Effect of UV radiation and elevated CO_2 on physiological attributes of canola (*Brassica napus* L.) grown under water stress

Efecto de la radiación UV y el CO_2 elevado sobre caracteres fisiológicos de canola (*Brassica napus* L.) cultivada bajo estrés hídrico

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Received: 07/21/2011 First reviewing ending: 02/08/2012 First review received: 02/18/2012 Accepted: 03/08/2012

ABSTRACT

Increased UV radiation on the earth's surface due to depletion of stratospheric ozone layer is one of the changes of current climate-change pattern. In addition, increase of atmospheric CO_2 concentration because of fossil fuel application increases annual average temperature in the world and affects plant growth and development. Therefore an experiment was carried out to study the effects of solar UV radiation, UV-B, UV-C radiation and elevated CO_2 on some physiological attributes of two canola cultivars (*Brassica napus* L.) under two irrigation regimes that are complete irrigation and limited irrigation in two consecutive years. Generally, elevated CO_2 increased leaf soluble carbohydrates, reducing sugars, glucosinolate and Fv/Fm ratio while carotenoids and soluble proteins decreased. In addition, UV radiation decreased leaf soluble carbohydrates, reducing sugars, chlorophyll, proline and Fv to Fm ratio and increased UV absorbing pigments, soluble proteins and glucosinolate. Leaf soluble carbohydrates, reducing sugars, chlorophyll, and Fv to Fm ratio dramatically decreased because of water deficit stress while other traits were increased due to induced water stress. There were significant differences between cultivars in terms of physiological attributes.

Key words: Canola, elevated CO_2, UV radiation, water deficit stress

RESUMEN

El aumento de la radiación UV sobre la superficie de la tierra debido al agotamiento de la capa estratosférica de ozono es uno de los cambios del patrón del cambio climático común. Además, el incremento de la concentración del CO_2 atmosférico debido a la aplicación de combustibles fósiles aumenta la temperatura media anual en el mundo y afecta el crecimiento y desarrollo de las plantas. Por lo tanto, se realizó un experimento en dos años consecutivos para estudiar los efectos de la radiación solar UV, UV-B, radiación UV-C y CO_2 elevado sobre algunos caracteres fisiológicos de dos cultivares de canola (*Brassica napus* L.) bajo dos regímenes de riego, los cuales fueron riego completo y riego limitado. En general, el CO_2 elevado incrementó los carbohidratos solubles de las hojas, los azúcares reductores, glucosinolatos y la relación Fv:Fm, mientras que los carotenoides y la proteína soluble se redujeron debido al CO_2 elevado. Además, la radiación UV disminuyó los carbohidratos solubles de las hojas, azúcares reductores, clorofila, prolina y la relación Fv:Fm e incrementó los pigmentos que absorben la radiación UV, proteínas solubles y glucosinolato. Los carbohidratos solubles de las hojas, azúcares reductores, la clorofila y la relación Fv: Fm disminuyeron drásticamente debido al estrés del déficit hídrico mientras los otros caracteres se incrementaron debido al estrés hídrico inducido. Hubo diferencias significativas entre los cultivares en términos de los caracteres fisiológicos.

Palabras clave: Canola, CO_2 elevado, radiación ultravioleta, estrés de déficit hídrico

INTRODUCTION

Depletion of stratospheric ozone can significantly increase the quantity of ultraviolet radiation reaching the earth's surface (Taalas *et al*., 2000). Elevated UV radiation causes a wide range of morphological, physiological and metabolic responses in plants. For example, increases in UV absorbing compounds such as flavonoids (Olson *et al*., 1999), anthocyanin, carotenoids and a decrease in the efficiency of photosystem II (Germ *et al*., 2005) due to chlorophyll degradation have been reported. Some of the mechanisms that could lead to this damage are damage to DNA (Bray and West, 2005). However, many plants are quite resistant to UV radiation. In contrast sensitive plants develop several repair and

adaptive mechanisms. The first and foremost adaptations are structural modifications such as thickening of cell walls, epicuticular wax formation (Day 1993), and synthesis of anthocyanin and flavonoid (Teramura 1983). One of the most important mechanisms is screening out UV radiation by accumulation of flavonoids, anthocyanins or other UV absorbing compounds in the leaf epidermis (Schmelzer *et al*., 1988).

The influence of other environmental factors such as water stress and increasing of CO_2 can also interact to alter the balance or consequences of the defence mechanisms described above. Current atmospheric levels of CO_2 may double from 340 µL L^{-1} to 680 µL L^{-1} by the middle of the 21st century (Gribbin, 1981). Simultaneous with CO_2 increasing an increase in photosynthesis and biomass can be expected in C_3 plants. In UV sensitive plants, photosynthetic capacity may be reduced directly by the effect of UV radiation on photosynthetic enzymes or disruption of PSII reaction centres, or indirectly by effects on photosynthetic pigments and stomatal function (Teramura, 1983). Both CO_2 and UV radiation are expected to increase simultaneously with future changes in global climate and drought stress is reportedly the most important limiting factor in agricultural production in the world. Thus an experiment was performed in order to study on these three environmental factors and their interaction on two canola cultivars. In this study, we investigated the effect of three environmental factors (water stress, different UV radiation and elevated CO_2) and their interaction on two canola cultivars that are Okapi and Talaye. In addition we analysed UV absorbing compounds, leaf soluble carbohydrates, reducing sugars, chlorophyll content, soluble proteins, glucosinolate, Fv/Fm and endogenous content of proline accumulated in the tissues as a results of water stress, UV radiation and CO_2 treatments.

