JARQ 51 (3), 241-250 (2017) https://www.jircas.go.jp

Lowbush Blueberry, Highbush Blueberry and Cranberry Extracts Protect Cucumber (*Cucumis sativus* L.) Cotyledons from Damage Induced by UV-B Irradiation

Seiji YAMASAKI*, Kenji MIZOGUCHI, Naohiro KODAMA and Joji ISEKI

Laboratory of Plant Physiology, Department of Science Education, Faculty of Education, University of Teacher Education Fukuoka (Munakata, Fukuoka 811-4192, Japan)

Abstract

We prepared water extracts from four dry berries, lowbush blueberry (LBB) (*Vaccinium angustifolium* L.), highbush blueberry (HBB) (*Vaccinium corymbosum* L.), cranberry (CB) (*Vaccinium macrocarpon* Ait.) and grape (*Vitis vinifera* L.). The four berry extracts were efficient absorbers of ultraviolet (UV) light, primarily UV-B. The treatment of cucumber (*Cucumis sativus* L.) cotyledons with LBB, HBB and CB extracts attenuated the damage induced by continuous UV-B irradiation (0.58 W m^{-2}) for 11 days and did not cause any side effects. Anthocyanin and polyphenol contents in the four berry extracts were analyzed using high performance liquid chromatography (HPLC) and spectrophotometry. Anthocyanins, which are major UV-absorbing compounds in higher plants, were not the primary polyphenolic compounds in the four berry extracts. A total of 51 specific polyphenols were identified. The LBB extract contained primarily chlorogenic acid, caffeic acid and protocatechuic acid; the HBB extract contained primarily chlorogenic acid, caffeic acid and syringic acid; and the CB extract contained primarily protocatechuic acid, myricetin and *p*-coumaric acid. These components might contribute to the protection of cucumber plants against UV-B-induced damage.

Discipline: Plant protection
Additional key words: antioxidants, caffeic acid, chlorogenic acid, polyphenolic compounds

Introduction

The depletion of the ozone layer in the stratosphere by chlorofluorocarbons (CFCs) increases the amount of solar ultraviolet-B (UV-B; 280-320 nm) irradiation passing through to the earth's surface (Rozema et al. 1997). In higher plants, UV-B irradiation is known to damage DNA and membranes, reduce photosynthetic activity, inhibit hypocotyl elongation, stunt growth, reduce leaf area, and induce leaf bronzing and necrosis (Teramura 1983, Ziska et al. 1992, Teramura & Sullivan 1994). UV-B irradiation is thus harmful to higher plants. In agriculture, the increase in solar UV-B reduces farm productivity. Protection against UV-B irradiation poses an important problem for higher plants including farm crops.

Mutants resistant to UV-B irradiation have been isolated in higher plants. The *Arabidopsis ultraviolet tolerant 1* (*uvt1*) and *radical-induced cell death1-2* (*rcd1-2*) mutants have increased the accumulation of UV-absorbing compounds such as flavonoids, thereby contributing to UV-B tolerance (Bieza & Lois 2001, Fujibe et al. 2004). The *Arabidopsis ultraviolet insensitive 1* (*uvi1*) mutant has enhanced photoreactivation activity for cyclobutane pyrimidine dimers (CPDs) and dark repair activity for (6-4) photoproducts (Tanaka et al. 2002). In the *Arabidopsis uvi4* mutant, enhanced endoreduplication leads to enhanced UV-B tolerance (Hase et al. 2006). Thus, the mechanism of UV-B resistance in higher plants has been studied and clarified in *Arabidopsis*. However, the introduction of homologous mutations to increase UV-B tolerance in other higher plants including farm crops is unrealistic. To protect many farm crops against UV-B irradiation, a new method other than gene mutation should be established.

Cucumber (*Cucumis sativus* L.) is a primary summer

*Corresponding author: e-mail yamasaki@fukuoka-edu.ac.jp
Received 12 April 2016; accepted 4 August 2016.

vegetable in Japan. Cucumber has frequently been used as a model plant for UV-B studies because of its high sensitivity to UV-B irradiation (Takeuchi et al. 1996, Krizek et al. 1997, Shinkle et al. 2004, Yamasaki et al. 2007). We have previously shown that continuous UV-B irradiation reduces the expression of cell cycle-related genes and the normal growth of cucumber cotyledons (Yamasaki et al. 2007, Yamasaki et al. 2015). Thus, cucumber cotyledons are suitable for studying the effects of UV-B irradiation in the laboratory.

Red and purple berries such as lowbush blueberry (LBB) (*Vaccinium angustifolium* L.), highbush blueberry (HBB) (*Vaccinium corymbosum* L.), cranberry (CB) (*Vaccinium macrocarpon* Ait.) and grape (*Vitis vinifera* L.) are rich sources of phenolic compounds (Singh et al. 2009, Ivanova et al. 2010, Rodriguez-Mateos et al. 2012). And because phenolic compounds absorb UV, berry extracts may possibly function in UV absorption for farm crops.

In the present study, we tried to develop in the laboratory a simple method of protecting farm crops against the damage caused by UV-B irradiation. To achieve that objective, we investigated whether water extracts [designated as 'berry extracts' in the present study, see Materials and methods, 1 (1) Preparation of berry extracts] containing natural compounds from LBB, HBB, CB and grape could confer protection against UV-B irradiation for cucumber cotyledons. Although there are substances with low recovery rates by water extraction, water extracts of natural resources are simple to prepare, easy to use, and provide a safe and biologically compatible matrix for bioactive compounds. We first examined the light absorption spectra of the four berry extracts. Then we treated cucumber cotyledons with the four berry extracts and investigated the subsequent effects of continuous UV-B irradiation on cucumber seedlings. Finally, we performed high performance liquid chromatography (HPLC) and spectrophotometric analyses to determine the amounts of total anthocyanins and total polyphenols, and identify the most effective natural compounds and polyphenols in the berry extracts for attenuating UV-B-induced damage in farm crops.

