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UV-B IRRADIATION EFFECTS ON BIOLOGICAL ACTIVITIES AND CYTOLOGICAL BEHAVIOR OF SAINFOIN (*ONOBRYCHIS VICIIFOLIA* SCOP.) GROWN *IN VIVO* AND *IN VITRO*

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Abstract

To investigate the feasibility of UV-B irradiation (312 nm), seeds of *Onobrychis viciifolia* were exposed to five different intensities for determining the effectiveness of cellular behavior, nutritional constituents and biological activities in *In vivo* and *In vitro* growth cultures. The atomic spectroscopy analysis confirmed that concentrations of two macronutrients (P and N) improved after UV-B exposure as compared with control plants. Near infrared radiation conducted on both *In vivo* and *In vitro* plants showed significant differences on dry matter digestibility (DMD) and crude fiber (CF). Flavonoid and phenolic compounds were increased in both growth cultures by 40% intensity of UV-B irradiation, although *In vitro* plants had the higher compounds than intact plants. Increasing the UV-B irradiation intensity was also found to yield positive effect on anthocyanin. Observations on cellular behavior such as determination of nuclear and cell areas, mitotic index and chromosomal aberrations were proven to be essential in deducing the effectiveness of UV-B irradiation to induce somaclonal variation in sainfoin.

Key words: Sainfoin, UV-B irradiation, Macronutrients, Pigment, Cytology, Phenolic, Flavonoid.

Introduction

Sainfoin (*Onobrychis viciifolia* Scop. syn. *Onobrychis sativa* L.) is one of the most important forage legumes, which is favoured by farmers due to high nutritional value properties. Forage quality is the ability of pasture to produce a desired livestock response, which has direct relation with nutritional constituents (Mohajer *et al.,* 2012). The potential of chlorophyll to absorb the light energy across a wide visible range helps the optimum photosynthesis efficiency in plants.

Approximately, 8-9% of total solar radiation consists of ultraviolet radiation (UV), which is a part of non-ionizing electromagnetic spectrum (Frederick, 1993). Plants are regularly exposed to UV irradiation by sunlight as a requirement for photosynthesis. It was also reported that UV-B irradiation could promote growth, morphological, physiological and biochemical responses in plants (Zhang *et al.,* 2003; He *et al.,* 2003). However, excessive UV-B irradiation has been proven to have a negative impact on most of the crops or plant species (Agrawal *et al.,* 2006). The high intensity of UV irradiation has been shown to perturb protein synthesis (Xiuzher, 1994; Kang *et al.,* 2012), and affect the balance of hormones (Rabie *et al.,* 1996), enzyme activity, water exchange (Stoeva *et al.,* 2001) and gas exchange in plant leaves (Stoeva & Bineva, 2001).

Some researchers have also demonstrated that exposure to UV light can result in significant changes in photosynthetic pigments by the composure of chlorophylls and carotenoids (Teramura & Ziska, 1996) and impairing their photosynthetic function (Grzymski *et al.,* 2001). Response to UV-B irradiation differs between species, whereby distinctive mechanisms were employed, such as inhibition of free radicals due to exposure of seeds to UV irradiation, involving peroxidase and antioxidants (Rogozhin *et al.,* 2000; Ahmad *et al.,* 2013). The inhibition of free radicals is a biochemical defense mechanism utilizing flavonoids and carotenoids. Liang *et al.* (2006) stated that flavonoids played a significant role to guard against UV-B damage in plants, whereby flavonoids would act as a UV filter by absorbing irradiation with wavelengths between 280-320 nm, while carotenoids function reacts as an internal filter.

Moreover, irradiation of UV-B was widely used in tissue culture systems, where it can be used to induce somatic variation and to deduce the varieties of interest and evaluation of genetic resource (Run *et al.,* 1999). UV light can also affect the cellular activity, such as changing the chromosomal function without resulting in cytoplasmic damage and shifting of chromosomes after exposure on cells (Bradshaw *et al.,* 1995). Along this line, the chromosomes were found to move passed the equator to the non-irradiated pole and lose the capacity of division (Sinha & Hader, 2002).

The present study was designed with the following objectives; to investigate the feasibility of UV-B irradiation on seeds to improve the expression of biological activities without mutation, to determine the appropriate irradiation intensity for induction of somaclonal variation; subsequently verified by means of cytological study.

Materials and Methods

UV-B treatment: Seeds of *Onobrychis viciifolia* were exposed to five different levels (100% down to 20%) of UV-B (312nm) irradiation by Spectroline Transilluminator (TVC-312R, six 15-watt UV-B tubes, 120V, 60Hz, 2.0 AMPS) for 15 min per day of a week.

