

## THE EFFECTS OF UV-A, UV-B AND UV-C ON PROTEIN AND ASCORBATE CONTENT, LIPID PEROXIDATION AND BIOSYNTHESIS OF SCREENING COMPOUNDS IN BRASSICA NAPUS\*

F. NASIBI AND KH. M-KALANTARI\*\*

Biology Department, Shahid Bahonar University of Kerman, Kerman, I. R. of Iran,  
E-mail: nasibi2002@yahoo.com  
E-mail: Kh\_kalantari@yahoo.com

**Abstract** – The role of the antioxidant defense system was studied in *Brassica napus*, and subjected to different supplementary bands of ultraviolet radiation (UV-A, UV-B and UV-C). This study showed that UV-B and UV-C induced oxidative stress as evidenced by an increase in lipid peroxidation and total ascorbate content. Under UV-B and UV-C treatments, flavonoids and anthocyanins were increased significantly compared with control and UV-A treated plants. These compounds act as internal filters and also have an antioxidant activity against active oxygen species such as H<sub>2</sub>O<sub>2</sub>. Results of this research showed that the protein content increased under UV-B and UV-C. These increments are probably related to the synthesis of defense proteins such as HSP, the expression of genes that are encoding the antioxidant enzymes, or those which contribute in UV-absorbing compound synthesis pathways such as PAL, CHS and CHI.

**Keywords** – UV radiation, ascorbic acid, lipid peroxidation, oxidative stress, flavonoids, anthocyanins, *Brassica napus*

### 1. INTRODUCTION

Stratospheric ozone depletion resulting from continual antropogenic release of chloroflourocarbons and other pollutants has led to an increase in the ultraviolet radiation reaching the earth's surface [1, 2]. UV radiation causes damage to living organisms [3]. Terrestrial plants are especially vulnerable due to their obligatory requirement of sunlight for photosynthesis [4, 2]. Many studies have shown deleterious UV effects such as reduced photosynthesis, biomass reduction, decreased proteins, impaired chloroplast function and damage to DNA [5]. UV radiation also produces oxidative stress [3], which arises from the deleterious effects of active oxygen species (AOS), which react with lipids, pigment, proteins and nucleic acid [1]. Plants are well adapted for minimizing damage that could be induced by AOS under natural growth conditions. However, oxygen toxicity emerges when the production of these AOS exceed the quenching capacity of protective systems due to environmentally adverse conditions such as drought, high light, high salinity, water logging, heavy metal toxicity, air pollutants and UV radiation[3, 1]. Recent studies have shown that enhanced UV radiation alters membranes. This can be seen by the increase in malondialdehyd concentration (MDA), reduced monogalagosyl diacylglycerol (MGDG), as well as an increase in ethylene and ethane concentration [1]. Protein degradation occurs under conditions of induced oxidative stress [6]. Some plants are more

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\*\*Corresponding author

tolerant to UV than others because they produce a variety of secondary metabolites including flavonoids and anthocyanins. These compounds often accumulate in the upper epidermis cells of leaves and effectively absorb UV radiation, preventing it from penetrating the leaf mesophyll cells [2]. Plants have developed a complex antioxidant system that includes reduced glutathione (GSH), ascorbic acid (ASA),  $\alpha$ -tocopherol, carotenoids and enzymes that protect plants against oxidative damage [3]. ASA is an ubiquitous soluble antioxidant in both plants and animals [7], and is an important compound in plant cells, as well as being one of the most important reducing substrates for  $H_2O_2$  detoxification [3].

However, the effects of different bands of UV radiation on membranes, proteins, UV-absorbing compounds and non enzymatic oxidative stress defense systems have not been compared. Thus the objectives of the present study are 1) to determine whether or not supplementary UV-A, UV-B and UV-C induces oxidative stress in *Brassica napus* leaves as indicated by MDA and other aldehydes contents. 2) to elucidate the effects of supplementary UV-A, UV-B and UV-C on ASA concentration as a non-enzymatic defense system. 3) investigation of the defense role of UV-absorbing compounds in these treatments.