Materials and methods

1. Optical absorption properties of the berry extracts

(1) Preparation of berry extracts

Commercially produced dry fruits of lowbush blueberry (LBB) (*Vaccinium angustifolium* L.) (Wild blueberry), highbush blueberry (HBB) (*Vaccinium corymbosum* L.) (Cultivate blueberry), cranberry (CB) (*Vaccinium macrocarpon* Ait.) and grape (*Vitis vinifera* L.) were purchased from a local Aeon supermarket (Topvalu; Aeon Co., Ltd., Chiba, Japan) in Fukuoka prefecture on 27 September 2012 and 11 July 2013, and then used before their expiry dates. For each of the four species, 5 g of dried berries was ground using a mortar and pestle at room temperature, and then 15 ml of distilled water (pH 7.5) was added. Samples were placed at 4°C and the ground tissue was extracted into distilled water for 14 h. The samples were then centrifuged at 6,000 rpm for 15 min. at 4°C. The crude supernatant was transferred to a new tube and centrifuged at 12,000 rpm for 10 min. at 4°C, and clear supernatant was collected. This clear supernatant is designated as the 'berry extract' in the present study. Brix of the four berry extracts was measured with a pocket sugar content meter (APAL-J; Atago Co., Ltd., Tokyo, Japan). The Brix values of the LBB, HBB, CB and grape extracts were 19.4%, 18.7%, 22.4% and 21.6%, respectively. The extraction rates [(weight of collected extract / weight of dry fruits) × Brix] of the LBB, HBB, CB and grape extracts were 45.7%, 42.4%, 43.2% and 56.1%, respectively. The four berry extracts were stored at 4°C during the experiment.

(2) Light absorption spectral analyses of the berry extracts

To examine the light absorption spectra of the four berry extracts, we prepared 1-5% (v/v) dilutions of the extracts for analysis with ultraviolet and visible spectrophotometry at wavelengths of 200-700 nm using a UVIDEC-4 instrument (Jasco Co., Tokyo, Japan). For comparison, we prepared aqueous solutions (in distilled water) of 4% (w/v) delphinidin (an anthocyanidin) and 2% (w/v) cyanidin-3-glucoside (an anthocyanin) and then analyzed the light absorption spectra at 200-700 nm.

2. Effects of berry extracts and subsequent UV-B irradiation on cucumber seedling growth

(1) Plant materials

Cucumber (*Cucumis sativus* L. cv. Santo-suyo No. 2) seeds were purchased from Nakahara Seed Product Co., Ltd. (Fukuoka, Japan). The seeds were germinated on wet filter paper in a Petri dish at 26°C in the dark for 2-3 days. Germinated seedlings were transferred to plastic pots containing the soil composite Kumiai-Engei-Baido (0.4 g kg^{-1} N, 1.2 g kg^{-1} P, 0.2 g kg^{-1} K; Seishin Sangyo Co., Ltd., Kitakyushu, Japan). Seedlings were grown under continuous fluorescent light (FLR40SW/M/36-B; Hitachi, Ltd., Tokyo, Japan) in an incubator (LH-200RDS; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) at 26°C. The photosynthetic photon flux density (PPFD) at the plant surface was approximately 213 μmol m^{-2} s^{-1}.

(2) Treatment of cucumber cotyledons with berry extracts

When cotyledon blades were approximately 1.5 cm

long, plants were transferred to a growth cabinet furnished with continuous fluorescent light (FLR40SW/M/36-B; Hitachi, Ltd.) at 25°C, with a PPFD at the plant surface of approximately 160 μmol m^{-2} s^{-1}. The berry extract [containing 0.1% (v/v) Tween 20] was then sprayed on cotyledons uniformly once a day for three days. Eight plants were used to test each of the four berry extracts. Plants that were sprayed with distilled water containing 0.1% (v/v) Tween 20 and grown similarly as the test plants were designated as 'Control'. The dose responses of the LBB, HBB and CB extracts were tested by spraying cucumber cotyledons with 12.5%, 25% and 50% (v/v) dilutions of the berry extracts. Eight plants were used to test each of these three doses for the three berry extracts.

(3) Cucumber cotyledons treated with berry extracts and later subjected to continuous UV-B irradiation

Continuous UV-B treatment [UV-B (+)] began immediately after the berry extract spray treatment on day 3; note that day 0 of UV-B (+) and day 3 of berry extract treatment are the same day. The method used for UV-B (+) was essentially the same as that described by Yamasaki et al. (2007). Briefly, a sunlamp (FL-20E; Tozai Densan, Ltd., Osaka, Japan) was suspended 7 cm above the cotyledons. For continuous UV-B (+), the sunlamp was fitted with a polyvinyl chloride film (Cutting Sheet 000C; Nakagawa Chemical Inc., Tokyo, Japan) that absorbed wavelengths below 290 nm. For treatment without continuous UV-B irradiation [UV-B (–)], plants were grown under another sunlamp covered with a polyester film (equivalent to Mylar film) that absorbs all wavelengths below 320 nm (Melinex 516; Imperial Chemical Industries PLC, London, UK). The films were replaced weekly due to declining transmittance. Continuous UV-B (+) or UV-B (–) was conducted for 11 days. The UV intensity was measured using digital UV intensity meters (UV-5.7, UV-6.2 and UV-8.0; MK Scientific, Inc., Yokohama, Japan). Table 1 summarizes the UV intensities. The average UV-B irradiation intensity was 0.58 ± 0.13 W m^{-2} for UV-B (+) plants. The average UV-A intensity was 0.36 ± 0.08 W m^{-2} for UV-B (–) plants, and 0.42 ± 0.06 W m^{-2} for UV-B (+) plants. Given the similar UV-A intensities in the UV-B (+) and UV-B (–) groups, the present study did not consider the effects of UV-A irradiation.

(4) Measurement of the area of cotyledons and first leaves in cucumber seedlings treated with berry extracts and subsequent continuous UV-B (+)

The first leaves did unfold in seedlings treated with berry extracts for three days and subsequent continuous UV-B (–) for 11 days; for these seedlings, the areas of cotyledons and first leaves were measured (n=16 cotyledons, n=8 first leaves, N=8 plants). In contrast, most of the first leaves did not unfold in seedlings treated with berry extracts for three days and subsequent continuous UV-B (+) for 11 days; for these seedlings, only the cotyledon areas were measured (n=16 cotyledons, N=8 plants). Seedlings were photographed with a digital camera (CX1; Ricoh Imaging Company, Ltd., Tokyo, Japan) under the same lighting and spatial conditions on days 0, 1, 3, 5, 7, 9 and 11 of UV-B (+) or UV-B (–). The cotyledon and first leaf areas were morphometrically calculated from the digital images using "PhotoMeasure" software (Kenis, Ltd., Osaka, Japan).