***In vitro* and *In vivo* growth conditions:** After UV-B exposure, sterilized seeds were germinated on MS medium supplemented with 30 g/l sucrose and 7.8 g/l agar at pH 5.8. Cultures were maintained at 25±1°C under 70% humidity and 16 h light photoperiod provided by fluorescent lamps. For *In vivo* assessment, the non-sterilized seeds were germinated in 45 pots (20 cm in diameter) containing 1:1 ratio of black soil (humus): red soil (clay) and transferred to a growth room. The *In vivo* grown plants were subsequently transferred to the greenhouse after 10 days. Biological parameters were assessed after 2 months and cytological characteristics were evaluated before secondary root appearance.

Measurement of pigment content: Two grams of *In vitro* and *In vivo* fresh leaves were homogenized using a chilled mortar and pestle containing 10 ml of 80% (v/v) methanol and $MgCO_3$ (10 g/l). The sample extract was collected and filtered using the Buchner funnel through Whatman filter paper No. 5. The extract volume was then topped up to 50 ml with 80% (v/v) methanol. Samples were centrifuged for 5 minutes at 3000 rpm. Absorbance of the extract was measured at 666 nm, 653 nm and 470 nm using a Shimadzu spectrophotometer. The contents of chlorophyll a (C_a), chlorophyll b (C_b) and carotenoid were determined according to the methanolic modified formulas by Lichtenthaler & Wellburn (1985) based on µg/g FW;

C_a=15.65 A_{666} - 7.340 A_{653}

C_b=27.05 A_{653} - 11.21 A_{666}

C_{x+c}= 1000 A_{470} - 2.860 C_a – (129.2 C_b/245)

For measurement of anthocyanin content, 0.1 g of *In vitro* and *In vivo* fresh leaves were grounded in 3 ml of acidified methanol (1% of HCl in 99% of methanol). Samples were then centrifuged at 12000 rpm for 20 min and the supernatant was kept in the dark at room, at 4°C for 24 hrs. The absorbance was recorded at 550 nm, and the anthocyanin content was calculated based on an extinction coefficient of 33000 $Mol^{-1}cm^{-1}$ (Hosseini *et al.,* 2008).

Total flavonoids determination: Aluminum chloride calorimetric method was employed for determination of flavonoids in fresh leaves of *In vitro* and *In vivo* grown plants (Chang *et al.,* 2002). Each methanolic extract (0.5 ml of 1:10 g mL^{-1}) was separately mixed with 1.5 ml methanol, 0.1 ml 10% aluminum chloride, 0.1 ml 1 M potassium acetate and 2.8 ml distilled water, respectively. The extracts were kept at room temperature for 30 min and the absorbance of the reaction mixture was measured at 415 nm using a Shimadzu spectrophotometer. The calibration curve was developed by measuring quercetin solutions at concentrations of 20 to 100 µg ml^{-1} in methanol.

Total phenol determination: Total phenols were determined by Folin Ciocalteu reagent in fresh leaves of *In vitro* and *In vivo* grown plants (McDonald *et al.,* 2001). A methanol diluted extract of each sample (0.5ml of 1:10 g mL^{-1}) was mixed with the Folin Ciocalteu reagent (5 ml, 1:10 dilution using distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and total phenols were determined by measuring the absorption at 765 nm using the Shimadzu spectrophotometer. The standard curve was prepared using 20 to 100 µg ml^{-1}solutions of gallic acid in methanol.

Quality traits assessment: After two months, plants were harvested from *In vitro* and *In vivo* cultures and dried at 70°C for 48 hrs in an oven. Percentage of crude fiber (CF), dry matter digestibility (DMD), water soluble carbohydrates (WSC), acid detergent fiber (ADF) and Ash of non-irradiated (control) and irradiated samples were found using near infrared light (NIR) spectroscopy. After calibration, percentage of quality traits were calculated using a method by Jafari *et al.* (2003).

Atomic absorption spectrometry: *In vitro* and *In vivo* dried plants were also analyzed for different elements of Mn, Cu, Ca, P and N (based on ratio of irradiated samples to control) according to methods described by Anon., (2003), using atomic absorption spectrometry (Young Lin AAS-8020). Atoms of an element were vaporized and atomized in the flame. Atoms were then absorbing the light at a characteristic wavelength, whereby the source of light was from a hollow cathode lamp, made up of the same element. Absorbed energy was measured by a photo-detector read-out system, whereby it was proportional to the concentration of elements in the samples.

Slide preparation methods and image analysis: Permanent slides of *In vitro* and *In vivo* were analyzed for the evaluation of cellular behaviour characteristics, such as mitotic index (MI), mean nuclear and cell areas and abnormality division. Just before secondary root appearance, root tip meristems obtained from seedlings were pre-treated in 8-hydroxyl-quinalin (2 mM) at 4°C for 5 hrs, then fixed in carnoys solution (1 glacial acetic: 3 chloroform: 6 ethanol) in a refrigerator an overnight. The samples were hydrolyzed in 5N HCL for 40 min at room temperature (cold method). After washing in distill water, root tips were placed in Feulgen`s reagent for 3hrs. Subsequently, roots were squashed on slides, mounted with 45% (v/v) acetic acid, and rinsed. Cover slides were then mounted on the slides by DPX (Di-N-Butyle Phthalate in Xylene). The experiments were conducted using a light microscope (Zeiss Axioscope, Germany) connected to a Sony video camera and, images were captured.

