WALAILAK JOURNAL
http://wjst.wu.ac.th Agricultural Technology and Biological Sciences

Enhancement of the Enzymatic and Non-Enzymatic Antioxidant Activities of Cultured *Sauropus androgynus* L. Merr. Shoots Treated with UV-C Irradiation

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Received: 11 September 2017, Revised: 24 December 2017, Accepted: 23 January 2018

Abstract

The stimulation of enzymatic and non-enzymatic antioxidant activities of *Sauropus androgynus* L. Merr. shoot cultures treated with UV-C irradiation was investigated. The shoots were first cultured on Murashige and Skoog (MS) medium containing 1 mg/L 6-benzyladenine (BA) for 6 weeks. Multiple shoot formations were then treated with UV-C irradiation for 0 min (control group), 5, 10 min (experimental groups), and cultivated field. After 3 weeks of culture the shoots were extracted with methanol and analyzed for enzymatic antioxidant activities (with superoxide dismutase; SOD and glutathione peroxidase; GPX) and non-enzymatic antioxidant activities (with DPPH, ABTS and FRP assays) and total phenolic and flavonoid compound contents. The result indicate that the shoot treated with UV-C irradiation for 5 min exhibited the highest enzymatic antioxidant activities (SOD and GPX values of 2.26, 2.37 unit/mg soluble proteins, respectively). Non-enzymatic antioxidant activities determined by using DPPH, ABTS and FRP assays were found to be IC_{50} values of 10.13 mg/mL, 166.48 mg TEAC/100 g fresh weight (FW), and 22.72 mM $FeSO_4$ /100 g FW, respectively. Total phenolic and flavonoid compound contents had values at 15.54 mg GAE/100 g FW and 63.64 mg QE/100 g FW, respectively. Antioxidant activities revealed a positive moderate high correlation (r^2 = 0.729 - 0.980).

Keywords: *Sauropus androgynus* L. Merr., enzymatic and non-enzymatic antioxidant activities, UV-C irradiation, tissue culture

Introduction

Vegetables and fruits are commonly composed of phytochemicals and nutrients which exhibit antioxidant activities. Antioxidants in plant material include vitamins A, B, C, and E, carotenoids and phenolic compounds, especially flavonoids, and antioxidant enzymes, such as superoxide dismutases and peroxidases, molecular components of the cell conserved throughout deep evolutionary time in all eukaryotic organisms, from protozoa to vertebrates [1,2]. These antioxidants scavenge radicals which inhibit chain initiation and disrupt chain propagation. They can be used to treat oxidative stress by preventing cell damage and ameliorate chronic conditions such as cardiovascular disease, diabetes, and cancer [1]. *Sauropus androgynus* L. Merr. (Euphorbiaceae) is an underutilized plant with high nutritional value and antioxidant activities, native to South and Southeast Asia [3]. In Thailand, *S. androgynus* is found in the Northern, Northeast, and Southern regions [3], and is very popular with consumers. The shoots and leaves are good sources of proteins, minerals, and vitamins A, B, C, and E [3-6]. Alcohol extracts from the shoots and leaves contain papaverine, which inhibits HIV-1 reverse transcriptase and T-lymphocyte cell function [4], and 3-0-β-D-glucosyl-kaempferol (GKK), which reduces blood cholesterol [4-6]. The alcohol extracts also contain phytochemicals that act as antioxidants, including phytol,

octadecatrienoic acid, ethyl ester, glycerin, coenzyme Q, and phenolic compounds [4-6]. Ethanol extracts of *S. androgynus* shoots have been shown to prevent HEK-293 cell death after treatment with hydrogen peroxide [4].

Plant tissue culture has been found to have high potential for the creation of secondary metabolites, such as alkaloids, terpenoids, steroids, saponins, phenolics, flavonoids, anthraquinone, anthocyanin, and tocopherol [8-10]. Biotechnologists have special interest in plant tissue culture for the large scale production of commercially important compounds. These include pharmaceuticals, flavors, fragrances, cosmetics, food additives, feed stocks, and antimicrobials. Murashige and Skoog (MS) medium containing 6-benzyladenine (BA) can be used to produce secondary metabolites that have the same chemical composition as those produced by natural plants [11-13]. Plant tissue culture can be adjusted to optimize the conditions to stimulate the production of enzymatic and non-enzymatic antioxidant activities, such as ultraviolet light and plant wounding [10,14,15].

