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ULTRAVIOLET–B INDUCED FLAVONOID PRODUCTION IN *IN VITRO* CULTURES OF SHALLOT (*Allium cepa* var. *Aggregatum* G. Don cv Batanes)

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ABSTRACT

Onions are a rich source of health-enhancing flavonols, however research in enhancing the levels of these compounds, especially in shallots, is limited. This study was undertaken to investigate the relationship between flavonol production and UV-B exposure in shallots in an *in vitro* system and at the greenhouse. The experiment was conducted from January 2006 to April 2007 at the Plant Tissue Culture Laboratory, Institute of Crop Science, College of Agriculture and Food Science, UPLB. UV-B was used as an elicitor of flavonol production at two different stages of shallot tissue culture. *In vitro* established plantlets of shallots (cv *Batanes*) were subjected to UV-B (~290 nm) at 0, 3, and 6 h per day for 7 and 14 days at shoot induction and bulbing stages. Bulb diameter and total flavonol content was measured and the profiles of the three predominant flavonoids: quercetin, myricetin, and kaempferol, were described using paper chromatography. These were grown *in vivo* under greenhouse conditions. UV-B exposure did not affect bulb diameter of *in vitro*-grown plantlets but had increased total flavonols. Cultures at the bulbing stage had higher total flavonol than in the shoot induction stage. Flavonoid accumulation tends to increase with prolonged exposure to UV-B but could not be generalized for the different growth stages. There were no differences in flavonol accumulation regardless of the duration or exposure to UV-B. Percent survival was highest among plants that did not receive UV-B treatment. Bulb weight, diameter, plant height, and flavonol accumulation were not affected by UV-B treatments. Quercetin was found to be the most abundant flavonol followed by myricetin and kaempferol in *in vitro* cultures and among plants grown in the greenhouse.

Key words: flavonol, quercetin, myrcetin, kaempferol

INTRODUCTION

Flavonoids are ubiquitous in higher plants and is a diverse family of aromatic molecules which are subdivided into six major subgroups: the chalcones, flavones, flavonols, flavandiols, anthocyanins, and proanthocyanidins, and a seventh group, the aurones, which are widespread but not ubiquitous (Winkel-Shirley 2001). Flavonoids are potent antioxidants, capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxy radicals (Miller 1996). The flavonoids are UV-absorbing compounds and this characteristic implies a direct role of the compound in UV photoprotection. Arguably, the flavonols are the most important flavonoids that participate in stress responses such as that of UV radiation. Flavonol compounds have been shown to be specifically induced by UV light across a wide range of species (Falcone Ferreyra et al. 2012).

Shallots as part of the genus *Allium* are naturally rich in flavonoids. Quercetin, a flavonol, is the most abundant flavonoid in onion that had attracted special interest (Price and Rhodes 1996) in

regard to human nutrition. Quercetin has been reported to reduce the risk of cardiovascular diseases and certain cancers (Patil and Pike 1995, Patil et al 1995b). It is believed to be mostly found or compartmentalized in cell vacuoles. In intact onion plants, total quercetin is concentrated on the drying skin or scale (Patil and Pike 1995, Takahama and Hirota 2000) and may be due in part to mobilization of the molecule towards the drying skin (Gubb and MacTavish 2002).

In vitro or tissue culture model systems are widely accepted as an indispensable tool to investigate biosynthesis and physiology of secondary metabolites. It provides a clearer view that could be used as an alternative to the whole plant (Luckner 1972, Thorpe 1981, Kyte 1987). Plant tissue culture systems provide easier manipulation when elicitors are applied since tissue culture-derived plant materials have simpler organization, grows in a controlled environment, has shorter growth cycles, and minimizes the complexity of the whole intact plants (Staba 1980). Thus, possibilities to obtain fundamental knowledge about relationships between primary and secondary metabolism could be obtained. Secondary metabolite profiles particularly flavonoids in shallot onion cultivar of the Philippines have not been strongly established specifically for in *in vitro* grown cultures. Since the production of flavonoids is highly regulated by UV activating the biosynthetic pathway of flavonoids, using UV as an abiotic elicitor is a strategy to increase total flavonoid content in plants. Thus, this study focused on the flavonoids that may be induced by ultraviolet-B (UV-B) irradiation in *in vitro* cultures of shallot, which could offer substantial basis for further biotechnological manipulation of the crop.