3. Component analyses of the berry extracts

(1) Quantitative spectrophotometric analysis of total polyphenols in berry extracts

The content of total polyphenols was analyzed according to the method of Folin-Denis (Ono & Huang 2001). Briefly, 50 μl of berry extract was added to 4 ml of distilled water and 1 ml of phenol reagent (a 5-fold dilution of Folin-Ciocalteu reagent with distilled water). After mixing, 1 ml of 10% (w/v) sodium carbonate solution was added and the reaction was mixed again. The samples were then allowed to stand in the dark for 1 h at room temperature. Sample absorbance at 760 nm was

Table 1. Intensity of UV irradiation received by cucumber cotyledons treated with UV-B (+) and UV-B (–), and intensity of a sunlamp without film (with all data measured 7 cm below the sunlamp).

	Intensity of UV irradiation		
	UV-C (W m^{-2})	UV-B (W m^{-2})	UV-A (W m^{-2})
	(246-262 nm)	(280-320 nm)	(320-400 nm)
Sunlamp	N.D.[z]	2.89	0.83
Sunlamp + polyester film [UV-B (–)]	N.D.[z]	N.D.[z]	0.36 ± 0.08[y]
Sunlamp + polyvinyl chloride film [UV-B (+)]	N.D.[z]	0.58 ± 0.13[y]	0.42 ± 0.06[y]

[z] N.D., not detected.

[y] Values represent the means ± SE at three different spots over a seven-day period.

measured with ultraviolet and visible spectrophotometry (UVIDEC-4; Jasco Corporation, Tokyo, Japan). A calibration curve of (+)-catechin solution was prepared, and total polyphenol contents were calculated with respect to the (+)-catechin calibration curve (μg g^{-1}). The value of total polyphenol contents in each berry extract was expressed as the average of three replicates.

(2) HPLC analysis of total anthocyanin contents in berry extracts

HPLC analysis of total anthocyanin contents in berry extracts was performed by Japan Food Research Laboratories (Tokyo, Japan) according to a previously published method (Cassinese et al. 2007). Briefly, 25 ml of 2.0% (w/v) hydrochloric acid in methanol was added to 2.5 g of berry extract and then mixed. Then, 10 ml of this sample was transferred to a new tube, with 10 ml of 10% (w/v) phosphoric acid being added. This solution was subjected to HPLC analysis using an LC-20AD instrument (Shimadzu Corporation, Kyoto, Japan) equipped with ultraviolet and visible spectrophotometry (SPD-20AV; Shimadzu Corporation, Kyoto, Japan). A Zorbax Extend-C18 (4.6 mm i.d. × 250 mm, 5 μm) column (Agilent Technologies Japan, Ltd., Tokyo, Japan) was used. The temperature was set at 30°C, injection volume at 10 μl, and flow rate at 1.0 ml min^{-1}. The mobile phase consisted of 10% formic acid in water [1 HCOOH : 9 H_2O (v/v)] as solvent A and methanol/formic acid/acetonitrile/water [9 CH_3OH : 4 HCOOH : 9 CH_3CN : 16 H_2O (v/v/v/v)] as solvent B. The linear gradient was 93% A and 7% B at 0 min., 75% A and 25% B at 35 min., 35% A and 65% B at 45 min., 100% B at 46 min., and then returned to 93% A and 7% B at 5 min. Chromatograms were acquired at 535 nm. Fifteen types of anthocyanins were identified by comparison with validated chromatographic data for bilberry anthocyanins (Cassinese et al. 2007). The contents of each of these fifteen anthocyanins in berry extracts were calculated based on the cyanidin-3-glucoside hydrochloride standard. Total anthocyanin contents for the extracts were calculated by adding the contents of individual anthocyanins.

(3) HPLC analysis of 51 polyphenols in berry extracts

HPLC analysis of 51 polyphenols (protocatechuic acid, syringic acid, vanillic acid, β-resorcylic acid, p-hydroxy benzoic acid, m-aminobenzoic acid, 2,3-dihydroxy benzoic acid, p-aminobenzoic acid, α-resorcylic acid, gentisic acid, γ-resorcylic acid, gallic acid, salicylic acid, o-aminobenzoic acid, chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, o-coumaric acid, eugenol, vanillin, syringaldehyde, chrysin, apigenin, luteolin, quercetin, myricetin, kaempferol, galangin, fisetin, eriodictyol, hesperetin, naringenin, daidzein, genistein, glycitein, gallo catechin, epigallo catechin, catechin, epicatechin, epicatechin gallate, gallocatechin gallate, catechin gallate, epigallocatechin gallate, theaflavn-3-gallate, theaflavn-3'-gallate, theaflavn-3, 3'-gallate, sesamol, ellagic acid, scopoletin, 7-hydroxy coumarin) in berry extracts was performed by BML, Inc. (Tokyo, Japan) according to a method reported by Sakakibara et al. (2003) with modifications. Briefly, 500 μl of berry extract was prepared by adding 10 μl of 500 μg ml^{-1} 17-alpha-estradiol internal standard and 90 μl of antioxidant solution [0.1% (v/v) ethylenediaminetetraacetic acid (EDTA) and 20% (v/v) ascorbic acid], and then the sample was extracted with 400 μl of 0.1 M sodium acetate buffer (pH 5.0). This sample was mixed for 1 min. and designated as solution A. The sample was centrifuged at 3,000 rpm for 5 min., the supernatant was recovered, and then 50 μl of the supernatant was subjected to HPLC analysis. Chromatographic analysis was performed on an ESA HPLC (ESA Inc., Chelmsford, MA, USA) equipped with CoulArray data station version 3.05 software and CoulArray detection system model 5600A (ESA Inc., Chelmsford, MA, USA) coupled to a 10-channel CoulArray detector. The potentials applied on the electrode were −80, 0, 80, 160, 240, 320, 400, 480, 560 and 640 mV. A Hypersil-Gold (4.6 mm i.d. × 250 mm, 5 μm) HPLC column was used (Thermo Fisher Scientific K.K., Yokohama, Japan). The temperature was set at 35°C, injection volume at 10 μl, and flow rate at 1.0 ml min^{-1}. The mobile phase consisted of 100 mM sodium phosphate (pH 3.3) / methanol [95:5 (v/v)] as solvent A and 100 mM sodium phosphate (pH 3.3) / acetonitrile / methanol [3:6:1 (v/v/v)] as solvent B. The gradient conditions were 100% A at 0 min., linear increase of B from 0% to 1% and linear decrease of A from 100% to 99% for 50 min., linear increase of B from 1% to 18% and linear decrease of A from 99% to 82% for the next 40 min., linear increase of B from 18% to 28% and linear decrease of A from 82% to 72% for the next 30 min., linear increase of B from 28% to 80% and linear decrease of A from 72% to 20% for the next 20 min., and linear increase of B from 80% to 90% and linear decrease of A from 20% to 10% for the last 10 min. A total of 51 polyphenols were identified based on reported retention times in chromatographic databases. The polyphenolic compounds were quantified based on external standards or previously generated calibration curves in the system library. When the retention time of a polyphenol detected in the berry extract did not match known retention times of said standards, the sample was subjected to hydrolysis according to a method reported by Hertog et al. (1992) with modifications. Briefly, 20 mg of β-glycosidase was added to solution A and the sample was heated at 43°C