UV-C irradiation is an effective method to decontaminate surfaces and disinfect water, and is also particularly beneficial in controlling cellular decay and increasing antioxidant activities in fruits and vegetables as a postharvest treatment. Several studies investigated UV-C treatment to delay postharvest senescence and stimulate the production of antioxidant activities in *Vitis vinifera* L., strawberries, and blueberries [10,15-18]. UV-C irradiation also stimulated enzymes associated with antioxidant activities, including superoxide dismutase (SOD) and glutathione peroxidase (GPX), as a non-chemical treatment to maintain the quality of Chinese kale [15,17,19,21]. UV-C irradiation inhibited the decline of vitamin C and carotenoids in Chinese kale[19] and was the most efficient in promoting the accumulation of most secondary metabolites with variation of incubation period, according to tissue culture technique [20,21]. This research, therefore, aimed to investigate the stimulation of enzymatic and non-enzymatic antioxidant activities of *S. androgynus* shoots through UV-C irradiation.

Material and methods

Plant collection

S. androgynus plants were collected from a cultivated field in Uttaradit Province, Thailand. The plant was identified by the author. A voucher specimen was kept at the Science and Technology Centre, Uttaradit Rajabhat University, Uttaradit, Thailand.

Plant sample handling and illumination with UV-C

Approximately 0.5 - 0.8 cm of *S. androgynus* shoot buds were sterilized and cultured on MS medium containing 1 mg/L BA[20] to induce multiple shoot formation. Culture conditions were 25±3 °C with 16 h photoperiodic lighting. After 6 weeks, the shoot buds were subjected to 3 UV-C illumination durations at 0 min (control group) and 5 and 10 min (experimental groups) with dosages (0, 2.03, and 4.06 kJ m^{-2}) using a UV lamp, and shoot buds of *S. androgynus* from a cultivated field were kept at the Science and Technology Centre, Uttaradit Rajabhat University (reference group).

UV-C illumination

The UV-C illumination method was modified from Erkan *et al*. [15] and consisted of an unfiltered germicidal UV lamp, 8 W (El series UV-C lamp, UVP model UVS-28, the Netherlands), located 29 cm (in laminar air flow) above the specimens (**Figure 1**). UV-C intensities at the irradiation area were determined using a UV-C light meter (UV-C-254SD, Lutron Electronic, Germany). Different UV-C illumination doses (0, 2.03, and 4.06 kJm^{-2}) were obtained by altering the duration of the exposure (5 and 10 min) at a fixed distance. A non-illuminated sample was used as the control. After illumination, samples were cultured at 25±3 °C with a 16 h photoperiod (40 μmol m^{-2} s^{-2}) for 3 weeks using mercury fluorescent lamps.

Extraction and assay of enzymatic antioxidant activities

Shoots of *S. androgynus* with a total weight of 1 g were sliced and homogenized with 15 mL of 50 mM sodium phosphate buffer (pH 7.0) and 1 % PVPP. The homogenate was centrifuged at 12,000×g for 10 min at 4 °C. The supernatant was collected and assayed for SOD and GPX activities.

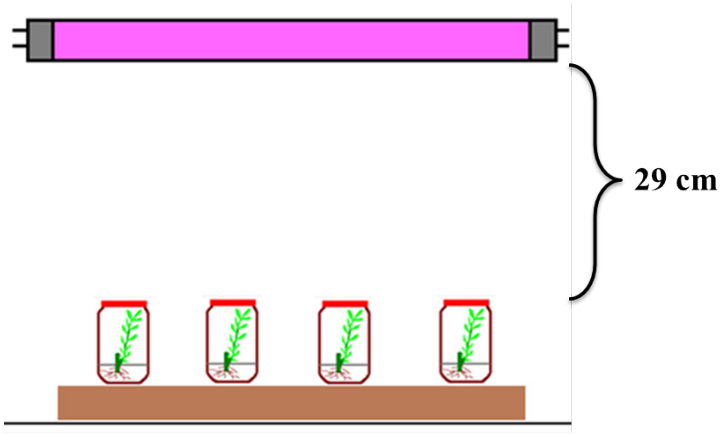

Figure 1 Diagram showing the UV-C treatment results.

