{-2}) and 5.7 (Wm^{-2}) for 2 weeks. Plants were treated with UV in their light period for 27 min per day for 14 days. The influence of UV-A, UV-B and UV-C radiation on chlorophyll, flavonoids, anthocyanin, proline, membrane permeability, lipid peroxidation and UV-absorbing compound was examined. Results showed that the contents of chlorophyll a, b and carotenoids of pepper leaves were reduced significantly in those plants which were exposed to UV-B and UV-C radiation and compared with control and UV-A treated plants. In contrast, UV-B and UV-C increased ($P < 0.05$) proline, quercetin, rutin and anthocyanin concentrations in leaves of *Capsicum annuum* L. Ultraviolet radiation induced oxidative stress in pepper by increasing lipid peroxidation and membrane permeability which indicating that limits of tolerance are much less than damaged caused by UV-radiation.

Key Words: Ultraviolet radiation, proline, lipid peroxidation, *Capsicum annuum* L.

Introduction

The spectrum of UV radiation reaching the earth 's surface has been divided into lower energy (UV-A, 320-400 nm), higher energy (UV-B, 280-320 nm) and UV-C (254-280 nm) regions. A given dose of radiation, of UV-A is less effective than UV-B and UV-C for induction of plant responses (Barta et al., 2004). UV radiation also produces oxidative stress (Costa et al., 2002), which arises from the deleterious effects of active oxygen species (AOS), which react with lipids, pigment, proteins and nucleic acid (Dai et al., 1997). Under conditions of normal healthy growth, plants possess a number of enzymatic and non-enzymatic detoxification mechanisms to efficiently scavenge either the AOS themselves or their secondary reaction products (Bartling et al., 1993). Since flavonoids and phenolics absorbe UV-B bands they represent a selective UV-B filter which protect plant tissue against harmful rays (Rozema et al., 2002).

This has led to the long-standing hypothesis that a primary adaptive advantage conferred by these compounds is that they absorb potentially harmful UV-B radiation at the leaf surface and protect underlying photosynthetic tissues. It had been argued that pigments localized in the epidermal cells (mainly flavonoids and anthocyannins) reduce epidermal penetration of UV-B radiation selectivelly protecting internal tissues of the UV-B irradiance without interfering photosynthesis (Caldwell et al., 1983).

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The study on *Sorghum vulgare* showed that UV-B irradiation induced decrease in chlorophyll and carotenoid concentrations (Ambasht & Agrawal, 1998). However, Strid and Porra (1992) suggested that UV-B irradiation in leaves of *Pisum sativum* has no specific effects on enzymes of the chlorophyll biosynthetic pathway but rather, influences the genetic regulation of chlorophyll binding protein, leading to destruction of chlorophyll.

It has been reported that UV-B stimulted the biosynthesis of UV-B absorbing compounds and carotenoids, which both perform a photoprotective function (Campos et al., 1991). The carotenoids are implicated in the direct protection of the photosystems against UV-B radiation (Middleton & Teramura, 1993). This is because UV-B radiation is a potential oxidative factor and carotenoids, as flavonoids have been shown to quench effectively active oxygen species (Larson, 1998).

By contrast, a dramatic induction of synthesis and accumulation of flavonoids is often observed in response to high (sun) light (Reay & Lancaster, 2001; Merzlyak & Solovchenko, 2002). Experiments with transgenic plants demonstrated an upregulation of the genes responsible for flavonoid (particularly, kaempferol and quercetin) biosynthesis under elevated UV-B conditions (Wang et al., 2000; Ryan et al., 2002).

There also a reposed which show that upon exposure to UV, plants increase the production of leaf flavonoids and anthocyanins (Krizek et al., 1998). Flavonoids have maximum absorption in UV region of light (Teramura & Sullivan, 1994; Krauss et al., 1997). Similar protective effects have also been suggested for anthocyanins (Burger & Edwards, 1996; Coley & Kursar, 1996; Woodall & Stewart, 1998).

Numerous studies have demonstrated that the accumulation of flavonoids and anthocyanins by plant provide a defence mechanism against UV-B radiation (Bieza & Lois, 2001). Rutin is a highly antioxidative active flavonoid. It can be found in many plants .There is some evidence that UV radiation increases the production of rutin in plants (Umek et al., 1999). Studies have shown that UV radiation alters membranes. This can be seen by the increase in malondialdehyd concentration (MDA), reduced monogalagtosyl diacylglycerol (MGDG), as well as an increase in ethylene and ethane concentration (Dai et al., 1997).