## 2. MATERIAL AND METHODS

### Plant growth and treatments

Seeds of *Brassica napus* were germinated at 30 °C for 24 h, and then sown in plastic pots containing 1 Kg of sand, silt, and humus (1:1:2). Plants were grown in the greenhouse at 25/20 °C (day/night), with a 16h light/8h dark photoperiod for 14 days. After 14 days of growth, selected plants were subjected to ultraviolet radiation with UV lamps. UV-A (320-390nm), UV-B (312nm) and UV-C (254nm) irradiation with a density of 6.1(Wm<sup>-2</sup>), 5.8 (Wm<sup>-2</sup>) and 5.7(Wm<sup>-2</sup>), respectively, (measured with UV sensor model: LEYBOLD DIDACTIC). UV-A, UV-B and UV-C lamps were purchased from the Philips Company.

Each pot treated with UV in their light period for 30 min per day for 21 days. We have three replicates for each treatment.

### a) Thiobarbituric acid reactive substances

0.2g 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 and Packer) [8], and at 455 nm for other aldehydes (Meir et.al., 1992) [9]. For the MDA and aldehyds calculation, an extinction coefficient (E) of  $1.56 \times 10^5 M^{-1} cm^{-1}$  was used at 532 nm for MDA and an E of  $0.457 \times 10^5 M^{-1} cm^{-1}$  was used at 455nm as the average of the E obtained for five other aldehyds (propanal, butanal, hexanal, heptanal and propanal-dimethyl acetal).

### b) Ascorbic acid, dehydroascorbic acid and total ascorbate

ASA and DHAS were determined as described by Mc de Pinto et.al., [10]. 0.5g of leaf tissue were homogenized in 10 ml metaphosphoric acid 5% and centrifuged for 15 minutes at 10000g.

300 $\mu$ l of supernatant was used for the ASA assay and these solutions were added to the extract respectively: 750 $\mu$ l potassium phosphate buffer and 300 $\mu$ l distilled water. 300 $\mu$ l of supernatant was used for the DHAS assay and the following solutions were added: 750 $\mu$ l potassium phosphate buffer (pH=7.2) and 150 $\mu$ l of 10mM dithiothreitol, the mixture was incubated at room temperature for 10 minutes, and then 150 $\mu$ l of 0.5% N-ethylmaleimide was added. Both samples were vortexed and incubated at room temperature for 10 minutes. To each sample 600 $\mu$ l of 10%(w/v)TCA, 600 $\mu$ l of 44%(v/v) H<sub>3</sub>PO<sub>4</sub>, 600 $\mu$ l of 4%(w/v)bipyridyl in 70%(v/v) ethanol and 10 $\mu$ l of 3% FeCl<sub>3</sub> were added. After vortex-mixing, samples were incubated at 40°C water bath for 20 minutes, and then samples brought up and vortexed again and incubated at 40°C water bath for 20 minutes. Absorbance of samples at 525 nm was recorded. A standard curve of ASA and DHAS was used for the calculation of ASA and DHAS concentration.

### c) UV-absorbing compounds

To determine the absorption by flavonoids, 0.1g of fresh leaf tissue were taken from the distal ends of an upper 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°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) [11].

To determine the concentration of anthocyanins, 0.1g 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<sup>-1</sup>cm<sup>-1</sup> [12].

### d) HPLC analysis of flavonoids

0.2 g fresh leaf tissue was taken. The homogenized tissue was incubated in 80%methanol, 20%filter purified water for 24h at 4°C. Methanolic(80%) leaf extracts were subjected to reversed phase HPLC (Agilent model:1100) and monitored at 340 nm with a diod array detector. A 20 $\mu$ l sample was injected into a 250 $\times$ 4.5mm C-18 column (Zorbax300sb) with a mobile phase of 91%(v/v)1mM phosphoric acid (pH=3), 9%(v/v) acetonitrile. A non-linear gradient to 100% acetonitrile was then run to elute the flavonoids (3min at 10% 1, 5 min at 11.5, 9min at 14%, 2 min at 19%, 9 min at 22%, and 6 min at 100% acetonitrile) [6]. The peaks were received from HPLC with Chemstation software.

### e) Protein determination

Protein concentration was evaluated by the method of Lowry et al. [13], using bovin albumin serum as a standard.

## 3. RESULTS

### a) Thiobarbituric acid reactive substances (TBARS)

TBARS formation in plants exposed to UV radiation is a reliable indicator of free radical formation in the tissue, and is currently used as an indicator of lipid peroxidation. The content of TBARS increased significantly in UV-B and UV-C treatments (150 and 200% over control, respectively) (Fig. 1).