MATERIALS AND METHODS

The experiment was conducted at (35º 59′ N latitude, 50º 75′ E longitude) in the 2008 and 2009 growing season. The experimental design was randomized complete blocks arrangement in factorial with three replicates. The first factor included two varieties of canola, while the second factor was irrigation regimes (complete irrigation and limited irrigation (60% field capacity). The third factor included two CO_2 levels (atmospheric concentration; 400 µL.L^{-1} and elevated concentration; 900 µL.L^{-1}) and the fourth factor was different levels of UV radiation (UV-A: wavelength > 320 nm or solar radiation, UV-B: 280-320 nm and UV-C: wavelength < 280 nm). In each experimental unit, an erected sheltered frame (1.5m×2.5m×2 m) covered with polyethylene plastic film to prevent CO_2 escaping was used. Disinfected canola seeds (Okapi and Talaye) were sown at a depth of 2-3 cm and irrigation was done immediately. All experimental units were irrigated at field capacity until seedling establishment after that in water stress units soil moisture was maintained at 60 percent of field capacity using Time-Domain Reflectometry (T.D.R, soil moisture, model 4593).

During water stress, UV-B and UV-C radiation were delivered on plants by UV lamps. Simultaneous with water stress and UV radiation, CO_2 concentration was increased to 900µL.L^{-1} for treated units. One CO_2 capsule was used and CO_2 concentration was elevated into covered frames. Carbon dioxide was adjusted to 900µL.L^{-1} by an electronical sensor (Testo Co. Germany). Nitrogen fertilizer (Urea) was applied in three stages; seed sowing, stem elongation and flowering. A systemic insecticide (Metasystox) was used at flowering stage of canola to protect plants against aphids.

Soluble carbohydrate

Soluble carbohydrates (glucose, xylose and mannose) were estimated according to the method of Dubois *et al* (1956). Leaf samples were homogenized in a mortar and pestle with 3 ml distilled water and homogenate was filtered by filter paper. 0.5 ml phenol (5%) and 2.5 ml sulfuric acid (98%) were added to the homogenate. After reaction, the test tubes were allowed to cool to room temperature. The amount of glucose, xylose and mannose was determined from the absorbance at 480, 485 and 490 nm, respectively. The sugar concentration was calculated from a glucose, xylose and mannose standard curve.

Reducing sugars

Reducing sugars were measured by dinitrosalicylic acid according to the method of Miller (1959). Sucrose was determined after incubation of 0.5 ml of the extract with acetate buffer (pH 4.5) containing 0.05 % invertase. The sucrose level was related to the difference in optical density values between the reactions with and without invertase. The

supernatant that remained after ethanol extractions was analysed for starch according to Dinar *et al*. (1983).

Chlorophyll and carotenoid assay

Chlorophyll was extracted in 80 % acetone from the leaf samples, according to the method of Arnon (1949). Extracts were filtrated and then absorbances of chlorophyll a, b and carotenoids were determined by spectrophotometer (UV-S, Sinco 2100) at 645, 663 and 470 nm. The content of chlorophyll was expressed as mg g^{-1} .FW.

Flavonoids assay

Flavonoids were estimated according to the method of Krizek *et al*., (1993). Leaf samples were homogenized in a mortar and pestle with 3 ml 1% acetic acid-ethanol solvent (1:99 v: v). The homogenate was centrifuged at 18000 g for 30 min, and then the supernatant was incubated in a water bath for 10 min at 80°C and then allowed to cool to room temperature. The amount of flavonoids was determined from the absorbance at 270, 300 and 330 nm. The content of flavonoids were determined using the extinction coefficient of flavonoids (ε=33000 mol^{-2} cm^{-1}). Flavonoid content was expressed as µmol cm^{-1}.

Anthocyanin assay

Anthocyanin content was estimated according to the method of Krizek *et al*. (1993). Leaf samples were homogenized in a mortar and pestle with 3 ml 1% HCl-methanol solvent (1: 99 v:v). The homogenate was centrifuged at 18000 g for 30 min at 4°C, and then the supernatant was filtered through Whatman #1 to remove particulate matter and stored in darkness at 5°C for 24 h. The amount of anthocyanin was determined from the absorbance at 550 nm. The content of anthocyanin was determined using the extinction coefficient of anthocyanin (ε=33000 mol^{-2} cm^{-1}). Anthocyanin content was expressed as µmol cm^{-1}.

Proline assay

Proline content of leaves was determined according to a modification of the method of Bates *et al*. (1973). Samples of leaves (0.5 g) were homogenized in a mortar and pestle with 10 ml sulphosalicylic acid (3% w/v), and then centrifuged at 18 000 g for 15 min. Two millilitres of the supernatant was then added to a test tube, to which 2 ml glacial acetic acid and 2 ml freshly prepared acid ninhydrin solution (1.25 g ninhydrin dissolved in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) were added. The test tubes were incubated in a water bath for 1 h at 100°C and then allowed to cool to room temperature. Four millilitres of toluene were then added to the tubes and then mixed on a vortex mixer for 20 s. The test tubes were allowed to stand for at least 10 min, to allow separation of the toluene and aqueous phases. The toluene phase was carefully pipetted out into a glass test tube and its absorbance was measured at 520 nm in a spectrophotometer. The content of proline was calculated from a standard curve.

Soluble proteins

The protein content of the crude extract was determined using bovine serum albumin (BSA) as a standard, according to the method of Bradford (1976). One millilitre of Bradford solution was added to 100 µl crude extract and absorbance recorded at 595 nm for estimation of total protein content. The protein concentration was calculated from a BSA standard curve.