The aim of this study was to study the importance of the accumulation of flavonoids (specially rutin and quercetin) and non-enzymatic detoxification mechanisms which develop during exposure of pepper plants to UV-A, UV-B and UV-C radiation. Additionally, the content of lipid peroxidation and membrane permeability were examined in pepper plants in aim to find if enhanced flavonoids biosynthesis can protect plants from the which damaged caused by UV radiation and repair the damage.

Material and Methods

Plant growth and treatments

Seeds of *Capsicum annuum* L. were sown in plastic pots containing 1 kg of coarse sand and vermiculite (2:1, V/V). Plants were grown in the greenhouse at 25/20 ^{o}C (day/night), with a 16h light/8h dark photoperiod for 35 days. After 35 days, selected plants were subjected to ultraviolet radiation with UV lamps. UV-A (320-390 nm), UV-B (312 nm) and UV-C (254 nm) irradiation with a density of 6.1 (Wm^{-2}), 5.8 (Wm^{-2}) and 5.7 (Wm^{-2}), respectively, (measured with UV sensor model: LEYBOLD DIDACTIC). UV-A, UV-B and UV-C lams were purchased from the Philips Company.

Plant were treated with UV in their light period for 27 min per day for 14 days. We have four replicates for each treatment.

Pigment analysis

To determine the absorption by flavonoids, 0.1 g of fresh leaf tissue were taken from the distal ends of the leaf and were extracted in 15 ml glass centrifuge tubes containing 10 ml ethyl alcohol: acetic acid (99:1 v:v). The samples were gently boiled for 10 minutes in a water bath at 80 ^{o}C and brought up to volume. Absorbance was measured at three wavelengths: 270, 300 and 330 nm with UV-VIS spectrophotometer WPA, (model: S2100 Diod Array) (Krizek et al., 1998).

To determine the concentration of anthocyanins, 0.1 g fresh leaves were taken and were extracted in 15 ml glass centrifuge tubes containing 10 ml of acidified methanol (methanol: HCl, 99: 1, v:v) and kept over night in the dark. The samples were brought up to volume, and the absorbance at 550 nm was determined.

Anthocyanin concentration was calculated using an extinction coefficient of 33000 mol^{-1} cm^{-1} (Wanger, 1979)

Total chlorophylls and carotenoids were extracted from leaf discs with 80% acetone and determined according to Lichtenthaler (1987).

HPLC analysis of flavonoids

0.2 g fresh leaf tissue was taken. The homogenized tissue was incubated in 80% methanol, for 24 h at 4 °C. Methanolic (80%) leaf extracts were subjected to reversed phase HPLC (Agilent model: 1100) and monitored at 355nm with a diod array detector. A 20 µl sample was injected into a 250 × 4.5 mm C-18 column (Zorbax 300 sb) with a mobile phase of mM phosphoric acid (pH = 3), and acetonitrile. A non-linear gradient of acetonitrile was then run to elute the flavonoids (3 min at 10% , 1.5 min at 11.5 , 9 min at 14%, 2 min at 19%, 9 min at 22%, and 6 min at 100% acetonitrile) (Greenberg et al., 1996). Rutin eluted at 7.3 min and the peak area was compared with the standard. Quercetin eluted at 12.5 min and the peak area was compared with the standard.

Thiobarbituric acid reactive substances (TBARS)

0.2 g of the leaf tissue of plants were homogenized in 10 ml of 0.1% (w/v) trichloroacetic acid (TCA), then centrifuged at 10000 g for 15 minutes. 1 ml of supernatant was then vortexed with 4 ml of 20% (w/v) TCA containing 0.5% (w/v) 2-thiobarbituric acid (TBA), and the solution was heated for 30 minutes at 95 °C. The samples were cooled on ice for 5 min and recentrifuged for 10 minutes at 10000 g. The non-specific absorbance of supernatant at 600 nm was subtracted from the maximum absorbance at 532 nm for the MDA measurement (Heath & Packer, 1969), and at 455 nm for other aldehydes (Meirs et al., 1992). For the MDA and aldehyds calculation, an extinction coefficient (ε) of 1.56×10^5 M^{-1} cm^{-1} was used at 532 nm for MDA and an ε of 0.457×10^5 M^{-1} cm^{-1} was used at 455 nm as the average of the ε obtained for five other aldehyds (propanal, butanal, hexanal, heptanal and propanal-dimethyl acetal).