for 3 h. Then, 500 µl of methanol was added, the sample was mixed for 1 min. and centrifuged at 3,000 rpm for 5 min. at 4°C, the supernatant was recovered, and then 50 µl of the supernatant was subjected to HPLC analysis.

4. Statistical analysis

The area of cotyledons and first leaves in cucumber seedlings are expressed as the means ± SE. Statistically significant differences were assessed using one-way analysis of variance (ANOVA) followed by Dunnett's test (http://www.gen-info.osaka-u.ac.jp/MEPHAS/dunnett.html, January 6, 2016).

Results

1. Optical absorption properties of the berry extracts

The light absorption spectra of berry extracts, 1-5% dilutions of berry extracts, 4% delphinidin and 2% cyanidin-3-glucoside were analyzed at 200-700 nm. The maximum absorption wavelengths of 4% delphinidin peaked at 291 and 564 nm (Fig. 1A), whereas those of 2% cyanidin-3-glucoside peaked at 279 and 517 nm (Fig. 1B). Thus, delphinidin and cyanidin-3-glucoside absorb both ultraviolet light (100-400 nm) and visible light (400-700 nm). Essentially all anthocyanins have maximum absorption wavelengths between 500 and 540 nm (Suzuki et al. 2002); therefore, it is conceivable that the cyanidin-3-glucoside maximum absorption wavelength at 517 nm is specific for anthocyanins (Fig. 1B). Water extracts of LBB, HBB, CB and grape had maximum absorption wavelengths at 284, 283, 276 and 281 nm, respectively (Fig. 1C-F). The berry extract spectra did not have significant absorption peaks at visible light wavelengths (400-700 nm) or at anthocyanin-specific wavelengths (500-540 nm). A slight maximum absorption was observed at 531-532 nm in the HBB spectrum (Fig. 1D) and at 505 nm in the CB spectrum (Fig. 1E). These results indicate that the berry extracts efficiently absorb UV wavelengths, primarily UV-B.

2. Effect of continuous UV-B (+) on the growth of cucumber seedlings pretreated with the berry extracts

First, the effects of berry extracts on the subsequent growth of farm crops were evaluated. The berry extracts were sprayed on cucumber cotyledons uniformly once a day for three days, and the seedlings were grown under UV-B (–) for 11 days. Cotyledon area gradually increased during 11 days for control plants (Fig. 2A). For plants treated with LBB, HBB and CB extracts, cotyledon areas were not significantly different from those of control plants during growth under UV-B (–) for 11 days (Fig. 2A). For plants treated with grape extract, cotyledon area declined during 7-11 days compared with that of control plants ($P < 0.01$) (Fig. 2A). Cotyledons became yellow, brown and necrotic during the course of the experiment after pretreatment with grape extract (data not shown). This was considered a side effect. These results suggest that the LBB, HBB and CB extracts did not affect cucumber cotyledon growth under UV-B (–). The first leaf area increased during 11 days for control plants (Fig. 2B). Areas of the first leaves in plants treated with LBB, HBB, CB and grape extracts did not differ significantly from those of control plants during 11 days of UV-B (–) (Fig. 2B). The berry extracts were sprayed on cucumber cotyledons; therefore, the side effects of grape extract that were observed on cotyledons were not observed on the first leaves. These results show that applications of LBB, HBB, CB and grape extracts on cucumber cotyledons do not affect the growth of first leaves in cucumber plants under UV-B (–).

Next, we investigated the effects of continuous UV-B (+) on the growth of farm crops that had been pretreated with berry extracts. Water extracts of the four berries were sprayed on cucumber cotyledons uniformly once a day for three days, and continuous UV-B (+)

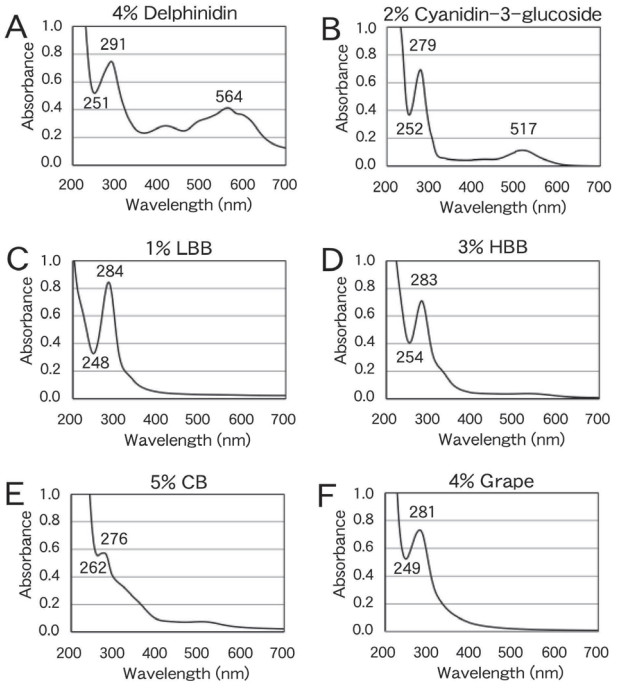

Fig. 1. Light absorption spectra between 200 and 700 nm of 4% delphinidin (A), 2% cyanidin-3-glucoside (B), and diluted extracts of LBB at 1% (C), HBB at 3% (D), CB at 5% (E) and grape at 4% (F). Numbers indicate maximum or minimum absorption wavelength.