Statistical analysis: The ANOVA was performed for each experiment and means were compared using Duncan's multiple range tests ($p<0.01$) through SAS (9.2) software.

Results

Increasing UV-B irradiation intensity was found to reduce the chlorophyll and carotenoid contents in *In vivo* and *In vitro* cultures (Table 1). *In vitro* grown plants exhibited an initial increase of chlorophyll (a & b) content, possibly due to the accumulation of UV-B absorbing pigments. Chlorophyll (a) and (b) contents were significantly reduced with increasing UV-B irradiation intensity in *In vivo* grown plants from 16.77 and 9.67 µg/g FW to 3.92 and 2.29 µg/g FW,

respectively. The carotenoid content also reduced gradually with increasing of UV-B irradiation intensity from 1.471 µg/g FW in control leaves to 0.502 µg/g FW in 60% intensity of UV-B irradiation (Table 1). Carotenoids played an important role in light harvesting and protection of chlorophyll from photo-oxidative damage; thus reduction in carotenoid content could have a serious impact on photosynthetic and chlorophyll pigments. Non-irradiated leaves of *In vivo* plants had lower anthocyanin content (11.15±0.19 µg/g FW) than control sample of *In vitro* plants (15.3±0.16 µg/g FW). However, UV-B irradiation was shown to improve the anthocyanin content significantly in *In vivo* and *In vivo* cultures, as shown by the higher anthocyanin levels depicted by UV-B irradiated *In vivo* plants compared to non-irradiated *In vivo* plants (Table 1).

The flavonoid content measured in terms of quercetin equivalent (y=0.148x-0.0242, R^2=0.991). Despite the fact that 80% and 100% intensities of UV-B irradiation showed negative effects on flavonoid content in both *In vivo* and *In vitro* samples, a remarkable increment in flavonoid content was observed in 40% and 60% intensities of UV-irradiated cells compared to non-irradiated (control) cells in *In vivo* and *In vitro* leaves, respectively (Fig. 1). The total phenolic content was measured by Folin Ciocalteu reagent in terms of gallic acid equivalent (y=0.1077x-0.0377, R^2= 0.979). *In vitro* non-irradiated plants were observed to contain higher phenolic content than *In vivo* non-irradiated plants. Phenolic content of *In vitro* leaves was also observed to increase gradually when subjected to 40% intensity of UV-B irradiation, while remarkably decreased from 60% to 100% UV-B exposure. Control leaves of *In vivo* plants had higher concentration of phenolic content significantly compared to irradiated leaves after exposure to 80% and 100% intensities of UV-B irradiation (Fig. 1).

The quality and nutritional value of sainfoin were directly correlated with crude protein (CP) and dry matter digestibility (DMD), whilst were inversely correlated with acid detergent fiber (ADF) and crude fiber (CF) (Mohajer *et al.,* 2012). Percentage of digestibility was significantly different in UV-B irradiated plants compared to non-irradiated plants in both growth cultures. Highest percentage of crude protein and digestibility were observed when the seeds were exposed to 100% UV-B irradiation in *In vivo* and *In vitro* culture. Percentage of water-soluble carbohydrates (WSC) was increased with increasing of the UV-B irradiation intensity, although no significant difference was analyzed in *In vivo* and *In vitro* plants. Moreover, percentage of acid detergent fiber (ADF) was gradually decreased with increasing of the UV-B irradiation doses. Significant difference was observed in terms of the crude fiber (CF) between *In vivo* and *In vitro* plants, despite the fact that UV-B irradiation did not have a significant influence on percentage of crude fiber (Table 2).

Furthermore, the ratio of trace metals (Mn and Cu) and macro-nutrients (Ca, P and N) in UV-irradiated to non-irradiated plants were determined using atomic absorption spectrophotometry (AAS). The ratio of trace metals was depicted in irradiated plants compared to control plants. Toxicity of copper was reduced in *In vivo* and *In vitro* irradiated plants at 100% intensity of UV-B irradiation. It was observed that exposure to 100% UV-B irradiation improved the N and P contents in *In vivo* and *In vitro* grown plants. In contrast, exposure to UV-B irradiation was shown to affect negatively the amount of Ca compared to the non-irradiated (control) sample. Although the amount of Ca, Cu and N were higher in *In vitro* grown plants, *In vivo* plants were more notable for P and Mn contents (Fig. 2).

Table 1. Effect of UV-B irradiation on chlorophyll, carotenoid and anthocyanin of *In vitro* and *In vivo* leaves.