Proline and membrane permeability determination

Free proline was extracted, derivatized with acid ninhydrin and absorbance read according to Bates et al. (1973) method. Membrane permeability of leaves was measured by electrolyte leakage (Dhindsa et al., 1981).

Leaf samples for all experiments were collected from the fully expanded third leaf below the tip of the stem.

Statistical analysis

Quantitive changes of different parameters were analysed through analysis of variance (ANOVA), with Duncan's multiple range test being used to determine significant differences among treatments.

Results

The experimental results showed that UV-B and UV-C irradiance caused the reduction of the contents of chla, chlb and (chla + chlb) of pepper leaves but in UV-A treated plants there was no significant decrease in these pigments. Significant decrease in chlorophyll a contents was observed in UV-B by 8% and by 15% UV-C treated plants, but in UV-A treated plants there was no significant decrease (1%) in comparison with the control samples (Figure 1). Chlorophyll b contents was significantly decreased, in UV-B and UV-C treated pepper plants by 12 and 21% respectively but in UV-A treated plants there was no significant decrease (3%) in comparison with the control (Figure 1). Total chlorophyll contents was significantly decreased, in UV-B and UV-C treated pepper plants by 9 and 17% respectively but in UV-A treated plants there was no significant decrease (2%) in comparison with the control (Figure 1)

Significant decrease in carotenoid contents was observed in UV-B and UV-C treated plants by 11 and 20%, but in UV-A treated plants there was no significant decrease (1%) in comparison with the control (Figure 2).

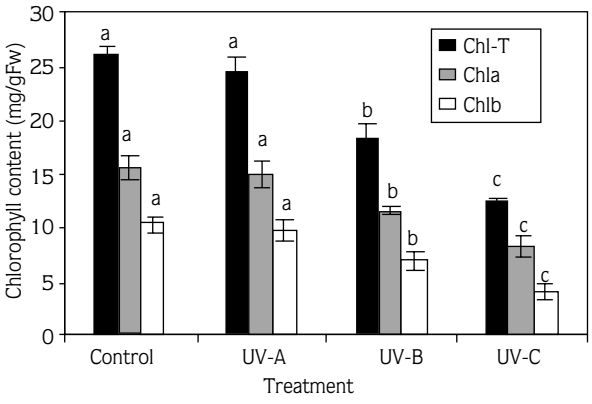

Figure 1. Levels of chla, chlb and chl (a+b) in leaves of *C.annuum* treated with supplementary UV-A, UV- B and UV-C. Values are the means of four replicates, and bars indicate SEM significant difference at $P < 0.05$ according to Duncan 's test, (chl = chlorophyll).

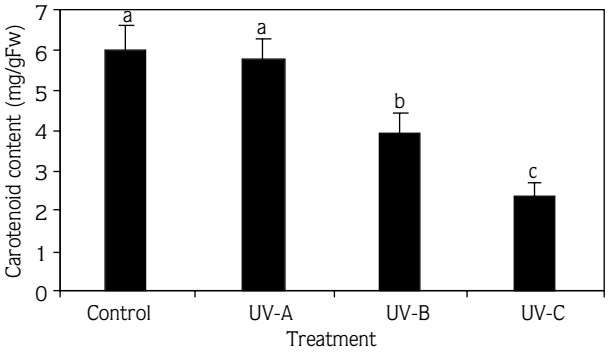

Figure 2. Effect of UV-A, UV-B and UV-C treatment on carotenoid content. Values are the means of four replicates, and bars indicates, and bars indicate SEM significant difference at P < 0.05 according to Duncan 's test.

Anthocyanin concentrations was significantly increased, in UV-B and UV-C treated pepper plants by 4 and 7% respectively but in UV-A treated plants there was only (1%) increase in comparison with the control (Figure 3).

This study revealed increases in flavonoid levels (270, 300 and 330 nm) in pepper leaves exposed to UV-A (respectively 6%, 4%, 5%), and UV-B (respectively 10%, 8%, 7%) and UV-C (respectively 12%, 8%, 8%) in comparison with control (Figure 4).