was conducted for 11 days starting on the same day as the third berry extract treatment (designated as day 0). Plant growth was determined by measuring the areas of cotyledons on days 0, 1, 3, 5, 7, 9 and 11 after continuous UV-B (+). For control plants, cotyledon area increased during the first five days and decreased during 5-9 days, and all seedlings perished at day 11 [Fig. 3A (a) and Fig. 4A-D (Control)]. For plants that were pretreated with LBB, HBB and CB extracts (Fig. 3B-D), only a few plants perished by day 11 [Fig. 3B (b), D (c)]. This trend was confirmed in two replicate experiments (data not shown). For plants pretreated with the LBB, HBB and CB extracts [Fig. 4A-C (Undiluted solution)], cotyledon areas increased for seven days and decreased slightly during 7-11 days. The cotyledon area during 3-5 days for plants pretreated with LBB extract [Fig. 4A (Undiluted solution)] was greater than that of control plants ($P < 0.05$) [Fig. 4A (Control)]. The cotyledon area at day 5 for plants pretreated with HBB extract [Fig. 4B (Undiluted solution)] was greater than that of control plants ($P < 0.05$) [Fig. 4B (Control)]. The cotyledon area at day 5 for plants pretreated with CB extract [Fig. 4C (Undiluted solution)] was greater than that of control plants ($P < 0.05$) [Fig. 4C (Control)]. The rate of cotyledon area decline during 7-11 days for plants pretreated with LBB, HBB and CB extracts [Fig. 4A-C (Undiluted solution)] was lower than that of control plants ($P < 0.01$) [Fig. 4 A-C (Control)]. For plants pretreated with grape extract [Fig. 4D (Undiluted solution)], cotyledon area increased for five days and declined thereafter, but the rate of decline was less than that for control plants ($P < 0.01$) [Fig. 4D (Control)]. However, cotyledons became yellow, brown and necrotic during the course of the experiment after pretreatment with grape extract [Fig. 3E (d)]. This was considered a side effect similar to that described in the previous section. To summarize, these experiments indicate that the LBB, HBB and CB extracts (Undiluted solution) protected cucumber cotyledons and attenuated subsequent UV-B-induced damage during 11 days of continuous irradiation. No adverse side effects of these three berry extracts were observed.

Third, the effects of continuous UV-B (+) on cucumber cotyledons pretreated with diluted extracts

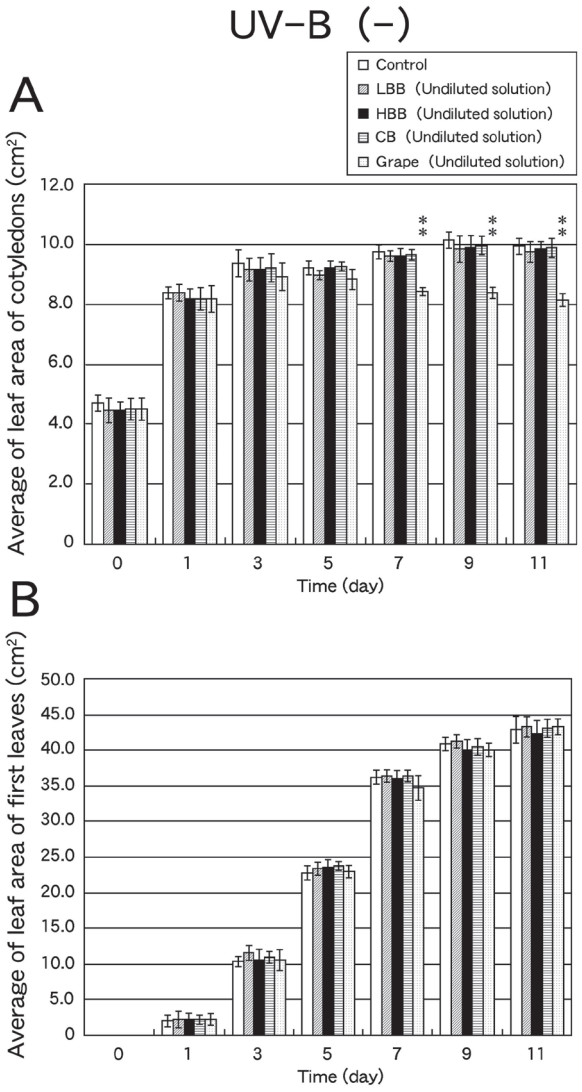

Fig. 2. Effect of LBB, HBB, CB and grape extracts on the leaf area of cotyledons (A) and first leaves (B) without continuous UV-B (+) for 11 days.
Values represent the average of sixteen cotyledons (A) and eight first leaves (B) from eight plants. Statistically significant differences were determined by Dunnett's test (**, P < 0.01 vs. Control).

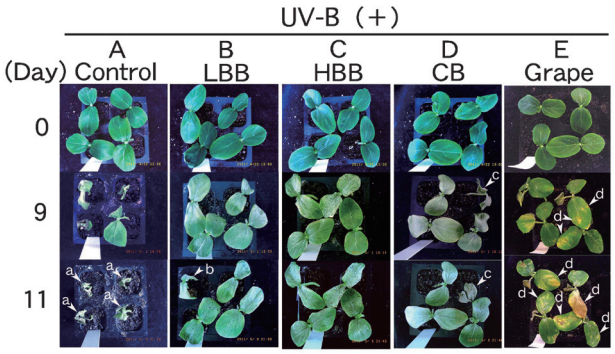

Fig. 3. Effect of 11-day continuous UV-B (+) on cucumber seedling growth.
Cotyledons were pretreated with distilled water containing 0.1% Tween 20 as control (A) or extracts of LBB (B), HBB (C), CB (D) and grape (E). UV-B-induced plant death is shown with arrowheads (a)-(c) on days 9 and 11: control (a), LBB extract (b) and CB extract (c). The cotyledons that become yellow, brown and necrotic are shown with arrowheads (d) on days 9 and 11: grape extract (d).