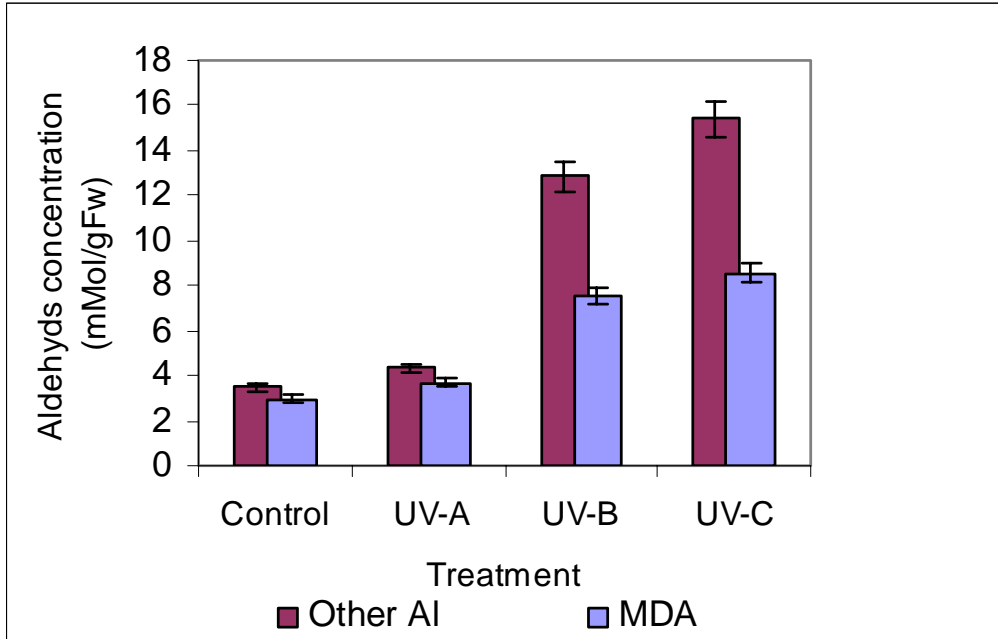


Fig. 1. Effect of UV-A, UV-B and UV-C treatment on lipid peroxidation, values are the means of three replicates, and bars indicate SEM significant difference at  $P < 0.05$  according to Duncan's test, (other al=other aldehyds)

**b) Ascorbic acid, dehydroascorbic acid and total ascorbate**

In this study, ASA, DHAS and total ASA content showed a significant increase in UV-B and UV-C treatments, but in UV-A treated plants there was no significant increase in these compounds. (Fig. 2).

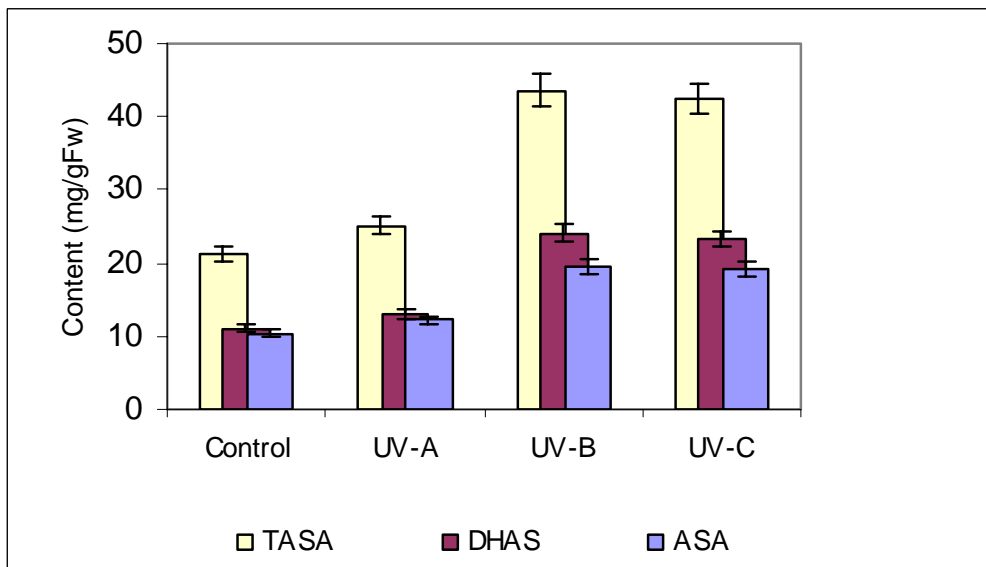


Fig. 2. Levels of ASA, DHAS and total ASA in leaves of *B. napus* treated with supplementary UV-A, UV-B and UV-C, values are the means of three replicates, and bars indicate SEM significant difference at  $P < 0.05$  according to Duncan's test