MATERIALS AND METHODS

Plant material

The shallot *Batanes* cultivar (*Allium cepa* var. *Aggregatum* G. Don cv Batanes) was used in this study (Fig. 1A). The bulbs are red to light purple in color and produce small oblate to round bulbs held in a common basal plate in clusters of two to six (Dahilig 1992).The shallots were purchased from Central Luzon State University (CLSU) in Muñoz, Nueva Ecija to assure purity of the cultivars. Dried leaves, scales and roots were trimmed and the good quality bulbs with the favorable sizes were selected. .

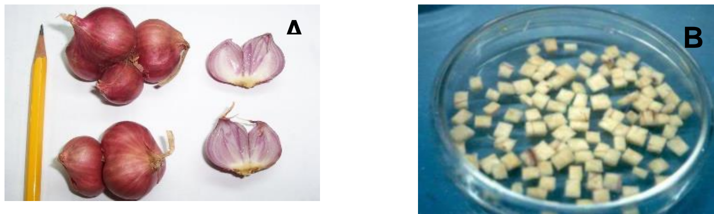

Fig. 1. Shallot used as source of explants are the small to medium sized bulbs of approximately1-2 cm diameter (A); and excised basal plates (B).

Culture media

The modified Murashige and Skoog (MS; 1962) nutrient medium was used as basal medium. Two different culture media were prepared: Shallot Induction Medium (SIM), for shoot multiplication; and Shallot Bulbing Medium (SBM), for bulb formation (Pateña et al. 1998). The pH of the culture media was adjusted to 5.8 using 1N KOH or 1N HCl before dispensing into 15 ml aliquots culture vessels. The culture media were autoclaved at 15 psi for 20 minutes.

Preparation of explants and establishment of sterile cultures

Bulblets with the outer scales removed were placed in a container, drenched with fungicide (benomyl) powder for at least 12 h and finally washed with running water. The bulblets were subsequently soaked in liquid detergent for at least 15 minutes and rinsed with water. Sterilization was done inside the inoculating chamber following the double sterilization regime (15 + 15 min). The

bulbs were initially sterilized with 10% sodium hypochlorite solution, followed by 5.25% hypochlorite commercial bleach (Zonrox®) for another 15 mins. In between and after sterilization procedures, the bulbs were rinsed thrice with sterile distilled water before dissection. The basal plates at approximately 3-4 mm^2 x 1-2 mm thick were excised (Fig. 1B) and sterilized with 1% sodium hypochlorite for 5 min, and rinsed with sterile distilled water three times. These were inoculated onto Shallot Induction Medium (SIM) for the establishment of sterile cultures and shoot multiplication. Adequate size of shoots was obtained three weeks after inoculation. These served as the initial cultures.

Culture maintenance and induction of flavonol production by UV-B radiation

The established cultures of shallots (cv *Batanes*) were either incubated unto SIM for shoot induction and SBM for bulbing. All cultures were incubated in culture room equipped with white cool fluorescent lamps at 8.86 $\mu mol/m^2/s$ PAR at 24 ± 4°C room temperature under 16-h photoperiod for shoot induction and 10-h photoperiod for bulb formation. Shallot cultures at shoot and bulb stages were exposed to UV-B radiation (≈290nm) at different exposure durations similar to the method described by Lavola (1998), Olsson et al. (1998) and Wilson et al. (1998). The treatments were as follows: T_1 – 0 UV-B for 7 days; T_2 – 3 h UV-B for 7 days; T_3 – 6 h UV-B for 7 days; T_4 – 0 h UV-B for 14 days; T_5 – 3 h UV-B for 14 days; T_6 – 6 h UV-B for 14 days

UV-B (280-320nm) lamps (Kandolite, India) were installed in separate culture cabinets enclosed with black cloth. Supplementary UV-B was provided with UV tubes (Kandolite FL 30W). Other wavelengths were filtered using 0.13 mm thick cellulose acetate which were replaced every other day. Sample tissues for the determination of flavonoids were gathered 24 h after the last day of exposure to UV-B. Plant tissue extracts were collected at the end of each treatment duration. The extracts were kept in the freezer (-20°C) until further analysis.