(12.5%, 25.0% and 50.0%) of LBB, HBB and CB were analyzed. Grape extract was not used due to its side effect [Fig. 3E (d)]. During the first five days under continuous UV-B (+), the cotyledon area of cucumber seedlings pretreated with 12.5% and 25.0% dilutions of LBB, HBB and CB extracts, and 50.0% dilution of HBB and CB extracts did not significantly differ from those of control plants (Fig. 4A-C). The cotyledon area at day 5 for plants pretreated with 50.0% dilution of LBB extract was greater than that of control plants ($P < 0.05$) (Fig. 4A). The rate of cotyledon area decline was lower in seedlings pretreated with 25.0% and 50.0% dilutions of LBB and HBB extracts and 50% dilution of CB extract during 7-11 days of continuous UV-B (+) compared with those of control seedlings ($P < 0.01$) (Fig. 4A-C). Thus, the rate of cotyledon area decline was lower in seedlings pretreated with diluted extracts of LBB, HBB or CB during 11 days of continuous UV-B (+) than in the control seedlings. The effect of each extract was dose-dependent. These results suggest that diluted extracts of LBB, HBB and CB protected cucumber cotyledons and attenuated subsequent UV-B-induced damage in a dose-dependent manner.

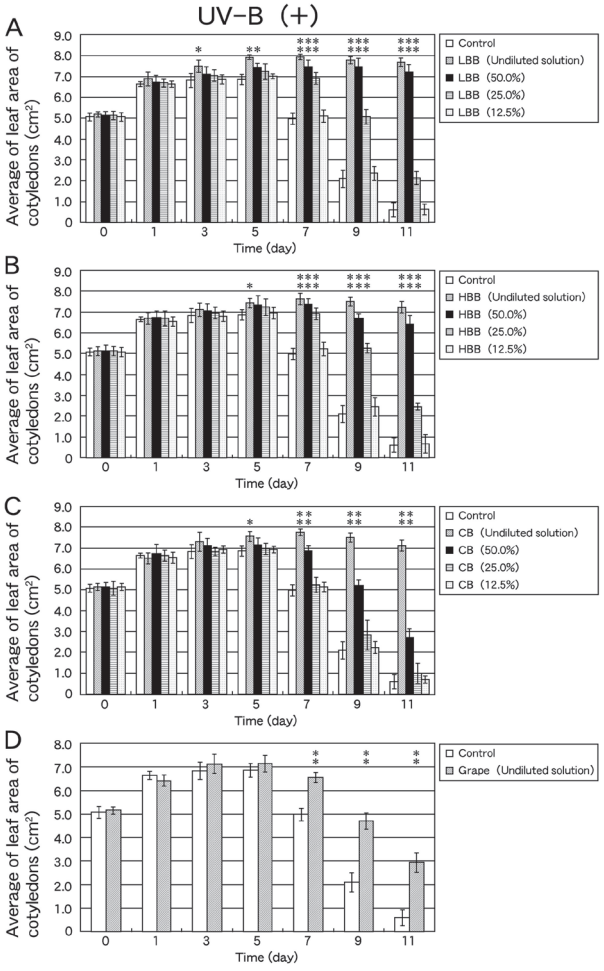

Fig. 4. Effect of 11-day continuous UV-B (+) on cotyledon area after pretreatment with pure and diluted (12.5%, 25.0% and 50.0%) extracts of LBB (A), HBB (B), CB (C) and grape (D).
Values represent the average of sixteen cotyledons from eight plants. Statistically significant differences were determined by Dunnett's test (*, $P < 0.05$ vs. Control; **, $P < 0.01$ vs. Control).

3. Component analyses of the berry extracts

To determine the components that absorb ultraviolet light in berry extracts, the contents of total polyphenols, total anthocyanins and 51 specific polyphenols were analyzed. The total polyphenol contents in the LBB, HBB, CB and grape extracts were 840.3 ± 9.4, 498.3 ± 8.6, 452.7 ± 12.6 and 458.3 ± 10.5 μg g^{-1}, respectively (Table 2). Therefore, the total polyphenol content in the LBB extract was 1.69-, 1.86- and 1.83-fold greater than those in the HBB, CB and grape extracts, respectively. The total anthocyanin content was analyzed by HPLC equipped with ultraviolet and visible spectrophotometry. Total anthocyanin contents in all berry extracts were lower than the detection limit (0.5 μg g^{-1}; Table 2). This result is consistent with the observation that none of the berry extracts had clear maximum absorption peak wavelengths between 500 and 540 nm, which are specific for anthocyanins (Fig. 1C-F). Next, the contents of 51 specific polyphenols in berry extracts were analyzed by HPLC equipped with the CoulArray detection system. Polyphenol classification was performed according to a previously published method (Sakakibara et al. 2003). In Table 2, the top three polyphenolic components in each berry extract are shaded in gray. The LBB extract contained primarily chlorogenic acid, caffeic acid and protocatechuic acid. The HBB extract contained primarily chlorogenic acid, caffeic acid and syringic acid. The CB extract contained primarily protocatechuic acid, myricetin and p-coumaric acid. The grape extract contained primarily protocatechuic acid, caffeic acid and p-coumaric acid. Chlorogenic acid was detected without performing hydrolysis, whereas caffeic acid was detected after performing hydrolysis. Therefore, caffeic acid contains the hydrolysis products of chlorogenic acid. The amounts of 42 other polyphenols in the four berry extracts were below the detection limit (0.1 μg g^{-1}; data not shown).

Discussion

In agriculture, the increase of solar UV-B reduces farm productivity. Therefore, protection against UV-B

Table 2. Contents of 10 specific polyphenols (9 polyphenols and total anthocyanins) and total polyphenols in LBB, HBB, CB and grape extracts.