HPLC analyses showed that rutin content was significantly increased, in UV-A, UV-B and UV-C respectively by 8, 17 and 27% in comparison with the control (Figure 5, 7).

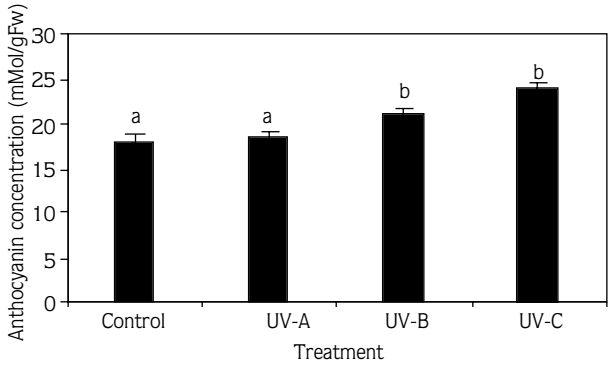

Figure 3. Influence of UV-A, UV-B and UV-C on concentration of anthocyanins at 550. Values are the means of four replicates, and bars indicate SEM significant difference at P < 0.05 according to Duncan 's test.

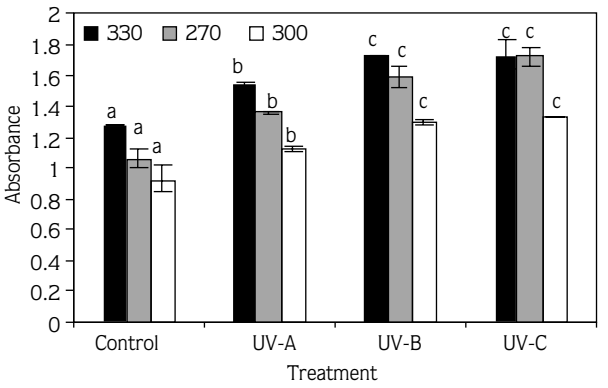

Figure 4. Influence of UV-A, UV-B and UV-C on the absorbance of the methanolic extract of leaves at 270, 300 and 330 nm. Values are the means of four replicates, and bars indicate SEM significant difference at P < 0.05 according to Duncan 's test.

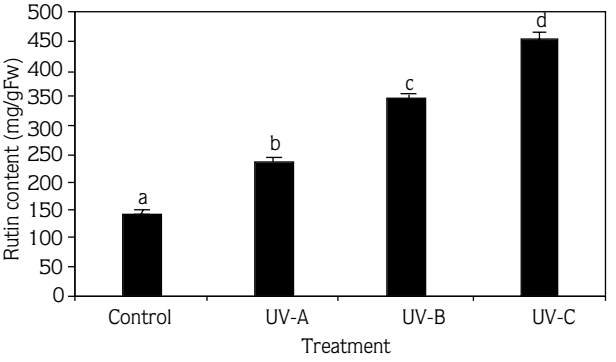

Figure 5. Influence of UV-A, UV-B and UV-C on the content of rutin. Values are the means of four replicates, and bars indicate SEM significant difference at P < 0.05 according to Duncan 's test.

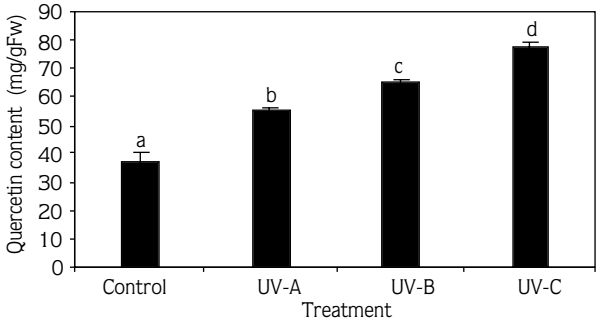

Figure 6. Influence of UV-A, UV-B and UV-C on the content of quercetin in pepper leaves. Values are the means of four replicates, and bars indicate SEM significant difference at P < 0.05 according to Duncan 's test.