Establishment of plant materials, maintenance and sampling

UV-B irradiated plantlets were acclimatized in the greenhouse for a week. After acclimation, the plantlets were washed, cleaned of agar and dipped in 0.1% fungicide (benomyl) solution for one minute. The plantlets were sown in individual pots containing (1:1:1) sterilized garden soil, compost, and carbonized rice hull (CRH) mixture. Bulbs from the non-irradiated cultures served as control. Four plants per pot were maintained for flavonoid analysis. Proper cultural management practices for the plants were employed simulating field conditions. Random destructive sampling was employed, washed with water, and blot dried. Composite samples of plants from each treatment were collected 50 days after transplanting for flavonoid analysis. Bulb diameter, weight, plant height and number of bulbs per cluster of the sample plants were measured.

Flavonol extraction

Extraction of flavonols was carried out using the method of Thompson et al (2005). Approximately 2 grams of tissue sample from each treatment were homogenized with 10 ml 80% ethanol. The homogenized samples were centrifuged at 5000 rpm for 15 minutes. The supernatant was collected and filtered using Whatman #5 filter paper. The filtrates were stored in screw-capped vials at –20°C for further analysis.

Total flavonol content

The extracts from each treatment in three trials, were warmed to room temperature prior to spectrophotometric analysis, and a 0.5-mL sample was diluted with 4.5 mL of 80% ethanol. The absorbance was determined at 362 nm (Shimadzu, UV-1800). Two technical replicates were performed on each sample and the average readings were taken. Quercetin dihydrate (Sigma) was used as a standard and different standard concentrations ranging from 0.00625 to 0.100 mg/mL were used to create a standard curve (absorbance vs concentration) using linear regression. Total flavonol

content was quantified on a wet matter basis using the equation for the best fitting line based on the standard curve.

Flavonol profile

Samples were taken from the composite of extracts from the different treatments in three trials. Paper chromatography was performed using the protocol of Markham (1982). Chromatographic paper strips (Whatman 3MM) were washed with acetone-water mixture (1:1 v/v) and air-dried before loading the samples. Three flavonol standards, quercetin, kaempferol, and myricetin, and the sample extract were loaded on each strip. The loaded strips were equilibrated for at least 12 h and developed by ascending chromatography using butanol: acetic acid: water (BAW; 4:1:5) and visualized by ammonia vapors. Each visible spot was eluted with ethanol and absorbance was determined at 362 nm.

Statistical design and analysis

Both tissue culture and greenhouse experiments were set-up in a 2 x 3 x 2 factorial in CRD. The factors were growth stage, time (h) exposure to UV-B, and duration (days) of UV-B treatment. The *in vitro* induction experiment had 20 replicates per treatment while the greenhouse study had five replicates. The Statistical Analysis Software (SAS) System (SAS Institute, Cary, NC, USA) was used to analyze all the data gathered following the general linear model (Proc GLM). Significant results of the ANOVA were further tested for difference among treatments using LSD at $\alpha = 0.05$

RESULTS AND DISCUSSION

Establishment of initial shoot cultures

Normal shoots that formed from basal plates are shown in Fig. 2A. The basal plates visibly enlarged 24 h after inoculation, while shoots started to protrude three days thereafter. An average of 92% of the total explants developed shoots with 4.4 shoots per explant. After 21 days of inoculation, 83.28% of the total number of cultures remained contamination-free. Contaminated cultures were immediately discarded and replaced to maintain the number of replicates in each treatment. Developed shoots were held at a common base. Leaf sheaths were white and gradually changed to green towards the leaf region. Explants that did not produce shoots and vitrified (Fig. 2B), formed flattened shoots but remained green (Fig. 2C) and those that assumed 'callus-like' appearance (Fig. 2D) were considered dead. Selected shoots were maintained and used as experimental materials in subsequent experiments.

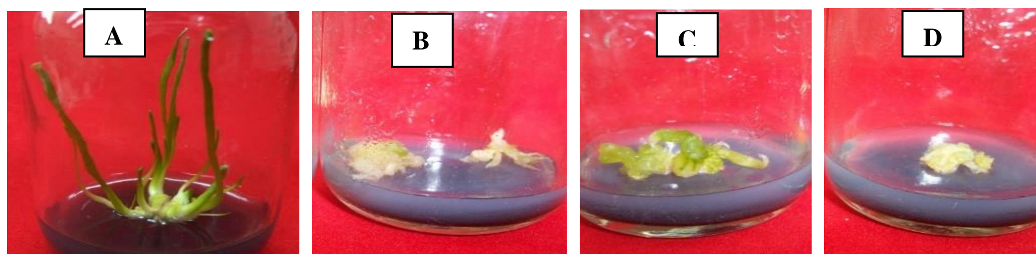

Fig. 2. Normal shoots formed from basal plates (A); dead cultures of shallot (cv *Batanes*) exhibiting vitrified and no shoot formation (B); flattened shoots (C); and 'callus-like' growth (D).