UV-B intensity	Control	20%	40%	60%	80%	100%
In vitro						
Chlorophyll a (µg/g FW)	$18.166^{a} \pm 0.12$	$18.490^{a} \pm 0.15$	$14.907^{b} \pm 0.21$	$9.847^{bc} \pm 0.21$	$9.217^{bc} \pm 0.19$	$8.471^{c} \pm 0.14$
Chlorophyll b (µg/g FW)	$11.576^{a} \pm 0.14$	$11.635^{a} \pm 0.12$	$8.716^{ab} \pm 0.15$	$7.074^{ab} \pm 0.11$	$6.146^{b} \pm 0.08$	$4.307^{b} \pm 0.05$
Carotenoid (mg/g FW)	$2.035^{a} \pm 0.05$	$1.829^{a} \pm 0.03$	$1.611^{ab} \pm 0.01$	$1.149^{b} \pm 0.01$	$0.907^{b} \pm 0.04$	$0.478^{c} \pm 0.01$
Anthocyanin (mMol/g FW)	$15.3^{a} \pm 0.16$	$16.5^{a} \pm 0.17$	$17.3^{a} \pm 0.14$	$13.5^{ab} \pm 0.14$	$12.4^{b} \pm 0.13$	$12.9^{b} \pm 0.15$
In vivo						
Chlorophyll a (µg/g FW)	$16.771^{a} \pm 0.14$	$12.412^{b} \pm 0.16$	$8.241^{bc} \pm 0.06$	$5.241^{c} \pm 0.24$	$4.580^{c} \pm 0.13$	$3.920^{c} \pm 0.09$
Chlorophyll b (µg/g FW)	$9.670^{a} \pm 0.08$	$7.380^{a} \pm 0.11$	$5.174^{ab} \pm 0.09$	$3.219^{b} \pm 0.02$	$2.755^{b} \pm 0.01$	$2.292^{b} \pm 0.05$
Carotenoid (mg/g FW)	$1.471^{a} \pm 0.01$	$1.153^{a} \pm 0.01$	$0.96^{ab} \pm 0.01$	$0.483^{b} \pm 0.01$	$0.502^{b} \pm 0.01$	$0.521^{b} \pm 0.01$
Anthocyanin (mMol/g FW)	$11.15^{b} \pm 0.19$	$20.5^{a} \pm 0.32$	$18.8^{a} \pm 0.15$	$16.8^{ab} \pm 0.21$	$13.1^{b} \pm 0.16$	$12.4^{b} \pm 0.14$

The means of treatments with same connotations were not significantly different as per Duncan's multi-range test at p≤0.01

Table 2. Mean comparison of nutritional value of *O. viciifolia* in *In vivo* and *In vitro* cultures.

Traits	*In vivo*			*In vitro*		
	Control	UV-B Intensity			UV-B Intensity	
		40%	100%	Control	40%	100%
CP%	$22.8^{a} \pm 1.1$	$25.1^{a} \pm 1.6$	$25.1^{a} \pm 1.4$	$23.2^{a} \pm 1.2$	$25.6^{a} \pm 1.5$	$26.3^{a} \pm 1.3$
DMD%	$69.6^{b} \pm 2.4$	$72.2^{ab} \pm 2.6$	$74.1^{a} \pm 2.1$	$71.3^{ab} \pm 2.3$	$73.2^{a} \pm 2.1$	$75.4^{a} \pm 2.8$
WSC%	$22.3^{a} \pm 1.1$	$23.1^{a} \pm 1.6$	$23.4^{a} \pm 1.6$	$23.8^{a} \pm 1.7$	$24.5^{a} \pm 2.1$	$24.9^{a} \pm 1.9$
ADF%	$26.4^{a} \pm 2.1$	$25.1^{a} \pm 2.3$	$23.9^{a} \pm 1.6$	$21.3^{ab} \pm 1.5$	$18.1^{b} \pm 1.8$	$16.4^{b} \pm 1.4$
CF%	$28.3^{a} \pm 1.1$	$28.5^{a} \pm 1.5$	$29.6^{a} \pm 1.3$	$22.1^{b} \pm 1.4$	$22.1^{b} \pm 1.4$	$22.4^{b} \pm 1.2$
ASH%	$6.3^{a} \pm 0.9$	$6.6^{a} \pm 0.9$	$6.1^{a} \pm 0.8$	$6.7^{a} \pm 0.9$	$7.3^{a} \pm 1.1$	$7.7^{a} \pm 1.1$

The means of treatments with same connotations were not significantly different as per Duncan's multi-range test at p≤0.01
Crude Protein (CP), Crude Fiber (CF), Acid Detergent Fiber (ADF), Dry Matter Digestibility (DMD), Water Soluble Carbohydrates (WSC)