SOD activity was assayed following the method of Weydert and Cullen [21] with some modifications. In this method, 2.7 mL aliquots of the reaction mixture (comprising 2.3 mL of 50 mM $NaCO_3^{\bullet}$ $NaHCO_3$ buffer pH 10.2, 0.1 mL of 1.0 mM NBT, 0.1 mL of 4.0 mM xanthine oxidase, 0.1 mL of 3.0 mM ethylene diamine tetraacetic acid (EDTA), and 0.1 mL of 0.15 % BSA (v/v)) were added to 0.1 mL of crude enzyme and incubated at 30 °C for 25 min; then, 0.2 mL of 8 mM $CuCl_2$ was added. The rate of reduction of cytochrome C was measured by the increase in absorbance at 560 nm. SOD activity was expressed as units/mg soluble protein. One unit SOD activity is defined as the amount of enzyme that can inhibit the rate of cytochrome C reduction by half under specific conditions.

GPX activity was assayed according to the method of Weydert and Cullen [21]. The 3 mL reaction mixture contained 10 mM potassium phosphate buffer (pH 7.0), 8 mM guaiacol, and 100 µL enzyme extract. The reaction was initiated by adding 2.75 mM H_2O_2. The increase in absorbance due to the formation of tetraguaiacol was recorded within 30 s at 470 nm. One unit of peroxidase activity was expressed as the change in absorbance per min, and the specific activity as enzyme units/mg soluble protein (extinction coefficient 6.39 mM/cm).

Extraction and assay of total antioxidant activities (TAA)

Shoots of *S. androgynus* weighing 1g were minced into small pieces and macerated in 10 mL of 80 % methanol for 72 h at room temperature (37±2 °C) with occasional shaking. The extracts were centrifuged at 12,000 g for 10 min. The supernatants were collected as a sample solution to determine the TAA using ABTS and DPPH radical scavenging and ferric reducing power (FRP) assays.

2,2 Diphenyl -1-picrylhydrazyl (DPPH) radical scavenging activity was determined using a procedure adapted from Vongsak *et al.* [22]. A total of 500 µL extract or standard was added to 500 µL DPPH in methanol solution (152 µM). After incubation at 37 °C for 20 min, the absorbance of each solution was determined at 517 nm using a UV-vis spectrophotometer (Perkin Elmer, USA). Corresponding blank readings were also taken, and the inhibition percentage was calculated as follows: % Inhibition = $(A_1-A_2)/A_1\times100$, where A_1 is the absorbance of the control reaction (containing all reagents except the test compound) and A_2 is the absorbance of the test compound. The sample concentration required for 50 % scavenging of the

DPPH free radical (IC_{50}) was determined from the linear equation of a standard curve prepared with Trolox and expressed as mg/mL.

2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was determined using a procedure adapted from Arnao *et al*. [23]. The stock solutions contained 7.4 mM $ABTS^{\bullet+}$ and 2.6 mM potassium persulfate. The working solution was prepared by mixing solutions of 7.4 mM $ABTS^{\bullet+}$ and 2.6 mM potassium persulfate (1:1) and allowing them to react for 12 h at room temperature in the dark. The mixed solution was then diluted with 1 ml $ABTS^{\bullet+}$ and 24 mL methanol to obtain an absorbance of 1.1±0.02 units at 734 nm, determined with a UV-vis spectrophotometer. Fresh $ABTS^{\bullet+}$ solution was prepared for each assay. Extracted samples (200 µL) were allowed to react with 2 mL $ABTS^{\bullet+}$ solution for 2 h in dark conditions. Absorbance was measured at 734 nm using a UV-vis spectrophotometer. The standard curve was linear between 25 and 600 mM Trolox. The results were expressed in milligrams (mg) of Trolox equivalent antioxidant capacity (TEAC)/100 g fresh weight (FW). Additional dilution was required if the measured ABTS value fell outside the linear range of the standard curve.