Growth stage and UV-B radiation exposure

UV-B exposure at the shoot and bulb stages did not affect bulb diameter in shallot (cv *Batanes*) cultures. In contrast, total flavonol accumulation was higher in cultures incubated in SBM (27.59 µg/g sample) than in SIM (18.08 µg/g sample) (Table 1). Induction of flavonol production in bulblets exposed to UV-B at bulbing stage is further evidenced by the presence of anthocyanin (Fig.

3B), also a flavonoid. Bulbs at shoot induction stage did not show anthocyanin pigmentation (Fig. 3A).

Table 1. Bulb diameter and total flavonol of *in vitro*-grown shallot (cv *Batanes*) as affected by the number of days and h per day of UV-B radiation exposure in different growth stages.

Exposure Time (Hrs)	[1]Bulb Diameter (mm)			[2]Total Flavonol (µg/g sample)		
	7	14	Mean	7	14	Mean
Shoot Induction Stage						
0	6.3a	6.6a	6.4a	20.13a	26.40a	23.27a
3	6.3a	6.3a	6.3a	13.23a	16.00a	14.62a
6	6.5a	6.4a	6.5a	16.03a	16.70a	16.37a
Mean	6.4a	6.4a	**6.4a**	16.47a	19.70a	**18.08**b**
Shallot Bulbing Stage						
0	6.4a	6.2a	6.3a	18.66a	17.40a	18.03a
3	6.4a	6.2a	6.3a	23.90a	35.37a	29.63a
6	6.0a	6.2a	6.1a	28.57a	41.67a	35.12a
Mean	6.3a	6.2a	**6.2a**	23.71a	31.48a	**27.59**a**
Standard Deviation			0.028			0.006
CV (%)			4.503			25.115

[1]-After acclimatization for 13 weeks in culture. Bulbing started at 9 weeks after inoculation.
[2]-Total flavonol was determined 24 h after the last days of exposure to UV-B
Means under each column and row heading having the same letters are not significant using LSD at α=0.05
** - Significant using LSD at α=0.01 level

Fig. 3. Bulblets of plants exposed to UV-B at shoot induction stage (A); and at bulbing stage (B) which show anthocyanin pigmentation.

Shallot is a bulb forming species of onion and the onset of bulb formation is affected by environmental factors such as photoperiod and temperature (Lancaster et al. 1996). In the absence of physical cues, specifically formulated tissue culture medium such as SBM may promote bulb formation (Pateña et al. 1998). One of the general responses of plants to UV-B radiation is reduced growth characteristics (Teramura 1983) and differs widely between genera, species and among cultivars (Teramura and Sullivan 1991, Jansen et al. 2001). UV, a minor component of sunlight, affects the accumulation of flavonoids. Several studies have demonstrated the change in flavonoid composition because of excess light or UV-radiation (Lois 1994, Olsson et al. 1998). Flavonoids and sinapate esters are UV-B screening pigments which were produced in response to elevated levels of UV-B in *Arabidopsis* (Li et al. 1993) and *Brassica napus* (Wilson et al. 2001).Enzymes and other precursors in the flavonoid biosynthetic pathway are limiting during the immature stage or specific developmental stage (Verhoeyen et al. 2002).In hydroponically and potting soil grown spring onions, total flavonol increased throughout the growing period (Thompson et al. 2005).Differences of nutrient components and total flavonol of onions were affected by age (Thompson et al. 2004) and nitrogen stress (Patil et al. 1995a),but ambient and elevated levels of carbon dioxide had no effect.