		Polyphenols	LBB	HBB	CB	grape
Simple Polyphenols						
	benzoic acids	protocatechuic acid	9.6	4.2	8.3	2.3
		syringic acid	6.5	4.3	N.D.[z]	N.D.[z]
		vanillic acid	1.7	N.D.[z]	1.4	N.D.[z]
	cinnamic acids	chlorogenic acid	44.7	53.7	4.8	N.D.[z]
		caffeic acid	35.4	27.5	2.9	2.3
		ferulic acid	2.8	4.1	2.2	0.9
		p-coumaric acid	1.0	1.7	6.3	1.0
Flavonoids						
	flavonols	quercetin	3.2	N.D.[z]	1.5	N.D.[z]
		myricetin	N.D.[z]	1.6	8.3	N.D.[z]
	total anthocyanins		N.D.[y]	N.D.[y]	N.D.[y]	N.D.[y]
Total Polyphenols			840.3±9.4	498.3±8.6	452.7±12.6	458.3±10.5

μg g-1

The top three components in each berry extract are shaded in gray. The amounts of 42 other polyphenols in the four berry extracts were below the detection limit (0.1 μg g^{-1}).
[z] N.D.: Not detected under the quantitative lower limit of 0.1 μg g^{-1}.
[y] N.D.: Not detected under the quantitative lower limit of 0.5 μg g^{-1}.

irradiation poses an important problem for higher plants including farm crops. Several mutants resistant to UV-B irradiation have been isolated in *Arabidopsis* (Bieza & Lois 2001, Tanaka et al. 2002, Fujibe et al. 2004, Hase et al. 2006). However, the introduction of homologous mutations to increase UV-B tolerance in other higher plants including farm crops is unrealistic. To protect many farm crops against UV-B irradiation, a new method other than gene mutation should be established. In the present study, we tried to develop in the laboratory a simple method of protecting farm crops against the damage caused by UV-B irradiation. To achieve this objective, we investigated whether LBB, HBB, CB and grape extracts could confer protection against UV-B irradiation for cucumber cotyledons. The treatment of cucumber cotyledons with LBB, HBB and CB extracts and their diluted extracts attenuated subsequent UV-B-induced damage in a dose-dependent manner without causing any observable side effects for 11 days (Fig. 3A-D, Fig. 4A-C). The LBB, HBB and CB extracts did not promote the growth of cucumber seedlings under UV-B (–) (Fig. 2A, B). Therefore, it is reasonable to consider that the LBB, HBB and CB extracts are effective in attenuating the UV-B-induced damage of cucumber cotyledons, but lack any effects to accelerate or decelerate the growth of cucumber plants. Thus, we could develop in the laboratory a simple method of protecting farm crops against the damage caused by UV-B irradiation. This simple method would help to inhibit the reduction of farm productivity caused by solar UV-B in agriculture. For practical use of this method, whether it is effective in protecting farm crops outdoors over a long term against solar UV-B irradiation must be investigated.

The LBB, HBB and CB extracts absorbed ultraviolet light, primarily UV-B, but did not absorb visible light (400-700 nm) (Fig. 1C-E). Therefore, it is conceivable that treatment of the three berry extracts applied to higher plants will not disturb photosynthesis that leads to plant growth. This idea is supported by the result that treatment of the LBB, HBB and CB extracts did not inhibit the growth of cucumber seedlings under UV-B (–) (Fig. 2A, B). Thus, the LBB, HBB and CB extracts are efficient absorbers of UV-B for higher plants.

To determine the components that absorb UV-B in berry extracts, the contents of total polyphenols, total anthocyanins and 51 specific polyphenols were analyzed. In higher plants, anthocyanins are flavonoids that are major UV-absorbing compounds (Bieza & Lois 2001, Hada et al. 2003, Fujibe et al. 2004). In the present study, the total anthocyanin contents in all four berry extracts were lower than the detection limit (0.5 μg g^{-1}; Table 2), and flavonoids were not the primary polyphenols in the

LBB, HBB and grape extracts (Table 2). Our protocol utilized a neutral solvent for extraction (distilled water, pH 7.5) because water extracts of natural substances are simple to prepare and safe for ingestion or topical use. Anthocyanins are stable and have maximum absorption wavelengths of 500-540 nm in acidic solvents, but are not stable in neutral and alkaline solvents (Ono et al. 2003). This could explain the undetectable levels of anthocyanins in our berry extracts. Thus, anthocyanins—major UV-absorbing compounds in higher plants—were not the primary polyphenolic compounds in the four berry extracts. The myricetin (a flavonoid) content was highest among 52 polyphenols (51 polyphenols and total anthocyanins) only in CB extract (Table 2). Myricetin may be the primary absorber of UV-B in CB extracts and may confer protection against UV-B-induced damage in cucumber cotyledons. The most abundant compounds in the extracts in addition to myricetin are chlorogenic acid, caffeic acid, *p*-coumaric acid, protocatechuic acid and syringic acid (gray cells in Table 2). These compounds can absorb UV-B (Lavola et al. 1997, Rozema et al. 2001, Cornard & Lapouge 2006, Sun et al. 2007, Zazza & Sanna 2010). Therefore, it is conceivable that these compounds are responsible for protecting cucumber cotyledons against UV-B-induced damage caused by UV-B absorption. In particular, because the contents of chlorogenic acid and caffeic acid are greater than those of other polyphenols, both are the main candidates for the absorption of UV-B.

Chlorogenic acid is an ester of caffeic acid and quinic acid. Chlorogenic acid and caffeic acid are nonflavonoid catecholic compounds that are present in many plants (Moridani et al. 2001). Catecholic acids are reported to have anti-inflammatory, antimutagenic, antioxidant and anticarcinogenic activities (Moridani et al. 2001). Therefore, in addition to UV-B absorption, the antioxidant activities of chlorogenic acid and caffeic acid might contribute to the attenuation of UV-B-induced damage in cucumber cotyledons. Further work is required to determine the exact mechanism of berry extract protection against UV-B-induced damage in cucumber plants.