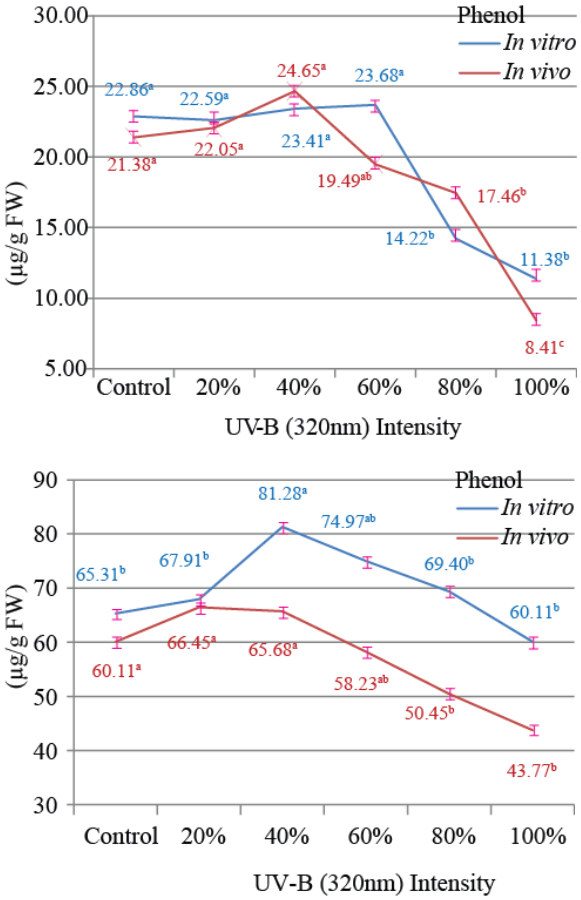

Fig. 1. Effect of UV-B irradiation on flavonoid and phenol compounds in fresh leaves of *In vivo* and *In vitro*.
The means of treatments with same connotations were not significantly different as per Duncan's multi-range test at p≤0.01.

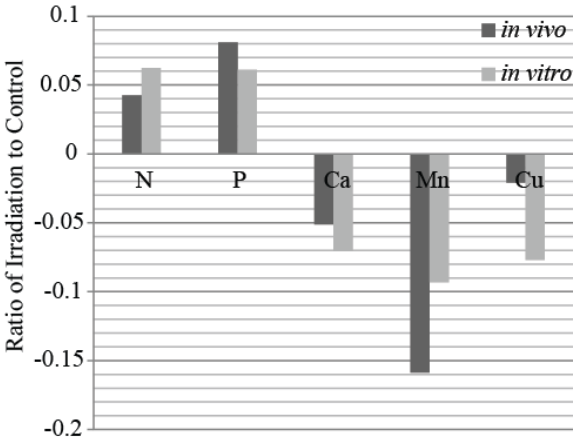

Fig. 2. Ratio of element contents in UV-irradiated samples to non-irradiated (control) samples.

Mitotic index (MI) is used as a parameter to describe cell activity and proliferation. It was observed that the MI of *In vivo* and *In vitro* meristematic root cells increased to 37.67% and 40.91% respectively by 100% intensity of UV-B irradiation. The majority of cells in irradiated and non-irradiated cells were found to be in interphase stage. However, significant increments were indicated in the metaphase and prophase stages after UV-B irradiation exposure (Table 3). In general, the average nuclear areas of *In vivo* and *In vitro* samples were found to expand with increasing of the UV-B irradiation intensities. UV-B irradiation had also positive effect on *In vitro* root cell areas more than *In vivo* cells (Table 4).

Most of the cells displayed regular cell cycle segregation. Nevertheless, some mitotic abnormalities or aberrations were also observed. The effect of different UV-B irradiation intensities on the mitotic irregularities of *In vivo* and *In vitro* root meristematic cells were measured in terms of laggard chromosome, binucleated cells, asynchronous nucleus, cytomixis, micronucleus, tripolar cells and fragmented chrosmosme (Table 5 and Fig. 3). Cytomixis (Fig. 3h) and aneuploidy were only observed in *In vitro* cells. The occurrence percentage of laggard/bridge chromosomes and micronucleus were also observed to increase with increasing of the UV-B irradiation intensity. Moreover, higher percentage of bridge chromosome was indicated in *In vivo* meristematic cells than *In vitro* cells. Laggards or non-oriented chromosomes that failed to reach the poles at the end of telophase could yield the formation of micronuclei (Utsunomiya *et al.,* 2002). UV-B irradiation was also found to induce the formation of tripolar cells in *In vitro* samples (Fig. 3g). Besides, exposure to high intensities of UV-B irradiation (80% and 100%) was also capable of inducing the formation of asynchronous nucleus and fragmented chromosome in both *In vivo* and *In vitro* cells (Fig. 3i). Indeed, UV-irradiated *In vitro* cells exhibited more mitotic irregularities compared to *In vivo* root meristematic cells.