Flavonol profile

In this study, the distribution of three flavonols in shallot extracts as influenced by exposure to UV-B radiation was determined. Qualitative analysis by paper chromatography identified seven distinct spots in the extracts. Quercetin was found to be the most abundant flavonol in *in vitro* cultures in both shoot induction and bulbing stages and across UV-B treatments (Fig 4). This finding is consistent with the estimates of Lombard (2000) that quercetin conjugates contribute 90 percentage to the total flavonol of bulb onions. Myricetin was the second most abundant flavonol followed by kaempferol. Quercetin was higher in cultures at the bulb stage than at the shoot induction stage, indicating that quercetin contributed most to the total flavonol accumulation (Fig. 4) and that UV-B exposure at relatively mature stage could have further enhanced its synthesis.

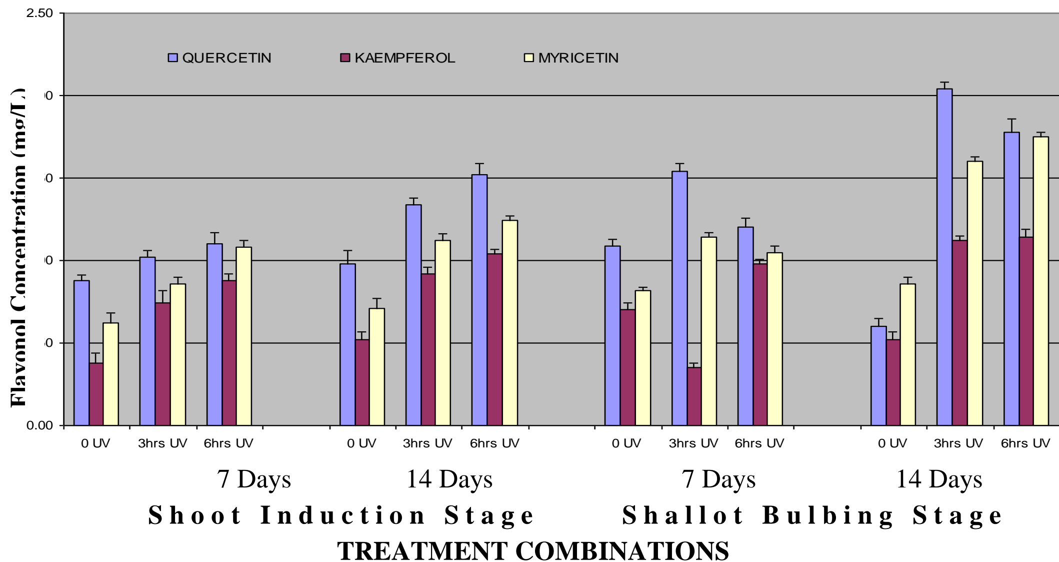

Fig. 4. Flavonoids concentration of *in vitro*-grown shallot (cv *Batanes*) as affected by the number of days and h per day of UV-B radiation exposure in different growth stages determined by paper chromatography and quantified through UV-Vis spectrophotometry. Error bars represent the standard error of the mean.

Quercetin, kaempferol, and myricetin are abundant in plants (Sellapan and Akoh 2002, Miean and Mohamed 2001, Hertog et al. 1992) and are commonly found in vegetables (Herrmann 1976). Flavonols, in general, are implicated in plant cells as UV protectant. However, these are also involved other in plant physiological activities. Quercetin and kaempferol (Vogt et al. 1995) are essential for pollen germination and tube growth in petunia (*Petunia hybrida*) and maize (Mo et al. 1992). Myricetin is suspected to play a role in co-pigmentation as its level increased with accumulation of anthocyanins in bilberries (Jaakola 2006). A number of studies showed that multiple forms of precursor enzymes exist in plants and that their expression patterns vary depending on the growth conditions as in the case of chalcone synthase (CHS), the key enzyme for flavonoid pathway. In barley leaves, CHS with a preference for ferrulic acid and caffeic acids instead of *p*-coumaric acid is induced by UV-B radiation (Christensen et al. 1998), which leads to quercetin synthesis over kaempferol biosynthesis (Ryan et al. 1998, Olsson et al. 1998, Wilson et al. 2001).

Detection and quantification of flavonols in i*n vitro*-derived plantlets grown *in vivo*.

Shallot cultures at both stages, that did not receive any UV-B treatment had the highest survival at the greenhouse. The lowest percentage survival was observed at 6 h UV-B exposure

(Table 2) regardless of the number of days of exposure. It is evident that there is a critical length of exposure period where UV-B damage is irreversible. Significant differences were observed in percentage survival between the control (43.13 %) and at 6 h per day of exposure to UV-B (35.65 %) but not with 3 h exposure. Increasing the UV-B exposure time resulted in a decrease in percentage survival of plantlets grown *in vivo*.