### c) Protein content

Decrease in protein content is a common phenomenon in UV stress. The reason for this is that the amino acid of proteins will absorb UV and will be degraded. But in this study, we observed an increase in protein content in UV-B and UV-C treatments. This increase is probably due to the synthesis of defense proteins and enzymes (Fig. 3).

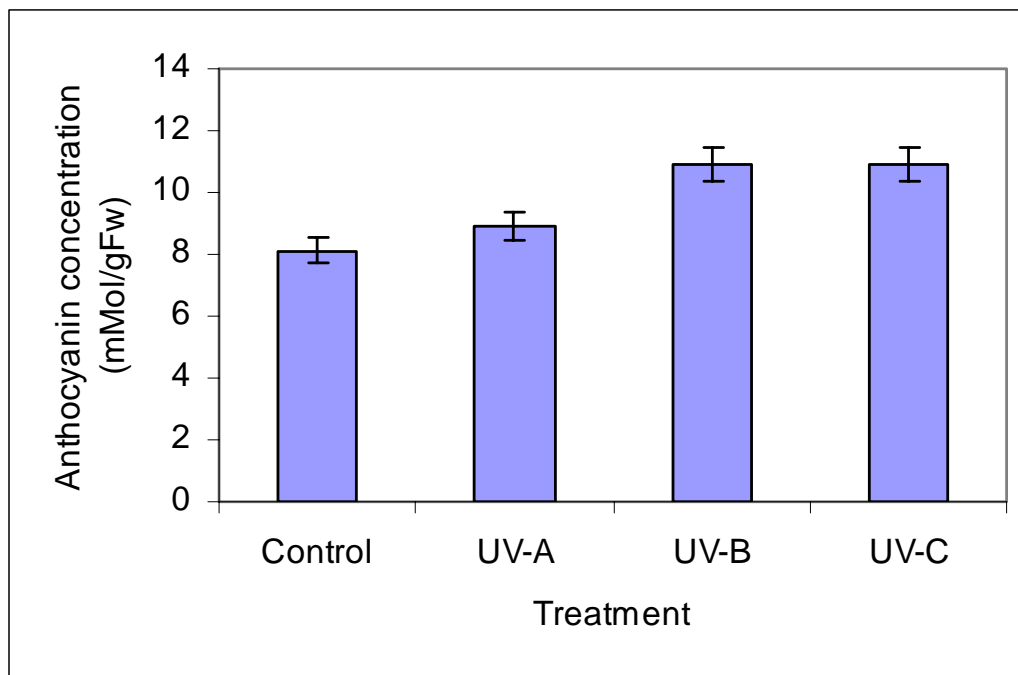


Fig. 3. Effect of UV radiation on protein content of *B.napus* which were grown 21 d under UV treatment, values are the means of three replicates, and bars indicate SEM significant difference at  $P < 0.05$  according to Duncan's test

### d) UV-absorbing compounds

One mechanism that could protect the sensitive tissue is an alteration in leaf transmittance properties. Epidermal UV-absorbing compounds are synthesized in most plant species in response to UV radiation. These compounds absorb UV radiation strongly, but do not absorb PAR. In this experiment, plants methanolic extracts grown under controlled conditions and UV-A treatment had a significantly lower absorbance of extract (presumably flavonoids) at 270, 300 and 330 nm than those of plants grown in the presence of UV-B and UV-C. (Fig. 4). Plants grown under UV-B and UV-C treatments showed a higher concentration of anthocyanins than control and UV-A treated plants (Fig. 5).