Glucosinolate assay

Glucosinolate content was measured according to the method of Embaby *et al*. (2010). Two hundred mg of canola meal were transferred to a test tube and heated in a water-bath at 75° C for 1 min. Two millilitres of boiling methanol solution (70% v/v) were added and 200 µl of 20 mmol/internal standard solution of sinigrin were added immediately. The heating at 75° C was continued for a further 10 min, shaking the tube at regular intervals. The tube was centrifuged at 3000g for 3 min and the supernatant was transferred to another tube. Two millilitres of boiling methanol solution were added to the tube containing the solid residue and the tube was reheated for 10 min, and then centrifuged for 3 min, as described above. The supernatant was added to the tube containing the first supernatant and the volume of the combined extracts was adjusted to 5 ml with water.

Pasteur pipettes were placed vertically on a stand and a glass wool plug placed in the neck of each pipette. Half a ml of suspension of ion exchange resin was transferred to each pipette. The pipettes were

rinsed with 2 ml of the imidazole formate solution (6 mol) followed with 1 ml portion of water. One millilitre of the glucosinolate extract was transferred to a prepared column and two 1 ml portions of sodium acetate buffer were added. The buffer was drained after each addition. Diluted purified sulfatase solution was added to the column (75 µl) and left to act overnight at ambient temperature. The second day, the desulfoglucosinolate was eluted with two 1 ml portions of water and collected in a tube placed under the column. Then the sample was ready for HPLC analysis.

The different glucosinolates in canola meal were determined by using High Performance Liquid Chromatography (HPLC). The desulfoglucosinolates were separated using a type C18 column with a flow rate of 0.5 ml/min at 30° C. Elution of desulfoglucosinolates from HPLC was performed by a gradient system of water (A) and acetonitrile/water (25:75, v/v, B). The total running time was 45 min with a gradient as follows: 100% A and 0% B for 5 min, then in 35 min to 0% A and 100% B and in 5 min back to 100%A and 0%B. An UV detector was used at a wavelength of 229 nm. Individual glucosinolates were identified in comparison with the retention time of siningrin standard. Quantification of individual glucosinolates was accomplished using the response factors as published in the ISO protocol (ISO Method, 1992).Total and individual glucosinolates are expressed as µmol g^{-1}.

Maximum photochemical efficiency

Maximum photochemical efficiency was determined by a portable fluorometer (PAM-2000, H WalsGmbH, Effeltrich, Germany). Before measurement, the leaves were dark adapted for 30 min. The maximum photochemical efficiency of PSII was determined from the ratio of variable (Fv) to maximum (Fm) fluorescence.

All data were analysed using SAS software and Duncan's Multiple Range Tests was used to measure statistical differences between treatments.

RESULTS AND DISCUSSION

Leaf soluble carbohydrates

Water deficit stress, carbon dioxide and UV radiation had significant effects on soluble carbohydrates in canola leaves, and these results were similar in both years of experiment (Table 1). Also we observed that canola cultivars differed in terms of leaf soluble carbohydrates in that leaf soluble carbohydrate in Talaye was more than Okapi. In addition, water deficit stress and UV radiation significantly decreased leaf soluble carbohydrates. In contrast elevated CO_2 increased leaf soluble carbohydrates (Table 2). Interaction between cultivar and other treatments; including water deficit stress, elevated CO_2 and UV radiation showed that, Talaye cultivar had the highest soluble carbohydrate in comparison to Okapi cultivar (Table 3).

The results showed that under conditions of complete irrigation or limited irrigation increasing CO_2 can increase soluble carbohydrate in leaves. Furthermore, regardless of presence of water deficit stress or elevated CO_2, UV radiation dramatically decreased leaf soluble carbohydrates (Table 3). Three way interactions on leaf soluble carbohydrates are shown in Table 4. The highest leaf soluble carbohydrates were observed in Talaye cultivars when these plants were grown under condition of complete irrigation and elevated CO_2 under natural sunlight (Table 5). UV-C radiation and water deficit stress significantly decreased leaf soluble carbohydrates in Okapi cultivars under condition of ambient CO_2 as this cultivar had the lowest leaf soluble carbohydrates. It is reported that thylakoid membranes can be damaged by oxygen free radicals induced by UV stress and then thylakoid membrane integrity would be decreased and thus photosynthetic process and energy production would be decreased (Mazza *et al*., 2000).

Additionally, several studies on the effects of UV radiation on plant carbohydrates have been carried out, some indicating increases in response to UV-B (Hilal *et al*., 2004) and others indicating decreases (Correia *et al*., 2005). This may be due to diversity of plant tissue or experimental conditions. In the present work, significant effects of UV radiation on total soluble carbohydrates was observed for both UV-B and UV-C radiation. Such increases have been reported in UV-B irradiated leaves of pea and corn (Santos *et al*., 1993; He *et al*., 1994).

Reducing sugars

Reducing sugar content was significantly affected by water deficit stress, elevated CO_2 and UV radiation. Although water stress and UV radiation decreased reducing sugars, elevated CO_2 increased

them (Table 2). The results showed that, Talaye cultivars had more sugar content than Okapi cultivar and these results were similar in both years of study. Alternatively, reducing sugars might increase during water stress, if sugar formation is a response to either osmotic regulation or respiration needs. Reducing sugars might increase after water stress due to failure in starch deposition (Hodgson *et al.*,, 1973) or the conversion of starch to sugars (Isherwood, 1973). Experimental evidence to support alternative hypotheses is sketchy. Total reducing sugar content was generally decreased by UV radiation. Decline in reducing sugar content due to UV radiation could be due to the damage caused to chloroplasts and photosynthetic systems. High levels of UV-B radiation have reportedly caused down-regulation of photosynthetic genes, leading to reduced levels of glucose in common bean leaves (Mackerness *et al*., 1997).