Discussion

In the present investigation, increasing of the UV-B intensity was found to reduce the chlorophyll content in both *In vitro* and *In vivo* grown plants, parallel to the findings by Kakani *et al.* (2003) and Agrawal & Rathore (2007) in various crop species. Reduction in chlorophyll content exhibited by irradiated samples was reported to correlate with inhibition of chlorophyll protein-coding genes called the cab gene (Strid *et al.,* 1990). Pal *et al.* (1999) observed an initial increase and subsequent decrease in chlorophyll content in *Vigna radiate*, which confirms the present results. It was indicated that carotenoid content was reduced with increasing UV-B irradiation intensity, similar to the findings by El-Mansy & Salisbury (1971). The synthesis of carotenoid might be inhibited or broken down when subjected to UV exposure, hence causing the decrease in carotenoid content (Stapleton, 1992).

Interestingly, anthocyanin concentration was increased with UV-B irradiation doses. Similar findings were depicted in several studies, when the anthocyanin content was increased over 171% and 275% in *Suaeda maritime* (Ravindran *et al.,* 2001) and maize (Ambasht & Agarwal, 1998), respectively. It was reported that the increase in anthocyanin levels was mainly due to UV-B irradiation effect (Ravindran *et al.,* 2001), or as a result of defense mechanism against damaging effect of UV-B, which involved the accumulation of anthocyanin to protect the photosynthetic apparatus.

Table 3. Effect of UV irradiation on mitotic behavior of root meristematic cells in *In vivo* and *In vitro*.

Sample	UV-B intensity	Mitosis stages					Mitotic index (MI)
		Interphase (%)	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	
In vivo	control	$74.41^{a} \pm 0.21$	$18.28^{b} \pm 0.18$	$3.67^{b} \pm 0.21$	$1.88^{a} \pm 0.01$	$1.77^{a} \pm 0.01$	$25.59^{b} \pm 0.35$
	20%	$64.63^{b} \pm 0.32$	$22.63^{ab} \pm 0.26$	$7.46^{b} \pm 0.21$	$2.69^{a} \pm 0.05$	$2.56^{a} \pm 0.01$	$35.37^{a} \pm 0.42$
	40%	$61.50^{b} \pm 0.12$	$19.50^{b} \pm 0.22$	$13.25^{a} \pm 0.14$	$3.51^{a} \pm 0.10$	$2.24^{a} \pm 0.03$	$38.51^{a} \pm 0.39$
	60%	$60.14^{b} \pm 0.05$	$20.86^{b} \pm 0.09$	$12.72^{a} \pm 0.18$	$3.14^{a} \pm 0.05$	$3.14^{a} \pm 0.05$	$39.86^{a} \pm 0.42$
	80%	$61.52^{b} \pm 0.14$	$26.13^{a} \pm 0.10$	$5.07^{b} \pm 0.24$	$3.43^{a} \pm 0.01$	$3.87^{a} \pm 0.03$	$38.48^{a} \pm 0.57$
	100%	$62.33^{b} \pm 0.23$	$26.83^{a} \pm 0.14$	$4.94^{b} \pm 0.08$	$2.78^{a} \pm 0.01$	$3.12^{a} \pm 0.01$	$37.67^{a} \pm 0.24$
In vitro	control	$73.47^{a} \pm 0.28$	$20.83^{a} \pm 023$	$3.22^{b} \pm 0.14$	$1.49^{a} \pm 0.01$	$0.97^{a} \pm 0.01$	$26.53^{b} \pm 0.62$
	20%	$61.83^{b} \pm 0.15$	$25.34^{a} \pm 0.12$	$9.37^{b} \pm 0.27$	$1.72^{a} \pm 0.01$	$1.64^{a} \pm 0.01$	$38.17^{a} \pm 0.41$
	40%	$61.53^{b} \pm 0.17$	$25.55^{a} \pm 0.14$	$9.25^{b} \pm 0.23$	$2.57^{a} \pm 0.03$	$1.10^{a} \pm 0.01$	$38.47^{a} \pm 0.35$
	60%	$62.24^{b} \pm 0.09$	$24.76^{a} \pm 0.08$	$9.12^{b} \pm 0.31$	$2.41^{a} \pm 0.02$	$1.47^{a} \pm 0.01$	$37.76^{a} \pm 0.19$
	80%	$59.76^{b} \pm 0.01$	$23.13^{a} \pm 0.24$	$12.68^{ab} \pm 0.24$	$2.72^{a} \pm 0.01$	$1.72^{a} \pm 0.01$	$40.24^{a} \pm 0.24$
	100%	$59.09^{b} \pm 0.01$	$20.13^{a} \pm 0.18$	$15.61^{a} \pm 0.26$	$3.26^{a} \pm 0.02$	$2.09^{a} \pm 0.01$	$40.91^{a} \pm 0.35$

The means of samples with same small letters were not significantly different as per Duncan's multi-range test at $p<0.01$

Table 4. Effect of UV-B irradiation on cell (C) and nuclear (N) areas of *In vivo* and *In vitro*.