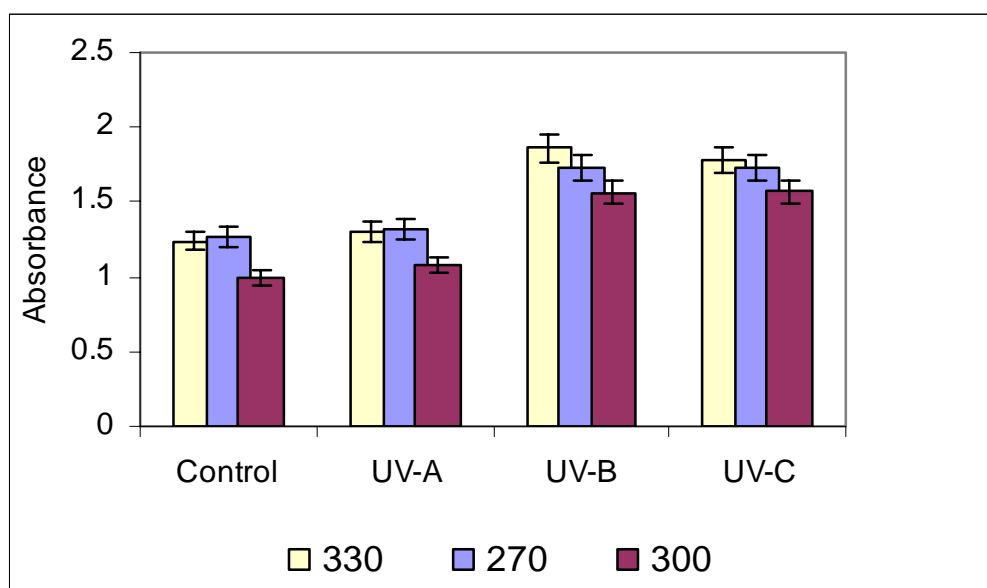


Fig. 4. Influence of UV-A, UV-B and UV-C on the absorbance of the methanolic extract of leaves at 270,300,330 nm, values are the means of three replicates, and bars indicate SEM significant difference at  $P<0.05$  according to Duncan's test

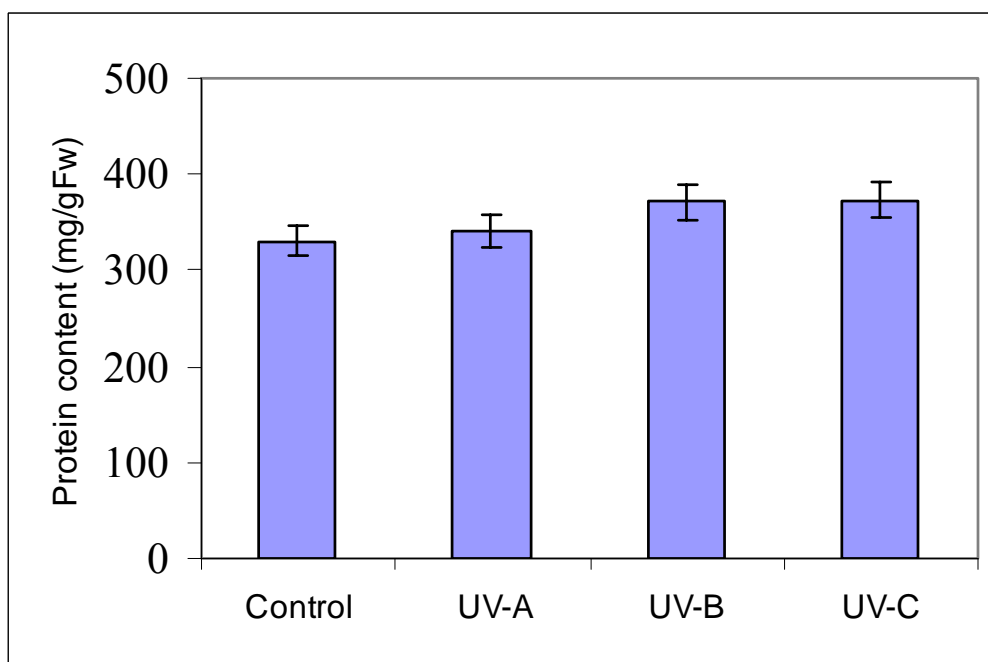


Fig. 5. Influence of UV-A, UV-B and UV on concentration of anthocyanins at 550, values are the means of three replicates, and bars indicate SEM significant difference at  $P<0.05$  according to Duncan's test

In addition, HPLC analysis at 340 nm showed a quantitative change in flavonoid content in UV-B and UV-C treatment, and confirmed the results of spectrophotometric analysis (Fig. 6).