Acknowledgements

This work was partially supported by the Japan Society for the Promotion of Science, a Grant-in-Aid for Scientific Research (C) (No. 15K07292 to S.Y.), the 5th Nissan Science Foundation and the Saito Gratitude Foundation.

References

Bieza, K. & Lois, R. (2001) An *Arabidopsis* mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. *Plant Physiol*., **126**, 1105-1115.

Cassinese, C. et al. (2007) New liquid chromatography method with ultraviolet detection for analysis of anthocyanins and anthocyanidins in *Vaccinium myrtillus* fruit dry extracts and commercial preparations. *J. AOAC Int*., **90**, 911-919.

Cornard, J. P. & Lapouge, C. (2006) Absorption spectra of caffeic acid, caffeate and their 1:1 complex with Al (III): Density functional theory and time-dependent density functional theory investigations. *J. Phys. Chem*., **110**, 7159-7166.

Fujibe, T. et al. (2004) A methyl viologen-resistant mutant of *Arabidopsis*, which is allelic to ozone-sensitive *rcd1*, is tolerant to supplemental ultraviolet-B irradiation. *Plant Physiol*., **134**, 275-285.

Hada, H. et al. (2003) Higher amounts of anthocyanins and UV-absorbing compounds effectively lowered CPD photorepair in purple rice (*Oryza sativa* L.). *Plant Cell Environ*., **26**, 1691-1701.

Hase, Y. et al. (2006) A mutation in the *uvi4* gene promotes progression of endoreduplication and confers increased tolerance towards ultraviolet B light. *Plant J*., **46**, 317-326.

Hertog, M. G. L. et al. (1992) Optimization of quantitative HPLC determination of potentially anticarcinogenic flavonoids in fruits and vegetables. *J. Agric. Food Chem*., **40**, 1591-1598.

Ivanova, V. et al. (2010) Determination of the polyphenol contents in Macedonian grapes and wines by standardized spectrophotometric methods. *J. Serb. Chem. Soc*., **75**, 45-59.

Krizek, D. T. et al. (1997) Inhibitory effects of ambient levels of solar UV-A and UV-B radiation on growth of cucumber. *Physiol. Plant*., **100**, 886-893.

Lavola, A. et al. (1997) The effect of u.v.-B radiation on u.v.-absorbing secondary metabolites in birch seedlings grown under simulated forest soil conditions. *New Phytol*., **137**, 617-621.

Moridani, M. Y. et al. (2001) Caffeic acid, chlorogenic acid, and dihydrocaffeic acid metabolism: glutathione conjugate formation. *Drug Metab. Dispos*., **29**, 1432-1439.

Ono, K. & Huang, S. A. (2001) Identification of anthocyains in Japanese black rice. *Res. Bull. Gifu City Women's College*, **51**, 135-138.

Ono, K. et al. (2003) An examination of optimal extraction solvents for anthocyanin pigments from black rice produced in Gifu. *Res. Bull. Gifu City Women's College*, **52**, 135-138.

Rodriguez-Mateos, A. et al. (2012) Procyanidin, anthocyanin, and chlorogenic acid contents of highbush and lowbush blueberries. *J. Agric. Food Chem*., **60**, 5772-5778.

Rozema, J. et al. (1997) UV-B as an environmental factor in plant life: Stress and regulation. *Trends Ecol. Evol*., **12**, 22-28.

Rozema, J. et al. (2001) UV-B absorbance and UV-B absorbing compounds (para-coumaric acid) in pollen and sporopollenin: the perspective to track historic UV-B levels. *J. Photochem. Photobiol. B*., **62**, 108-117.

Sakakibara, H. et al. (2003) Simultaneous determination of all

polyphenols in vegetables, fruit, and teas. *J. Agric. Food Chem*., **51**, 571-581.

Shinkle, J. R. et al. (2004) Growth and morphological responses to different UV wavebands in cucumber (*Cucumis sativum*) and other dicotyledonous seedlings. *Physiol. Plant*., **120**, 240-248.

Singh, A. P. et al. (2009) Isolation of specific cranberry flavonoids for biological activity assessment. *Food Chem*., **116**, 963-968.

Sun, J. et al. (2007) Screening non-colored phenolics in red wines using liquid chromatography/ultraviolet and mass spectrometry/mass spectrometry libraries. *Molecules*, **12**, 679-693.

Suzuki, K. et al. (2002) Survey on quality of exiting food additives used as ingredients of health food (Tomato color, anthocyanin colors, psyllium seed gum). *Ann. Rep. Tokyo Metr. Res. Lab. P.H*., **53**, 169-172.

Takeuchi, Y. et al. (1996) Adaptive alterations in the activities of scavengers of active oxygen in cucumber cotyledons irradiated with UV-B. *J. Plant Physiol*., **147**, 589-592.

Tanaka, A. et al. (2002) An ultraviolet-B-resistant mutant with enhanced DNA repair in *Arabidopsis*. *Plant Physiol*., **129**, 64-71.

Teramura, A. H. (1983) Effects of ultraviolet-B radiation on the growth and yield of crop plants. *Physiol. Plant*., **58**, 415-427.

Teramura, A. H. & Sullivan, J. H. (1994) Effects of UV-B radiation on photosynthesis and growth of terrestrial plants. *Photosynth. Res*., **39**, 463-473.

Yamasaki, S. et al. (2007) Continuous UV-B irradiation induces morphological changes and the accumulation of polyphenolic compounds on the surface of cucumber cotyledons. *J. Rad. Res*., **48**, 443-454.

Yamasaki, S. et al. (2015) Continuous UV-B irradiation suppresses the expression of cell cycle-related genes in cucumber (*Cucumis sativus* L.) cotyledons. *Saito Ho-on Kai Mus. Nat. Hist. Res. Bull*., **79**, 37-46.

Zazza, C. & Sanna, N. (2010) Photoabsorption spectra of a natural polyphenol compound for therapeutic applications: the protocatechuic acid in dilute water solution at room temperature. *Phys. Chem. Chem. Phys*., **12**, 3859-3863.

Ziska, L. H. et al. (1992) Physiological sensitivity of plants along an elevational gradient to UV-B radiation. *Am. J. Bot*., **79**, 863-871.