Chlorophyll

Significant effects of treatments and the changes in total chlorophyll content due to different treatments are shown in Tables 1, 2, 3, 4 and 5.

Significant depressing effects of water stress and UV radiation on total chlorophyll content, compared to the control treatments, indicate adverse effects of these abiotic stresses on the plants. It is worth mentioning that, no significant difference in total chlorophyll content was observed between ambient CO_2 and elevated CO_2 concentration. A similar result was found when two canola cultivars were compared in terms of chlorophyll content.

Exposure of canola plants to increasing UV-B and UV-C intensity reduced the content of chlorophyll. The lowest chlorophyll content was obtained from Okapi plants grown under ambient CO_2

Table 1: Analysis of variance on some physiological attributes of two canola cultivars affected by water stress, carbon dioxide and UV radiation.

S.O.V	df	LSC	RS	Chlor	Carot	Flav	Anthocy	Proline	SP	Gluc	Fv/Fm
Year	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
R (Year)	4	*	ns	*	ns	ns	ns	ns	ns	ns	ns
V	1	**	**	ns	**	ns	ns	**	**	ns	ns
W	1	**	**	**	**	**	**	**	**	**	**
C	1	**	**	ns	**	ns	ns	ns	**	**	**
U	2	**	**	**	**	**	**	**	**	**	**
V*W	1	ns	*	ns	ns	ns	*	ns	**	ns	**
V*C	1	ns	ns	**	ns	ns	*	ns	ns	**	ns
V*U	2	**	ns	ns	**	**	ns	ns	ns	ns	**
W*C	1	**	**	**	**	*	ns	ns	ns	**	ns
W*U	2	ns	**	**	**	**	**	ns	**	**	**
C*U	2	**	ns	ns	**	**	**	ns	**	**	**
VWC	1	ns	ns	**	**	*	ns	*	ns	**	*
VWU	2	**	**	ns	**	**	**	ns	ns	*	**
WCU	2	**	**	ns	**	ns	ns	**	**	**	**
VCU	2	**	**	ns	**	ns	ns	ns	ns	ns	ns
VWCU	2	**	ns	**	**	**	**	ns	ns	ns	**
Year (V)	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Year (W)	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Year (C)	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Year (U)	2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
C.V		5.97	8.09	8.05	4.94	10.19	23.54	17.92	10.80	6.41	4.66

S. O. V.: Source of variation; df: Degree of freedom; R: replication; V: variety; W: water stress; C: carbon dioxide; U: UV radiation; LSC: Leaf soluble carbohydrates ($mg.g^{-1}$ FW); RS: Reducing sugars ($mg.g^{-1}$ FW); Chlor: Chlorophyll ($mg.g^{-1}$ FW); Carot: Carotenoids ($mM.cm^{-1}$); Flav: Flavonoids ($mM.cm^{-1}$); Anthocy: Anthocyanin ($mM.cm^{-1}$); Proline (mg.g-1 FW; SP: Soluble proteins ($mg.g^{-1}$ FW); Gluc: Glucosinolate (as μmol g^{-1}) y ratio Fv/Fm.

*, ** significant at the 0.05 and 0.01 probability levels, respectively and, ns not significant.

concentration and subjected to water deficit stress and UV-C radiation (Table 5).

Chlorophylls play a central part in the energy capturing system of plants and so any significant alteration in their concentrations is likely to cause a marked effect on the plants' life (Shweta and Agrawal, 2006).

Damage to pigments and plastids, as well as decreased chlorophyll content because of water stress has been reported (Castrillo and Turujillo, 1994). The researchers also found that water stress also increases the speed of chlorophyll severance (Schutz and Fangmeier, 2001).

Reduction in chlorophyll contents by excess UV-B radiation has been reported in sessile oak (*Quercus petraea* L.) (Mészáros *et al*., 2001). A diminished chlorophyll concentration is a more common symptom of UV radiation stress. This can be attributed to inhibition of biosynthesis of pigments under UV exposure (Musil *et al*, 2002). Mackerness *et al*. (1999) suggested that under UV-B stress plants sacrifice their chloroplasts in order to protect the rest of the cell.

UV absorbing pigments

Carotenoids, flavonoids and anthocyanin concentration showed an increasing trend with decreasing of UV wavelength and water deficit stress. Elevated CO_2 had no significant effect on UV absorbing pigments except a little decline in carotenoid content. Also there was no significant difference between canola cultivars and result were similar in both years of experiment (Table 2, 3, 4 and 5).

Increase of UV absorbing pigments due to UV radiation points to the photo-protection role of these pigments in photosynthetic systems by dissipating excess excitation energy through the xanthophylls cycle (Demming Adams and Adams, 1992).

Accumulation of UV absorbing pigments such as carotenoids, flavonoids and anthocyanins is one of the ways by which plants alleviate the harmful effects of UV stress. Increase in flavonoid content is in support of the results obtained by Shweta and Agrawal (2006) in spinach (*Spinacia oleracea* L.), by Hilal *et al*. (2004) in quinoa (*Chenopodium quinoa* Willd.) and by Rathore *et al*. (2003) in wheat

Table 2: Main effects year, variety, water stress, carbon dioxide and UV radiation on some physiological attributes.