Ferric reducing power (FRP) method was determined using a procedure adapted from Ferreira *et al*. [24]. A volume of 250 µL of sample extract was mixed with 250 µL 0.2 M sodium phosphate buffer and 250 µL 1 % (w/v) potassium ferric cyanide solution, and then incubated at 50 °C for 20 min. The mixture was added to 1 mL of 10 % (w/v) trichloroacetic acid and centrifuged at 12,000×g for 10 min. The supernatant (250 µL) was collected and mixed with 250 µL deionized water and 50 µL 0.1 % (w/v) ferric chloride solution. The absorbance of this mixture was measured at 700 nm using a UV-VIS spectrophotometer. The Fe^{2+} content was evaluated and expressed as mM $FeSO_4$/100 g FW.

Determination of non-enzymatic antioxidant activities

The total phenolic content (TPC) was determined following a method adapted from Vongsak *et al.* [22] using Folin-Ciocalteu reagent. Each sample (400 µL) of 0.04 g/ml (w/v) was mixed with 250 µL of Folin-Ciocalteu reagent (diluted 1:10) in deionized water and 400 µL 7.5 % (w/v) sodium bicarbonate solution. The mixture was allowed to stand for 30 min at room temperature with intermittent shaking. The absorbance was measured at 765 nm using a UV-vis spectrophotometer. TPC was expressed as milligrams of gallic acid equivalents (GAE)/100 g FW.

The total flavonoid content (TFC) was determined following a method adapted from Vongsak *et al.* [22] using aluminum chloride. Each sample (400 µL) of 0.04 g/mL (w/v) was mixed with 400 µL of 2 % aluminum chloride solution. The mixture was allowed to stand for 10 min at room temperature with intermittent shaking. Absorbance was measured at 415 nm against a blank sample without aluminum chloride using a UV-vis spectrophotometer. TFC was expressed as milligrams of quercetin equivalents (QE)/100 g FW.

Data collection and analysis

Each treatment was carried out with 3 replicates using completely randomized design (CRD). Data were evaluated using analysis of variance (ANOVA), followed by Duncan's multiple range test for mean comparisons.

Results and discussion

Plant tissue culture stimulates the production of antioxidant activities and plant growth regulators such as 6-benzylamino-purine (BA) and α-naphthalene acetic acid (NAA), which can induce antioxidant activities and antioxidant substances in callus culture of plants [7-10]. In addition, plant tissue culture produces secondary metabolites that have the same chemical composition as those produced by natural plants [12,13,17]. UV-C radiation has proven particularly beneficial in controlling cellular decay and in increasing antioxidant activities in fruits and vegetables as a postharvest treatment [15-19].

Table 1 shows that UV-C irradiation for 5 min stimulated enzymatic and non-enzymatic antioxidant activities in shoot cultures of *S. androgynus* more actively than in the cultivated field, control, or the 10 min UV-C irradiation groups. Enzymatic antioxidant activities determined by using SOD and GPX were

found to be 2.26 and 2.37 unit/mg soluble proteins, respectively. Non-enzymatic antioxidant activities determined by using DPPH ABTS and FRP assays were found to be IC_{50} values of 10.13 mg/ml, 166.48 mg TEAC/100 g FW, and 22.72 mM $FeSO_4$/100g FW. The total phenolic and flavonoid compounds were 15.54 mg GAE/100 g FW and 63.64 mg QE/100 g FW. Enzymatic and non-enzymatic antioxidant activities and total phenolic and flavonoid compounds contents of shoots cultured from *S. androgynus* extracts were significantly different among treatments ($p < 0.05$). Due to low dosages, UV-C irradiation referred to hormesis induced a number of changes in fruits and vegetables [16], and UV-C irradiation for 10 min caused damaged cells and was harmful. There were 2 mechanisms for the UV-C irradiation. In the first mechanism, UV-C could also stimulate enzyme SOD and GPX, which acts as an inactivating precursor for molecules of free radicals by reacting with superoxide ($2O^{2-}$) changed to hydrogen peroxide. After that, GPX enzyme reacted with hydrogen peroxide with the created SOD and produced hydrogen peroxide changed to H_2O and O_2. This can prevent and reduce cellular and DNA damage from oxidative stress [15,17,19]. The second mechanism, UV-C can stimulate an increase of some secondary metabolites in fruits and vegetables, for example, Polyphenolic compounds, Flavonoids, vitamins C and E, carotenoids, and other organic acids [17,20]. In **Table 1**, the results show that UV-C irradiation for 5 min stimulates the production of antioxidant activities in shoot cultured *S. androgynus* to be higher than cultivated field, control group, and UV-C irradiation for 10 min. Similar results in **Table 2** show that enzymatic antioxidant activities and non-enzymatic antioxidant activities revealed a positive moderate to high correlation ($r^2 = 0.729 - 0.980$) and significant difference at $p < 0.01$. Several studies reported that UV-C irradiation of fruits and vegetables could be performed as a postharvest treatment to inhibit the decline of vitamin C and carotenoids in papaya, blueberry, strawberry, and Chinese Kale [15,17,25,26]. However, this study showed that UV-C irradiation enhanced enzymatic and non-enzymatic antioxidant activities in plant tissue culture.