Table 2. Percentage survival of *in vitro*-derived plantlets of shallots (cv *Batanes*) grown *in vivo*.

UV Exposure Time (Hrs/ day)	Percentage Survival (%)						
	Shoot Induction Stage			Shallot Bulbing Stage			Overall % survival (Mean)
	7	14	Mean	7	14	Mean	
0	44.44a	46.30a	45.37a	39.81a	37.95a	38.89a	43.13a*
3	37.96a	37.96a	37.96a	36.11a	37.96a	37.04a	37.50ab
6	34.26a	37.96a	36.11a	35.19a	35.19a	35.19a	35.65b*
Mean	38.89a	40.74a	39.81a	37.04a	37.04a	37.04a	**38.46**
S.D.							5.801
CV (%)							15.096

Means under each column and row heading having the same letters are not significant at 0.05 level (LSD test)
* Significant using LSD at α=0.05 level

Control plants tended to be more vigorous with greener and larger leaves (Fig. 5A) than plants exposed to UV-B in both growth stages at 30 days after potting out (Fig. 5). Necrotic lesions on the leaves were evident on plants exposed to UV-B radiation for 7 days at shoot induction stage (Fig. 5B) and bulbing stage (Fig. 5D), but more pronounced among plants exposed for 14 days to UV-B at both growth stage (Fig 6C and 6E). However, while these abnormalities were observed among UV-B treated plants, their overall growth were comparable with control plants. There were no significant differences on bulb diameter, bulb weight, plant height, and the number of bulbs per cluster, although there is a decreasing trend in their values as UV-B exposure is prolonged (Table 3).

Fig. 5. Growth of *in vitro*-derived shallots (cv *Batanes*) under greenhouse conditions after 30 days of transfer: control (A); exposed to 3 h UV-B radiation at shoot induction stage for 7 days (B); and for 14 days (C); exposed to 3 h UV-B radiation at bulbing stage for 7 days (D) and for 14 days (E).

As in the *in vitro* stage, quercetin was the most abundant flavonol in shallots (Fig. 6). There is an increasing trend in the levels of quercetin, myricetin, and kaempferol in both culture stages previously subjected to UV-B treatment. It seemed that age of the tissue and the accumulation of flavonols as a response to UV-B are directly related.

Flavonol profile using paper chromatography

The *in vitro*-derived plantlets of shallot were grown *in vivo* to determine the effect of UV-B radiation on the accumulation of flavonols in onion. Among the three flavonols determined through paper chromatography, quercetin was consistently the highest, followed by myricetin and kaempferol in plant previously exposed to UV-B radiation in both growth stages (data not shown). These were consistent with the results obtained in the *in vitro* cultures.

Table 3. Bulb diameter, bulb weight, plant height, and number of bulb per cluster of *in vitro*-derived plantlets of shallots (cv *Batanes*) grown *in vivo*.

UV-B Exposure Time (Hrs per day)	**Bulb Diameter (mm)**			**Bulb Weight (g)**			**Plant Height (cm)**			**Number of Bulbs per Cluster**		
	7	**14**		**7**	**14**		**7**	**14**		**7**	**14**	
Shoot Induction Stage												
0	83.0a	83.6a	83.3a	0.580a	0.533a	0.557a	12.68a	12.60a	12.64a	1.23a	1.23a	1.23a
3	82.7a	79.7a	81.2a	0.523a	0.513a	0.518a	12.14a	12.12a	12.13a	1.13a	1.17a	1.15a
6	76.7a	80.3a	78.5a	0.523a	0.557a	0.540a	11.64a	11.66a	11.65a	1.20a	1.13a	1.17a
Mean	80.8a	81.2a	81.0a	0.542a	0.534a	0.538a	12.15a	12.13a	12.14a	1.19a	1.18a	1.19a
Shallot Bulbing Stage												
0	82.2a	85.7a	83.9a	0.550a	0.543a	0.547a	12.60a	12.55a	12.58a	1.23a	1.23a	1.23a
3	82.5a	79.7a	81.1a	0.537a	0.537a	0.537a	11.95a	11.49a	11.72a	1.13a	1.13a	1.13a
6	82.1a	80.0a	81.0a	0.513a	0.520a	0.517a	11.63a	11.54a	11.58a	1.13a	1.10a	1.12a
Mean	82.3a	81.8a	82.0a	0.533a	0.533a	0.533a	12.06a	11.86a	11.96a	1.17a	1.16a	1.17a
Standard Deviation			0.069			0.040			0.682			0.097
CV (%)			0.815			7.484			5.662			8.290

Means under each column and row heading having the same letters are not significant (LSD test).