Treatments	Levels	LSC	RS	Chlor	Carot	Flav	Anthocy	Proline	SP	Gluc	Fv/Fm
Year	First	18.80a	22.50a	2.38a	0.80a	0.81a	0.64a	0.06a	0.63a	20.22a	0.41a
	Second	18.43a	22.13a	2.42a	0.76a	0.83a	0.67a	0.07a	0.61a	20.19a	0.39a
Variety	Okapi	17.51b	20.76b	2.39a	0.74b	0.82a	0.64a	0.06a	0.65a	20.39a	0.41a
	Talaye	20.10a	24.25a	2.38a	0.78a	0.80a	0.64a	0.05b	0.61b	19.99a	0.41a
Water stress	Complete	24.64a	28.50a	3.34a	0.67b	0.74b	0.53b	0.04b	0.46b	18.77b	0.45a
	Limited	12.97b	16.51b	1.43b	0.85a	0.88a	0.75a	0.07a	0.80a	21.61a	0.36b
Carbon dioxide	400 ppm	17.62b	21.30b	2.36a	0.77a	0.80a	0.62a	0.05a	0.69a	18.93b	0.39b
	900 ppm	19.98a	23.70a	2.41a	0.75b	0.82a	0.66a	0.06a	0.57b	21.45a	0.42a
UV radiation	A	21.15a	25.01a	2.55a	0.63c	0.57c	0.24c	0.04c	0.44c	14.52c	0.49a
	B	18.28b	22.37b	2.40b	0.80b	0.89b	0.72b	0.06b	0.67b	21.39b	0.43b
	C	16.98c	20.12c	2.20c	0.85a	0.97a	0.96a	0.07a	0.77a	24.66a	0.30c

LSC: Leaf soluble carbohydrates ($mg.g^{-1}$ FW); RS: Reducing sugars ($mg.g^{-1}$ FW); Chlor: Chlorophyll ($mg.g^{-1}$ FW); Carot: Carotenoids ($mM.cm^{-1}$); Flav: Flavonoids ($mM.cm^{-1}$); Anthocy: Anthocyanin ($mM.cm^{-1}$); Proline (mg.g-1 FW); SP: Soluble proteins ($mg.g^{-1}$ FW); Gluc: Glucosinolate (as $\mu mol\ g^{-1}$) y ratio Fv/Fm

Means within a column (levels) at the same treatment with similar letters are not significant at the 5% probability level according to Duncan's Multiple Range Tests.

(*Triticum aestivum* L.). In this study, UV absorbing pigment concentrations were significantly increased in leaves of canola plants exposed to UV-C radiation. Although water deficit stress had additive effects on these pigments the effect of UV radiation, especially UV-C radiation, was more noticeable.

Proline

Water deficit stress and UV radiation stress significantly increased proline content in leaves of both canola cultivars while elevated CO_2 had no significant effect. Accumulation of proline due to water deficit stress as a water status regulator amino

Table 3: Two way interaction between treatments on some physiological attributes.

Variety	WS	LSC	RS	Chlor	Carot	Flav	Anthocy	Proline	SP	Gluc	Fv/Fm
Okapi	Complete	23.33b	26.41b	3.36a	0.64d	0.75b	0.50b	0.05c	0.46c	18.98a	0.44b
	Limited	11.69d	15.10d	1.42b	0.84b	0.89a	0.78a	0.07a	0.84a	21.80a	0.37c
Talaye	Complete	25.94a	30.58a	3.33a	0.69c	0.73b	0.55b	0.04d	0.46c	18.56b	0.46a
	limited	14.26c	17.91c	1.44b	0.87a	0.87a	0.72a	0.06b	0.76b	21.42a	0.36d
Variety	CD										
Okapi	400 ppm	16.16c	19.54d	2.31b	0.75b	0.82a	0.65ab	0.06a	0.72a	19.65c	0.39b
	900 ppm	18.86b	21.97c	2.46a	0.72c	0.82a	0.64ab	0.06a	0.58c	21.14b	0.42a
Talaye	400 ppm	19.09b	23.06b	2.40ab	0.79a	0.78b	0.59b	0.05b	0.66b	18.21d	0.39b
	900 ppm	21.11a	25.43a	2.36b	0.78a	0.83a	0.68a	0.05b	0.56c	21.77a	0.42a
Variety	UV radiation										
Okapi	UV-A	19.22b	22.88c	2.52ab	0.59e	0.62c	0.28c	0.05c	0.48c	14.88c	0.50a
	UV-B	17.37d	20.59d	2.44bc	0.79c	0.89b	0.73b	0.06b	0.69b	21.30b	0.42c
	UV-C	15.93e	18.80e	2.20d	0.84b	0.96a	0.93a	0.07a	0.78a	25.00a	0.31d
Talaye	UV-A	23.09a	27.13a	2.59a	0.67d	0.53d	0.21c	0.04d	0.41d	14.16c	0.49a
	UV-B	19.18b	24.15b	2.35c	0.81c	0.89b	0.71b	0.05c	0.65b	21.48b	0.44b
	UV-C	18.03c	21.45d	2.21d	0.86a	0.98a	0.98a	0.06b	0.76a	24.33a	0.30d
WS	CD										
Complete	400 ppm	23.19b	26.84b	3.39a	0.65d	0.71c	0.48c	0.04b	0.51c	16.14b	0.44b
	900 ppm	26.09a	30.15a	3.30a	0.68c	0.77b	0.57b	0.04b	0.41d	21.41a	0.46a
Limited	400 ppm	12.06d	15.76d	1.33c	0.88a	0.89a	0.76a	0.07a	0.86a	21.72a	0.35d
	900 ppm	13.88c	17.25c	1.53b	0.82b	0.87a	0.75a	0.07a	0.73b	21.50a	0.38c
WS	UV radiation										
Complete	UV-A	26.97a	30.63a	3.41a	0.50d	0.45d	0.11d	0.03e	0.28d	12.97d	0.56a
	UV-B	24.16b	29.37b	3.43a	0.74c	0.87b	0.69b	0.04d	0.47c	18.65b	0.46b
	UV-C	22.78c	25.50c	3.19b	0.75c	0.91b	0.78b	0.06c	0.63b	24.70a	0.33e
Limited	UV-A	15.33d	19.39d	1.70c	0.75c	0.70c	0.38c	0.06c	0.61b	16.07c	0.43c
	UV-B	12.40e	15.38e	1.37d	0.86b	0.90b	0.75b	0.07b	0.87a	24.13a	0.39d
	UV-C	11.18f	14.75e	1.21e	0.95a	1.04a	1.13a	0.08a	0.91a	24.63a	0.27f
CD	UV radiation										
400 ppm	UV-A	20.31b	23.85b	2.51ab	0.59e	0.57d	0.25d	0.04c	0.48e	13.91e	0.49b
	UV-B	17.57d	21.57c	2.37c	0.82b	0.90bc	0.76bc	0.06b	0.73b	19.08c	0.40d
	UV-C	14.99e	18.48d	2.19d	0.89a	0.93b	0.85b	0.07a	0.85a	23.81b	0.29f
900 ppm	UV-A	22.00a	26.17a	2.60a	0.66d	0.58d	0.24d	0.04c	0.41f	15.14d	0.50a
	UV-B	18.98c	23.18b	2.43bc	0.78c	0.87c	0.68c	0.06b	0.60d	23.70b	0.45c
	UV-C	18.97c	21.77c	2.21d	0.81b	1.01a	1.07a	0.07a	0.69c	25.52a	0.31e