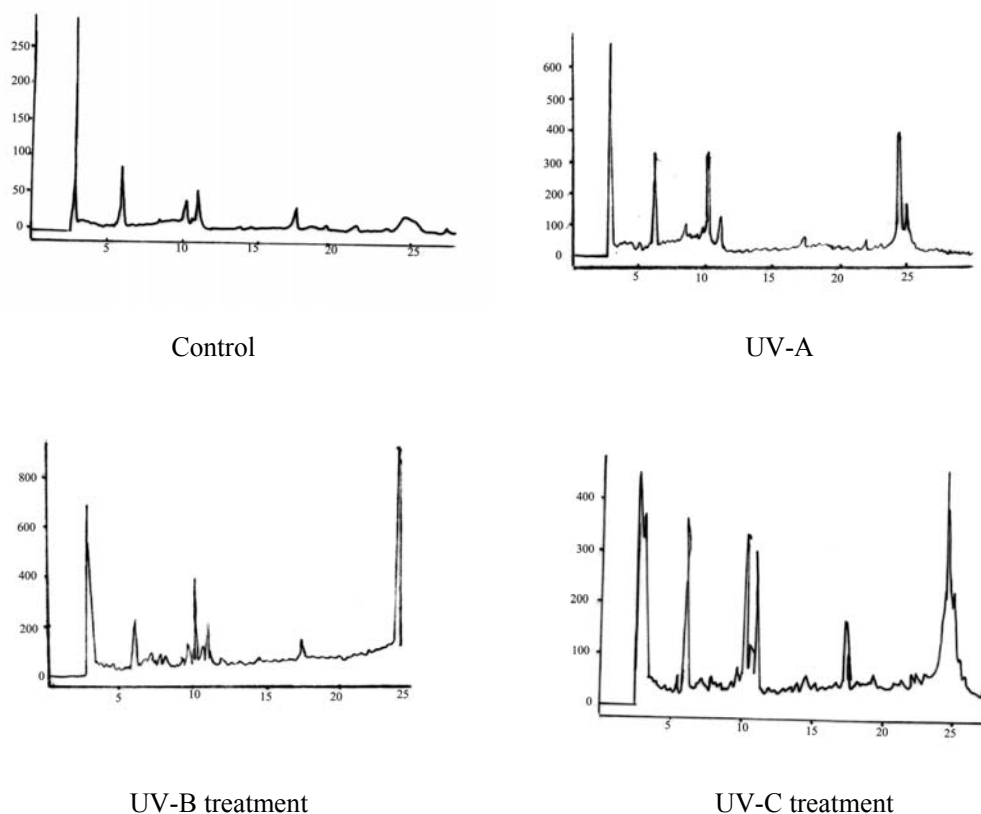


Fig. 6. Chromatograms (HPLC 340 nm) of methanolic extracts of leaves of *B. napus* grown under control, UV-A, UV-B and UV-C treatments, respectively. 14 old plants were subjected to ultraviolet rays with UV lamps

These results revealed that flavonoids and other UV-absorbing compounds are synthesized in large amounts in response to UV.

#### 4. DISCUSSION

Although the mechanism of a generation of active oxygen species in UV irradiation in plants is not known, it is clear that UV causes oxidative stress in plants [14]. Unsaturated lipids can be photochemically modified by UV absorbance. Oxidative damage can be detected by lipid peroxidation. Hydroxyl radicals and singlet oxygen can react with lipids and form lipid peroxy radicals and hydroperoxide [15, 16].

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 [16, 17]. The increase in TBARS content is more precisely an indicator of general UV-induced oxidative damage, due to the impairment of cell defense system [18, 3]. Change in TBARS (especially MDA) was the first evidence that under our experiment, UV (UV-B and UV-C) induced oxidative stress (Fig. 1).