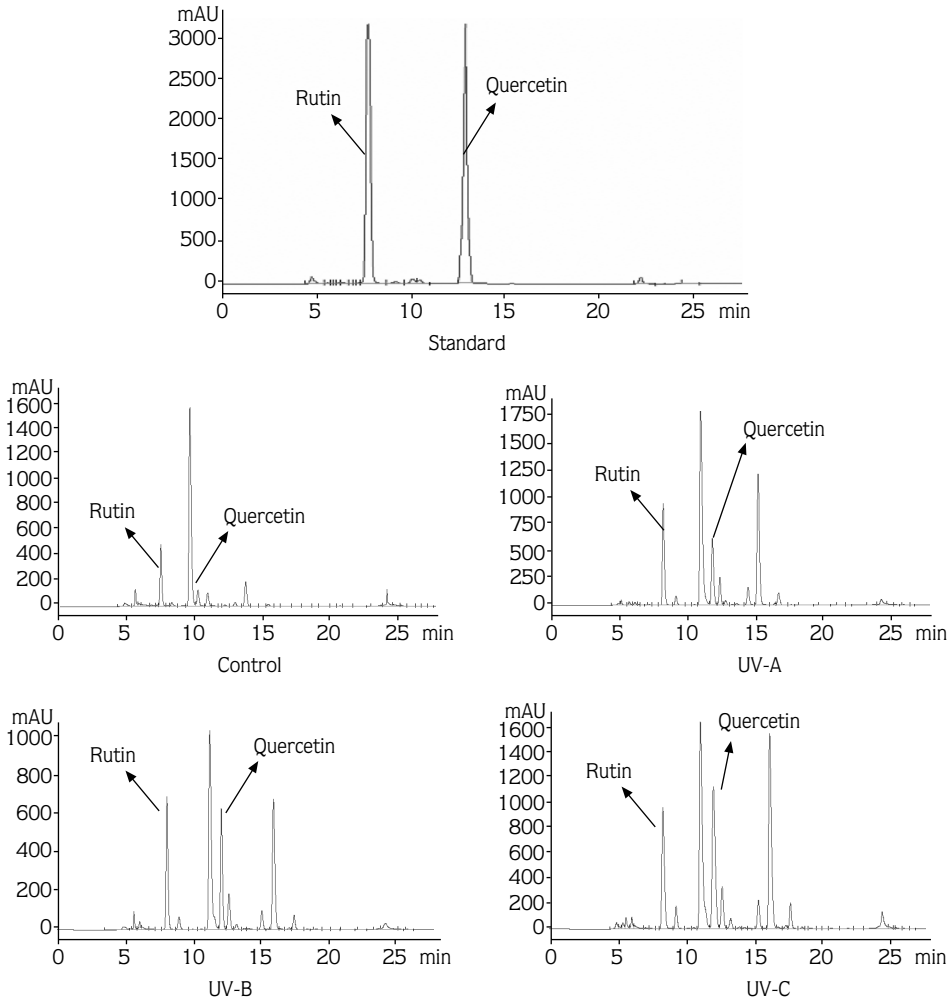

Figure 7. An HPLC-chromatogram of pepper (*Capsicum annuum* L.) leaf extract at 355 nm grown under control, UV-A, UV-B and UV-C treatments, respectively. Rutin eluted at 7.3 min and the peak area was compared to the standared. Quercetin eluted at 12.5 min and the peak area was compared to the standard.

Our results by HPLC analyses revealed that quercetin content is synthesized in large amounts in response to UV-A, UV-B and UV-C respectively by 7, 12 and 17% in comparison with the control (Figure 6,7).

Malonedialdehyde concentration was significantly increased, in UV-B and UV-C treated pepper leaves by 13 and 23% respectively but in UV-A treated plants there was no significant increase (1%) in comparison with the control. Malonedialdehyde concentration was significantly increased, in UV-A, UV-B and UV-C treated pepper roots by 2, 16 and 38% respectively in comparison with the control. Other aldehyde concentration was significantly increased, in UV-B and UV-C treated pepper leaves by 27 and 31% respectively and in UV-A treated plants there was no significant increase (2%) in comparison with the control. Other aldehyde concentration was significantly increased, in UV-B and UV-C treated pepper roots by 13 and 22% respectively but in UV-A treated plants there was no significant increase (5%) in comparison with the control (Figure 8).

Leakage of electrolytes was significantly increased, in UV-A, UV-B and UV-C treated pepper leaves by 10, 20 and 38% respectively in comparison with the control (Figure 9).