LSC: Leaf soluble carbohydrates (mg.g^{-1} FW); RS: Reducing sugars (mg.g^{-1} FW); Chlor: Chlorophyll (mg.g^{-1} FW); Carot: Carotenoids (mM.cm^{-1}); Flav: Flavonoids (mM.cm^{-1}); Anthocy: Anthocyanin (mM.cm^{-1}); Proline (mg.g-1 FW); SP: Soluble proteins (mg.g^{-1} FW); Gluc: Glucosinolate (as μmol g^{-1}) y ratio Fv/Fm

Means within a column (two factor interaction) with similar letters are not significant at the 5% probability level according to Duncan's Multiple Range Tests. Water stress (WS) and carbon dioxide (CD).

Table 4: Three way interaction between treatments on some physiological attributes.

V	or	WS	or	CD or R	LSC	RS	Chlor	Carot	Flav	Anthocy	Proline	SP	Gluc	Fv/Fm
Okapi		Complete		400 ppm	21.59c	24.74c	3.42a	0.64ef	0.75b	0.51c	0.05d	0.51d	16.38e	0.43c
				900 ppm	25.07b	28.08b	3.29a	0.63f	0.75b	0.50c	0.05d	0.41e	21.59bc	0.46b
		Limited		400 ppm	10.73g	14.34g	1.20d	0.86b	0.90a	0.79a	0.07b	0.92a	22.92a	0.36e
				900 ppm	12.64f	15.86f	1.63b	0.81c	0.89a	0.78a	0.08a	0.75c	20.69d	0.38d
Talaye		Complete		400 ppm	24.78b	28.94b	3.35a	0.66e	0.68c	0.46c	0.03e	0.51d	15.91e	0.45b
				900 ppm	27.10a	32.23a	3.30a	0.73d	0.79b	0.64b	0.04e	0.40e	21.22cd	0.47a
		Limited		400 ppm	13.40e	17.18e	1.45c	0.91a	0.88a	0.72ab	0.06c	0.80b	20.52d	0.34f
				900 ppm	15.12d	18.64d	1.42c	0.82c	0.86a	0.73ab	0.06c	0.71c	22.31ab	0.38d
Okapi		Complete		UV-A	25.00b	28.78b	3.37ab	0.41g	0.52e	0.16e	0.03de	0.29f	13.80d	0.56a
				UV-B	22.72cd	26.71c	3.48a	0.74e	0.85c	0.63c	0.05c	0.47e	18.37b	0.44c
				UV-C	22.26d	23.74d	3.22bc	0.77e	0.89bc	0.72bc	0.07b	0.63c	24.79a	0.33e
		Limited		UV-A	13.44f	16.98f	1.67d	0.76e	0.71d	0.40d	0.06b	0.68c	15.97c	0.44c
				UV-B	12.03g	14.47gh	1.40e	0.84d	0.93b	0.83b	0.08a	0.90a	24.22a	0.39d
				UV-C	9.59h	13.85h	1.18f	0.91b	1.04a	1.13a	0.08a	0.93a	25.22a	0.28f
Talaye		Complete		UV-A	28.94a	32.47a	3.45a	0.59f	0.38f	0.06e	0.02e	0.27f	12.15e	0.56a
				UV-B	25.59b	32.02a	3.37ab	0.74e	0.90bc	0.75bc	0.04d	0.46e	18.93b	0.49b
				UV-C	23.29c	27.26c	3.16c	0.74e	0.93b	0.84b	0.05c	0.64c	24.62a	0.33e
		Limited		UV-A	17.23e	21.79e	1.73d	0.74e	0.69d	0.36d	0.05c	0.54d	16.18c	0.43c
				UV-B	12.77fg	16.29f	1.34ef	0.87c	0.88bc	0.68c	0.06b	0.84b	24.04a	0.38d
				UV-C	12.77fg	15.65fg	1.25ef	0.99a	1.04a	1.13a	0.08a	0.89ab	24.03a	0.27g
Complete		400 ppm		UV-A	25.05b	28.30c	3.48a	0.42g	0.41g	0.10g	0.04e	0.29g	11.61g	0.55b
				UV-B	23.44c	27.72cd	3.45a	0.75de	0.89d	0.72de	0.04de	0.57e	13.12f	0.44d
				UV-C	21.06d	24.50e	3.23bc	0.79d	0.85d	0.62e	0.05d	0.68d	23.69c	0.33g
		900 ppm		UV-A	28.89a	32.95a	3.34ab	0.59f	0.48f	0.12g	0.02f	0.27g	14.33e	0.57a
				UV-B	24.87b	31.01b	3.40a	0.73e	0.86d	0.66e	0.04de	0.36f	24.17bc	0.49c
				UV-C	24.49b	26.50d	3.15c	0.72e	0.97bc	0.94c	0.06c	0.59e	25.71a	0.33g
Limited		400 ppm		UV-A	15.56e	19.40f	1.54e	0.77d	0.73e	0.40f	0.05d	0.67d	16.21d	0.43d
				UV-B	11.71g	15.42h	1.29fg	0.89b	0.92cd	0.80d	0.07bc	0.90b	25.04ab	0.36f