Sample	UV-B variable intensity	Nuclear (μm^2)	Cell (μm^2)	N/C
In vivo	Control	$106.21^{b} \pm 6.3$	$493.63^{b} \pm 13.3$	0.215
	20%	$130.43^{a} \pm 9.5$	$755.71^{a} \pm 14.2$	0.172
	40%	$142.75^{a} \pm 11.2$	$771.98^{a} \pm 14.5$	0.184
	60%	$140.81^{a} \pm 10.4$	$772.45^{a} \pm 16.1$	0.181
	80%	$132.52^{a} \pm 10.2$	$728.83^{a} \pm 17.2$	0.181
	100%	$130.66^{a} \pm 9.5$	$711.64^{a} \pm 14.5$	0.182
In vitro	Control	$141.84^{b} \pm 8.4$	$651.83^{b} \pm 17.3$	0.216
	20%	$166.29^{a} \pm 10.2$	$941.73^{a} \pm 16.6$	0.176
	40%	$170.68^{a} \pm 15.2$	$1019.03^{a} \pm 16.3$	0.167
	60%	$167.71^{a} \pm 17.1$	$989.93^{a} \pm 17.7$	0.168
	80%	$172.83^{a} \pm 12.4$	$979.07^{a} \pm 18.2$	0.175
	100%	$165.23^{a} \pm 12.3$	$925.59^{a} \pm 15.4$	0.178

The means of samples with same small letters were not significantly different as per Duncan's multi-range test at $p \leq 0.01$

Table 5. Effect of UV irradiation on mitotic aberrations found in root meristematic cells of *In vivo* and *In vitro*.

Sample	UV-B intensity	Cytomixis (%)	Fragmented (%)	Bridge/ laggard (%)	Micronucleus (%)	Asynchronous nucleus (%)	Tripolar cells (%)	Binucleated cells (%)	Aneuploidy (%)
In vivo	Control	-	-	0.18	0.09	-	-	0.07	-
	20%	-	-	0.27	0.27	-	-	0.48	-
	40%	-	-	0.35	0.41	-	-	0.52	-
	60%	-	-	0.46	0.42	-	-	0.43	-
	80%	-	-	0.33	0.38	0.09	-	0.72	-
	100%	-	0.07	0.29	0.35	0.12	-	0.69	-
In vitro	Control	-	-	0.09	0.12	-	-	0.11	-
	20%	-	-	0.21	0.32	-	-	0.45	-
	40%	-	-	0.14	0.36	-	-	0.67	-
	60%	-	0.08	0.22	0.47	-	-	0.52	-
	80%	-	0.09	0.23	0.42	0.08	0.05	0.54	0.06
	100%	0.04	0.14	0.29	0.44	0.07	0.06	0.78	0.07

$\pm$ SD< 0.001

Due to their chemical structure, flavonoids have been excellent UV-absorbers and act as protective screen-savers for plants (Middleton & Teramura, 1993). Flavonoids mainly existed in leaves, but can also be found in other organs (Tao *et al.,* 2006; Karioti *et al.,* 2008). Flavonoids played an important role in plants' protective mechanism, where they were found to accumulate in the leaf epidermis and act UV-absorbing compounds when exposed to dangerous doses of UV-B irradiation (Tevini *et al.,* 1983; Caldwell *et al.,* 1998). Increasing flavonoid concentration has been found to reduce the penetration of UV-B and able to aid in the protection of the photosynthetic apparatus (Feng *et al.,* 2007). Evidently, flavonoids are highly dependent on the energy input presented by the UV irradiation. Similar to our present results, it has also been reported that 40% and 60% UV-B irradiation had led to a subsequent increase in flavonoid contents in different tissues (Tommasini *et al.,* 2004).