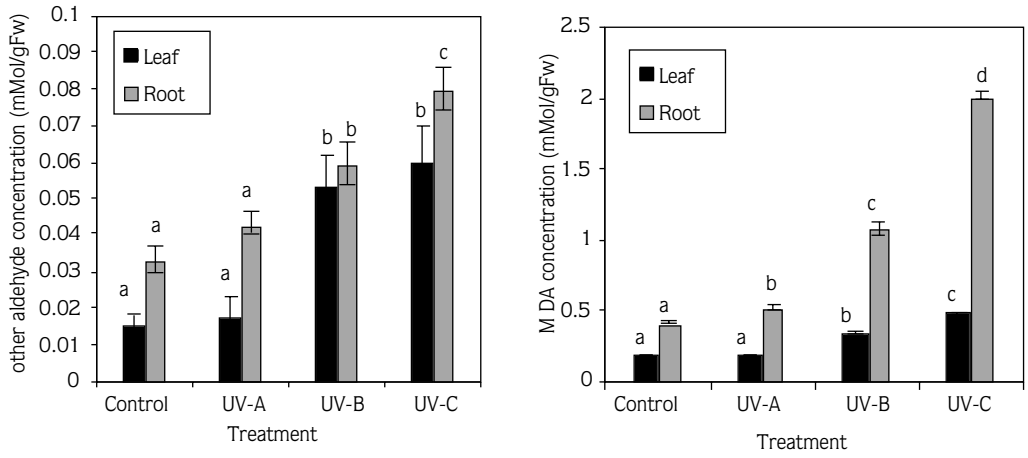

Figure 8. Effect of UV-A, UV-B and UV-C treatment on lipid peroxidation. Values are the means of four replicates, and bars indicate SEM significant difference at $P < 0.05$ according to Duncan 's test. The comparison between the mean for leaf and root content of these aldehydes were not shown.

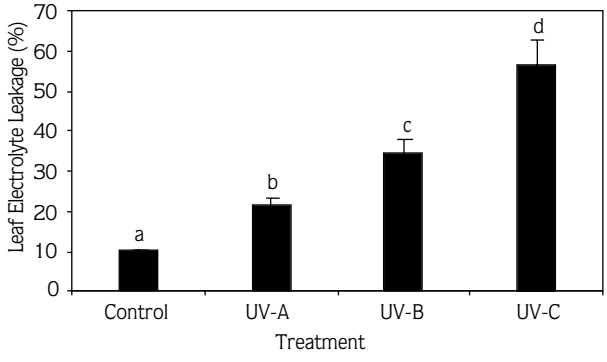

Figure 9. Effect of UV-A, UV-B and UV-C treatment on leakage of electrolytes. Values are the means of four replicates, and bars indicate SEM significant difference at $P < 0.05$ according to Duncan 's test.

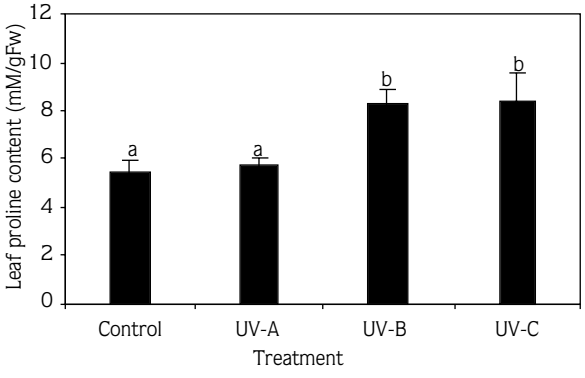

Figure 10. Proline content of leaves of *Capsicum annuum* L. treated with supplementary UV-A, UV-B and UV-C. Values are the means of four replicates, and bars indicate SEM significant difference at $P < 0.05$ according to Duncan 's test.

In this study, proline content was significantly elevated, in UV-B and UV-C treated pepper leaves by 10 and 10% respectively but in UV-A treated plants there was no significant increase (1%) in comparison with the control (Figure 10).

Discussion

The experimental results showed that increase UV-B and UV-C irradiance also caused the reduction of the contents of chlorophyll a, b and (a + b) of pepper leaves. The reduction of the chlorophyll content has a negative effect on plant photosynthetic efficiency. Since it has been reported that photosynthesis is dependent on the light harvesting properties of the chlorophylls (Gao et al., 2004). UV-B induced reduction in chlorophyll may be expected to result in lower levels of biomass accumulation, and hence be a useful indicator of UV-B sensitivity (Smith et al., 2000). Recent studies have shown that carotenoids serve a protective function against UV-B (Rau et al., 1991) and UV-C (Campos et al., 1991) radiation.