				UV-C	8.92h	12.47i	1.15g	1.00a	1.02ab	1.07b	0.08a	1.01a	23.92c	0.26i
		900 ppm		UV-A	15.11e	19.38f	1.86d	0.73e	0.68e	0.35f	0.06c	0.55e	15.94d	0.44d
				UV-B	13.09f	15.34h	1.45ef	0.83c	0.89d	0.70de	0.07bc	0.84c	23.22c	0.41e
				UV-C	13.44f	17.03g	1.28g	0.89b	1.06a	1.20a	0.08ab	0.80c	25.33a	0.29h
Okapi		400 ppm		UV-A	18.65de	22.38de	2.45ac	0.57i	0.61e	0.27f	0.05de	0.52e	14.73e	0.49b
				UV-B	16.08f	19.74g	2.39bc	0.81cde	0.92bcd	0.81cd	0.06c	0.76b	19.55d	0.40e
				UV-C	13.75g	16.50h	2.09d	0.88b	0.94bc	0.87bc	0.07ab	0.86a	24.67ab	0.30gh
		900 ppm		UV-A	19.79c	23.39cd	2.59a	0.60h	0.62e	0.28f	0.05ef	0.44f	15.04e	0.51a
				UV-B	18.67de	21.44ef	2.49ab	0.77f	0.86d	0.65e	0.07bc	0.61d	23.04c	0.43d
				UV-C	18.11e	21.09efg	2.31c	0.80def	0.98ab	0.99b	0.08a	0.69c	25.33ab	0.32f
Talaye		400 ppm		UV-A	21.96b	25.32b	2.56a	0.61h	0.53f	0.23f	0.03g	0.43fg	13.09f	0.49b
				UV-B	19.07cde	23.39cd	2.35bc	0.83c	0.89cd	0.71de	0.05de	0.71c	18.61d	0.40e
				UV-C	16.24f	20.46fg	2.29c	0.91a	0.92bcd	0.82cd	0.06cd	0.83a	22.94c	0.29h
		900 ppm		UV-A	24.21a	28.95a	2.61a	0.72g	0.54f	0.19f	0.04gf	0.38g	15.23e	0.50ab
				UV-B	19.29cd	24.92bc	2.36bc	0.78ef	0.89cd	0.71de	0.05ef	0.60d	24.36b	0.47c
				UV-C	19.83c	22.44de	2.12d	0.82cd	1.04a	1.15a	0.06bc	0.70c	25.71a	0.31fg

LSC: Leaf soluble carbohydrates (mg.g^{-1} FW); RS: Reducing sugars (mg.g^{-1} FW); Chlor: Chlorophyll (mg.g^{-1} FW); Carot: Carotenoids (mM.cm^{-1}); Flav: Flavonoids (mM.cm^{-1}); Anthocy: Anthocyanin (mM.cm^{-1}); Proline (mg.g-1 FW); SP: Soluble proteins (mg.g^{-1} FW); Gluc: Glucosinolate (as µmol g^{-1}) y ratio Fv/Fm

Means within a column (three factor interaction) with similar letters are not significant at the 5% probability level according to Duncan's Multiple Range Tests. Variety (V), water stress (WS), carbon dioxide (CD) and UV radiation (R).

acid has been known previously (Moradshahi *et al*., 2004; Din *et al*, 2011). In this study, proline content was increased too. According to Saradhi *et al*. (1995), free proline might have the capacity to scavenge and/or reduce the production of free radicals and could be an essential tool in UV protection as well as the relative contribution of other mechanisms to the overall tolerance of plants to UV radiation. Thus we concluded that, proline accumulation in plants subjected to UV radiation may be attributed to regulator effect of proline in cell water status.

Soluble proteins

The results showed that soluble proteins were increased due to water deficit stress and UV radiation while increase of CO_2 decreased soluble proteins in canola leaf tissues. Also Okapi cultivar had high level of proteins compared to Talaye cultivar (Table 2).

It seems that, water stress or UV radiation leads to protein breaking down and soluble protein content would be increased in plant tissues.