In the shoot induction stage, quercetin accumulation increased with prolonged UV-B exposure time and days of exposure. In contrast, the level of kaempferol and myricetin varied with different lengths of exposure time and days of exposure to UV-B. In the bulbing stage, the level of quercetin peaked at three h exposure and declined with prolonged exposure time and days of exposure to UV-B. A similar trend was also observed on kaempferol accumulation, however, much lower than quercetin. In contrast, myricetin accumulation was directly proportional to the length of exposure time at 14 days of exposure but not at seven days of exposure. It was also noted that accumulation of myricetin was relatively higher at 14 days than at seven days of exposure to UV-B. These results conformed with the findings of Lavola et al. (1998) on birch tree exposed to UV radiation.

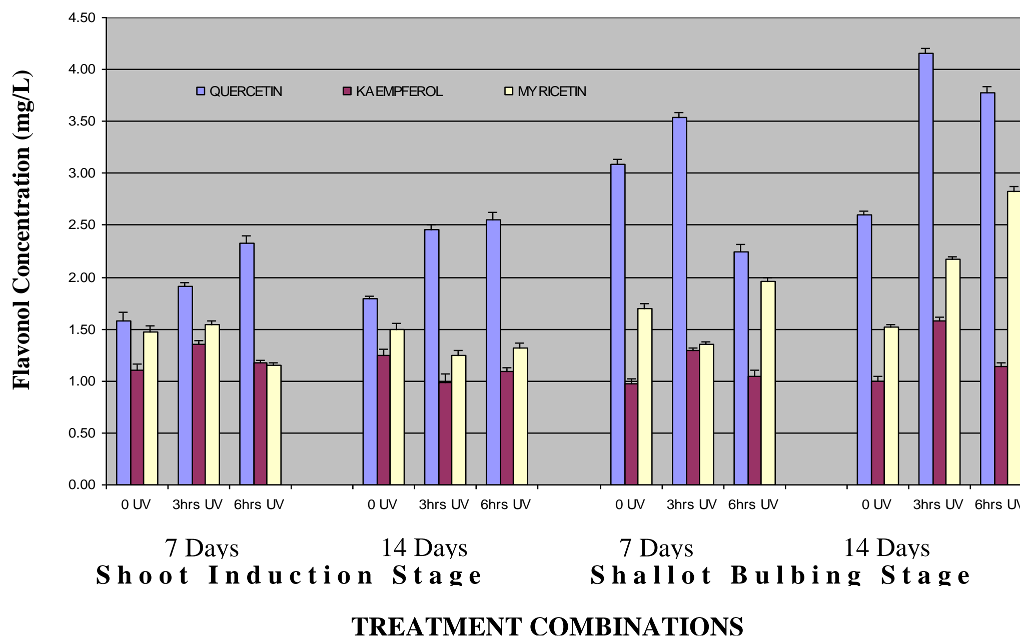

Fig. 6. Flavonol concentration of *in vitro*-derived plantlets of shallot (cv *Batanes*) grown *in vivo* determined by paper chromatography and quantified through UV-Vis spectrophotometry. Error bars represent the standard error of the mean.

CONCLUSION

Collectively, this study demonstrated that accumulation of flavonols (quercetin, myricetin and kaempferol) in *in vitro* conditions was regulated by UV-B. Specifically, UV-B enhanced the accumulation of quercetin while it had minimal effect on the accumulation of myricetin and kaempferol. This preliminary result established the flavonoid profile of shallot (cv. *Batanes*) specifically for the *in vitro* grown cultures. While UV-B affected quercetin accumulation, it did not affect growth characteristics such as bulb diameter, bulb weight, plant height and number of bulbs per cluster. Important considerations for future experiments may include the age of culture and number of days of UV-B exposure for *in vitro*-grown shallots (cv *Batanes*).

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