The efficacy of carotenoids in protecting the photosystems is likely due to their function as efficient quenchers of high energy short wave radiation. The

mechanism by which this is accomplished was first proposed to involve a photochemical state change of singlet oxygen to triplet form by interaction with carotenoids, removing the potentially dangerous oxygen radicals produced in photo oxidative processes (Krinsky, 1979).

In the present study pepper leaves responded to the UV-B treatment by increasing significantly their flavonoid and anthocyanin contents, which presumably offers protection from the high UV-B level thus suggesting that pepper leaves are not well protected from exposure to high UV-B and need to synthesize additional pigments to protect them from the UV-B. Measuring rutin was an attempt to estimate the significance of phenolic compounds, accumulated in response to UV radiation (Figure 5). The flavonoids play many defensive roles in plants, and interception of UV-B by epidermal flavonoids often proposed as an adaptive mechanism preventing UV-B from reaching the mesophyll and affecting photosynthesis (Liu et al., 1995). Thus, the pepper plants may activted a defence mechanism against UV damages by increasing non-photosynthetic pigments.

The involvement of flavonols in the UV-B response has been reported for several plant species, including legume such as soybeans (*Glycine max*) (Middleton & Teramura, 1993).

However, the antioxidant function of flavonoids is complex and depends on a variety of factors, including compartmentalization, redox potential, presence of double bands, glycosylation and hydroxylation (Bors et al., 1995; Rice et al., 1996; Cooper-Driver & Bhattacharya, 1998).

This complexity therefore also needs to be taken into account in the consideration of possible antioxidant functions for increased flavonoid levels under UV treatment.

In this research the results of spectrophotometric and HPLC analysis showed an increase in flavonoids under UV treatment, especially UV-B and UV-C treatments that probably means either increment in flavonoid is the role of these compounds in the protection of oxidative stress in plants, or preventing the penetration of destructive bands of UV light to the most sensitive tissue.

Oxidative damage can be detected by lipid peroxidation. Hydroxyl radicals and singlet oxygen can react with lipids and form lipid peroxy radicals and hydroperoxide (Blokhina et al., 2003; Hollosy 2002). The peroxy radicals can abstract hydrogen from other unsaturated fatty acids, leading to a chain reaction of peroxidation. The peroxidation of membrane lipids leads to the breakdown of their structure and function (Hollosy 2002; Yuan et al., 2000). The increase in TBARS content is more precisely an indicator of general UV-induced oxidative damage, due to the impairment of cell defense system (Costa et al., 2002; Barka et al., 2000). Change in `TBARS (especially MDA) was the first evidence that under our experiment, UV (UV-B and UV-C) induced oxidative stress (Figure 8).

In pepper, an increase of leaf membrane-permeability was considerable in comparison with the control. Indirect evidence from many experiments suggests that UV-B and UV-C exposure generates free radicals, which increase the lipid peroxidation and disruption of membrane integrity (Kramer et al., 1991). In the present study, a marked increase in proline in UV-B and UV-C treatment (Figure 10) represents adaptive responses against oxidative damage induced by UV radiation.

Proline is known to be involved in alleviating cytosolic acidic associated with several stresses (Kurkdjian & 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 and UV-C induced damage. This lead us to believe that UV radiation induced proline accumulation protects plants against UV radiation promoted peroxidation processes.

Secondary metabolite analysis in our experiment showed increases in quercetin in pepper leaves under UV-A, UV-B and UV-C radiation (Figure 6, 7). Correlations between quercetin concentrations and lipid peroxidation levels indicated an antioxidant role of secondary metabolites in pepper leaves exposed to UV-A, UV-B and UV-C radiation. These findings indicate an important role of UV-B and UV-C radiation in quercetin synthesis. It seems that applied doses of UV radiation exert a state of stress, where limits of tolerance are exceeded and adaptive capacity is overtaxed, that possibly results in a disturbance of quercetin synthesis.

Conclusion

By considering to obtained results in this study we concluded that UV-A often harmful in plant, but UV-B and UV-C have serious effects on plant.

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