Glucosinolate

Glucosinolate content increased under conditions of water deficit stress, elevated CO_2 and UV radiation. There was no significant difference between cultivars on glucosinolate content. Enhancement of glucosinolate content was parallel with decrease of UV wavelength so that in those plants which were subjected to UV-C radiation glucosinolate content was at maximum amount (Table 2). Interaction among different treatments showed that the highest glucosinolate content was observed in those plants which received UV radiation and high CO_2 concentration and a water deficit stress. There are few studies about glucosinolate accumulation in response to water stress, although the previous studies

Table 5: Four way interaction between treatments on some physiological attributes.

V	WS	CD	R	LSC	Chlor	Carot	Flav	Anthocy	Fv/Fm
Okapi	Complete	400ppm	A	23.43e	3.51a	0.37i	0.53i	0.17gh	0.54cd
			B	20.72f	3.49a	0.75g	0.86de	0.67cd	0.42g
			C	20.63f	3.27abcd	0.81ef	0.87cd	0.69cd	0.32j
		900ppm	A	26.58b	3.23bcd	0.46h	0.51i	0.14gh	0.58a
			B	24.73de	3.47ab	0.73g	0.84de	0.60cde	0.45e
			C	23.90de	3.17cd	0.72g	0.90cd	0.76cd	0.34ij
	Limited	400ppm	A	13.88hi	1.39h	0.77fg	0.70gh	0.37f	0.45ef
			B	11.44lm	1.30hi	0.87cd	0.98abc	0.96b	0.37h
			C	6.87n	0.91j	0.94b	1.02ab	1.06ab	0.27k
		900ppm	A	13.00ijk	1.94e	0.75g	0.73fgh	0.43ef	0.44efg
			B	12.61ijkl	1.51gh	0.82ef	0.88cd	0.70cd	0.42g
			C	12.32jklm	1.45h	0.87cd	1.06a	1.21a	0.29k
Talaye	Complete	400ppm	A	26.68b	3.45ab	0.46h	0.30j	0.03h	0.56bc
			B	26.17bc	3.41abc	0.76g	0.92bcd	0.78c	0.45e
			C	21.50f	3.20cd	0.76g	0.82def	0.56de	0.34ij
		900ppm	A	31.21a	3.45ab	0.73g	0.46i	0.10gh	0.56ab
			B	25.01cd	3.34abcd	0.73g	0.89cd	0.72cd	0.52d
			C	25.09cd	3.13d	0.73g	1.03a	1.11ab	0.33j
	Limited	400ppm	A	17.24g	1.68fg	0.77fg	0.76efg	0.44ef	0.42g
			B	11.97klm	1.29hi	0.91bc	0.86d	0.650cd	0.36hi
			C	10.97m	1.39h	1.06a	1.02a	1.08ab	0.24l
		900ppm	A	17.22g	1.78f	0.72g	0.63h	0.28fg	0.44efg
			B	13.57hij	1.39h	0.84de	0.89cd	0.71cd	0.41g
			C	14.57h	1.11ij	0.91bc	1.06a	1.19a	0.29k

LSC: Leaf soluble carbohydrates (mg.g^{-1} FW); RS: Reducing sugars (mg.g^{-1} FW); Chlor: Chlorophyll (mg.g^{-1} FW); Carot: Carotenoids (mM.cm^{-1}); Flav: Flavonoids (mM.cm^{-1}); Anthocy: Anthocyanin (mM.cm^{-1}); Proline (mg.g-1 FW); SP: Soluble proteins (mg.g^{-1} FW); Gluc: Glucosinolate (as µmol g^{-1}) y ratio Fv/Fm

Means within a column (four factor interaction) with similar letters are not significant at the 5% probability level according to Duncan's Multiple Range Tests. Variety (V), water stress (WS), carbon dioxide (CD) and UV radiation (R).

indicate that environmental factors such as light (Engelen-Eigles *et al*., 2006), temperature (Velasco *et al*., 2007) and heavy metals (Tolra *et al*., 2006) alter the glucosinolate content. Increase of glycerinate in response to water deficit stress may be a strategy to increase plant resistance to water stress. In addition, it has been suggested that high concentrations of organic solutes in the cytoplasm, including proline, sucrose, glycine betaine and secondary metabolites, such as glucosinolates, contribute to the osmotic balance (López- Berenguer *et al*., 2009). Glucosinolates may have a potential role in osmotic adjustment and might be an adaptive component of salt tolerance (López-Berenguer *et al*., 2009). Other studies have reported that mechanical impacts also increase glucosinolate concentration in Brassica vegetables (Bodnaryk, 1992). Some abiotic stress factors, such as UV-B (Schreiner *et al*., 2009) and water stress (Zhang *et al*., 2008), lead to increased glucosinolate concentration in nasturtium and turnip.

Ratio Fv/Fm

Maximum photochemical efficiency decreased due to water stress and UV radiation in contrast, elevating of CO_2 increased Fv to Fm ratio (Table 2). The decline in the Fv/Fm ratio is a good indicator of photoinhibitory damage caused by light or other environmental stresses. In this study we found that water stress and UV radiation had strong effect on Fv to Fm ratio and decreased this index. Chlorophyll fluorescence that decreased under UV radiation at both ambient and elevated CO_2 indicates that UV radiation might have damaged the D_1 and D_2 proteins of PS II (Olsson *et al*., 2000) and degraded chlorophyll, which might have resulted in reduced quantum efficiency or lower photosynthetic capacity. In the case of photosynthesis, chlorophyll has a crucial role in the production of assimilates. Also, we observed that, increasing of CO_2 concentration improved maximum photochemical efficiency; it seems that, elevated CO_2 can improve photosynthesis efficiency via increase of CO_2 accessibility.

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