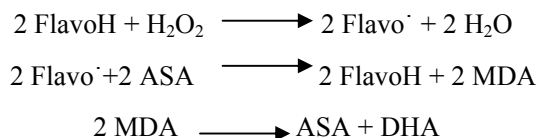
In the present study, a marked increase in ASA, DHAS and total ASA in UV-B and UV-C treatment (Fig. 2) represents adaptive responses against oxidative damage induced by UV radiation.

An increase in both ASA and DHAS that lead to an increase in total ascorbate showed that the ascorbate-glutathione cycle in this condition is active and has a key role in the detoxification of AOS. In the ascorbate-glutathione cycle, APX catalysis is the first step of the H<sub>2</sub>O<sub>2</sub> scavenging pathway by oxidizing ASA and producing MDHAS which can dismutate spontaneously to ASA and DHAS or be enzymatically reduced to ASA by GSH- dependent DHAR [19].

In addition, ASA can react similarly to SOD, dismutate superoxide anion and produce hydrogen peroxide [20]. Researchers report that the exogenous addition of ASA demonstrated significant protection against the oxidative stress, lipid peroxidation, DNA breakage, photosynthetic damage and chlorophyll bleaching [21, 5]. UV radiation causes not only modification or destruction of amino acids, but leads to the inactivation of proteins and enzymes. The reason for this is that the aromatic amino acid and disulfide groups strongly absorb UV radiation [16].

Protein degradation also occurs under conditions that induce oxidative stress. A number of reports have shown that cells exhibit increased rates of proteolysis following exposure to oxidative-inducing agents [6]. In this study we observed an increase in protein content under UV-B and UV-C treatments (Fig. 3). This increment may be related to defense proteins and enzymes which are probably synthetic during stress [22]. It was been reported in the *Arabidopsis* plant, oxidative stress leads to induction of antioxidant enzymes such as GR, PXs, CAT and SOD, and also leads to the expression of defense genes that encode HSP proteins. HSPs have a main role in detoxification of degraded proteins [23]. In addition, in wild types of *Petunia* it has been shown that UV-B induced an up regulation of genes coding key enzymes in the phenylpropanoid pathway including PAL, CHS and CHI. [24].

Phenolic compounds were accumulated in vacuoles of epidermal cells and act as optical filters, preventing penetration of this radiation to sensitive mesophyll tissue and damage to cellular apparatus, but these screening compounds have an antioxidant role in plant cells [4, 25, 26]. UV irradiation induces oxidative stress, therefore it is likely that polyphenolic pigments protect the cell from UV damage by an indirect means such as H<sub>2</sub>O<sub>2</sub> scavenging in addition to their absorption properties. In most plant cells vacuols have a basic peroxidase that may be localized in the inner surface of the tonoplast membrane. This spatial distribution enables vacuolar peroxidase to scavenging H<sub>2</sub>O<sub>2</sub> leaked out from other organel [12]. Unlike other active oxygen species, H<sub>2</sub>O<sub>2</sub> is able to diffuse across membranes due to its high stability and membrane-permeability. H<sub>2</sub>O<sub>2</sub> toxicity itself is weak compared with other reactive oxygen species, but in the presence of transitional metals such as Fe and Cu, will produce hydroxyl radicals, the most reactive oxygen. Thus scavenging of H<sub>2</sub>O<sub>2</sub> is essential to avoid oxidative damage of plant cells. When phenolic function as an antioxidant, either by an enzymatic or direct radical scavenging mechanism are univalently oxidized to phenoxy radicals, they can be reduced to their parent compounds by a non-enzymatic reaction with ascorbate [7].



MDHAS and DHAS are reduced to ASA in the ascorbic-glutathione cycle [12]. In this research our 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.

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### NOMENCLATURE

AOS	active oxygen specie
APX	ascorbate peroxidase
ASA	ascorbic acid
CAT	catalase
CHI	chalcone isomerase
CHS	chalcone synthase
DHAR	dehydroascorbate reductase
DHAS	dehydroascorbic acid
GR	glutathione reductase
GSH	reduced glutathione
HPLC	high performance liquid chromatography
HSP	heat shock protein
MDHAR	monodehydroascorbate reductase
MDHAS	monodehydroascorbate
PAL	phenyl alanin ammonia lyase
PAR	photosynthetic active radiation
SOD	superoxide dismutase
SEM	standard error of mean
TBARS	thiobarbituric acid reactive substances
UV	ultraviolet

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