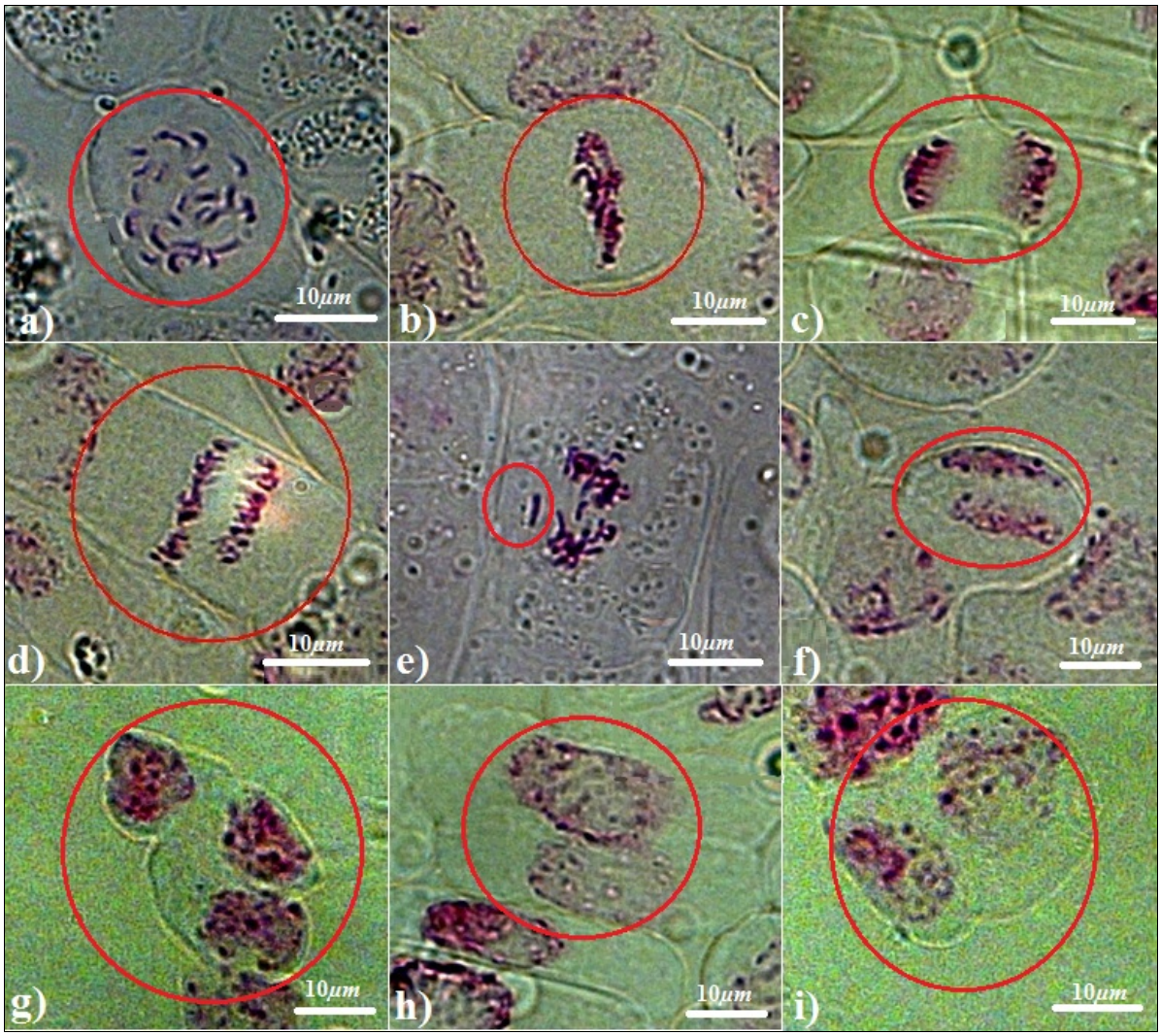

Fig. 3. Root meristematic cells of *O. viciifolia,* showing normal and abnormal mitosis: (a) Prophase, (b) Metaphase, (c) Anaphase, (d) Telophase, (e) Fragmented chromosome, (f) Binucleated cells, (g) Tripolar cells, (h) Cytomixis, (i) Asynchronous nucleus. Bars = 10 *μm*.

Phenol concentration was also increased in UV-B treated samples. Phenolic compounds are also involved in plants' protective mechanism against harmful effects of UV irradiation, where they act as selective UV-B filters (Rozema *et al.,* 2002). Phenolic compounds can also aid to protect plant cells from the oxidative damage induced by free radicals (Wada & Ou, 2002; Khan *et al.,* 2012). In the present study, we had successfully demonstrated that exposure of seeds (pre-sowing) to optimal selected doses of UV-B irradiation could be very beneficial to increase the amount of phenol compounds.

Furthermore, UV irradiation can also cause DNA mutations and formations of pyrimidinedimers, which in turn would impair the hydrogen bonds between the double-stranded DNAs and impede normal DNA replication. Increment of nuclear and cell areas confirmed that various mutagens had different mutagenic potential. In spite of the fact that mutant plants can be identified in M_2 generation, there is a correlation between M_1 treated plant and the frequency of mutation in M_2 induced by ionizing radiations. Probably, that is the reason, a quantitative determination of M_1 destruction can be indicated in mutation breeding, especially for plant species such as Sainfoin that has not been investigated extensively for crop improvement. The assessments of the mitotic cycle in shoot or root meristem cells offered a reliable test to determine the influence of the mutagens in M_1 (Gaul, 1977).

In the present investigation, it was found that chromosome and cellular aberrations had occurred in both *In vitro* and *In vivo* grown plants exposed to UV-B irradiation. The aberration frequency and mitotic index (MI) were found to enhance with increasing of the UV-B irradiation intensity. Bara & Odetta (2005) reported correlations of decreasing mitotic index with reduction in UV wavelength. It was also observed the scattered fragmented chromosomes in UV-B irradiated cells, similar to findings by Bradshaw *et al.* (1995) who reported the presence of membrane-bound micronucleus in irradiated cells. Similar to present assessment, Kim *et*

al. (1996) and Leal *et al.* (1999) reported that UV irradiation had yielded several cells to have two pronuclei of equal sizes, while multiple pronuclei of different sizes were also observed when irradiation was increased.

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