Table 1 Levels of enzymatic and non-enzymatic antioxidant activity of cultures of *S. androgynus* shoot extracts in cultivated field, control, and experimental groups after UV-C irradiation.

Treatment	Cultivated field	Control	UV-C 5 min	UV-C 10 min
Enzymatic antioxidant activities				
SOD (unit/mg soluble protein)	1.46±0.03^{b}	1.52±0.04^{b}	2.26±0.06^{a}	1.55±0.05^{b}
GPX (unit/mg soluble protein)	0.56±0.28^{c}	1.05±0.06^{b}	2.37±0.72^{a}	1.18±0.09^{b}
Non-enzymatic antioxidant activities				
DPPH assay (IC_{50}) (mg/mL)	18.77±2.83^{b}	15.24±1.37^{b}	10.13±0.91^{a}	16.21±1.53^{b}
ABTS assay (mg TEAC/100 g FW)	64.06±4.31^{d}	133.17±8.69^{b}	166.48±5.35^{a}	100.15±4.70^{c}
FRP assay (mM $FeSO_4$/100 g FW)	12.72±0.81^{b}	17.28±1.63^{b}	22.72±4.27^{a}	15.33±1.36^{b}
Total phenolics (mg GAE/100 g FW)	6.02±0.49^{c}	9.56±1.28^{b}	15.54±1.56^{a}	9.08±0.46^{b}
Total flavonoids (mg QE/100 g FW)	41.99±1.49^{b}	50.11±6.35^{b}	63.64±3.67^{a}	45.62±3.77^{b}

^{a,b,c,d}Different letters in the same row indicate significant difference at $p < 0.05$ using one-way ANOVA

Table 2 Pearson correlation coefficients of enzymatic and non-enzymatic antioxidant activities in cultures of *S. androgynus* shoot extracts for cultivated field, control, and experimental groups after UV-C irradiation.

Trait	r value						
	DPPH	**ABTS**	**FRP**	**Phenolic**	**Flavonoid**	**SOD**	**GPX**
DPPH	1						
ABTS	0.895**	1					
FRP	0.791**	0.815**	1				
Phenolic	0.980**	0.883**	0.825**	1			
Flavonoid	0.900**	0.796**	0.888**	0.921**	1		
SOD	0.922**	0.729**	0.746**	0.917**	0.847**	1	
GPX	0.973**	0.856**	0.811**	0.987**	0.894**	0.937**	1

**Correlation is significant at the 0.01 level

Conclusions

This study showed that shoot culture of *S. androgynus* treated with UV-C irradiation for 5 min had higher enzymatic and non-enzymatic antioxidant activities than control or cultivated field. These results suggest that UV-C irradiation enhances antioxidant activities in plant tissue culture. Further investigation can be utilized to improve the antioxidant activities of cultured *S. androgynus* shoots for raw materials that can be used in cosmetics, functional food, and tea.

Acknowledgements

This research was financially supported by Uttaradit Rajabhat University, and laboratory support was provided by King Mongkut's University of Technology Thonburi, Thailand.

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