JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 467−474

pubs.acs.org/JAFC

Development of Betalain Producing Callus Lines from Colored Quinoa Varieties (*Chenopodium quinoa* Willd)

Paula Henarejos-Escudero,[†,§] Berenice Guadarrama-Flores,[†,§] M. Alejandra Guerrero-Rubio,[†] Luz Rayda Gómez-Pando,[‡] Francisco García-Carmona,[†] and Fernando Gandía-Herrero*[,†]

[†]Departamento de Bioquímica y Biología Molecular A, Unidad Docente de Biología, Facultad de Veterinaria. Regional Campus of International Excellence "Campus Mare Nostrum". Universidad de Murcia, 30100 Murcia, Spain

[‡]Cereal Research Program, National Agricultural University La Molina, Lima, Peru

Supporting Information

ABSTRACT: Betalains are water-soluble plant pigments of hydrophilic nature with promising bioactive potential. Among the scarce edible sources of betalains is the grain crop quinoa (*Chenopodium quinoa* Willd), with violet, red, and yellow grains being colored by these pigments. In this work, callus cultures have been developed from differently colored plant varieties. Stable callus lines exhibited color and pigment production when maintained on Murashige and Skoog medium supplemented with the plant growth regulators 6-benzylaminopurine (8.88 μM) and 2,4-dichlorophenoxyacetic acid (6.79 μM) with a reduction of the nitrogen source to 5.91 mM. Pigment analysis by HPLC-DAD and ESI-MS/MS fully describes the content of individual pigments in the cell lines and allows the first report on the pigments present in quinoa seedlings. Phyllocactin and vulgaxanthin I are described as novel pigments in the species and show the potential of *C. quinoa* culture lines in the production of compounds of nutritional value.

KEYWORDS: *betalains, bioactive, cell culture, pigments, quinoa, secondary metabolism*

INTRODUCTION

Betalains are nitrogenous plant pigments which are characteristic of plants belonging to the order Caryophyllales. They are divided into two structural groups: the yellow betaxanthins and the violet betacyanins. Both groups share betalamic acid as the structural and chromophoric unit. It is condensed with amines and amino acids in betaxanthins and with *cyclo*-DOPA (*cyclo*-dihydroxyphenylalanine) in betacyanins.[1] Glycosylation and acylglycosilation of one or two hydroxyl groups are possible in betacyanins, and complex pigment structures can be obtained. In contrast, no glycosylation has ever been reported in betaxanthins. Betacyanins present absorbance spectra centered at wavelengths around λ_m = 536 nm, and the absorbance spectra of betaxanthins are centered at wavelengths around λ_m = 480 nm.[2] The joint presence of both types of pigments makes orange and red shades found in nature along with the pure yellow and violet colors. Betalains substitute anthocyanins and their roles in the colored tissues of the plant families that synthesize them being both families of water-soluble pigments mutually exclusive.[3]

Betalains have shown promising bioactive potential in recent years.[4] The first investigations that demonstrated a radical scavenging capacity in betalains were carried out separately with betacyanins and betaxanthins, extracted from *Beta vulgaris*.[5] Subsequent research revealed the existence of an intrinsic activity present in all betalains that is modulated by structural factors.[2,6] Recent work with purified compounds have shown a strong cancer chemopreventive activity[7] that support the previous studies with different cancer cell lines.[8,9] Also, experiments in vivo have shown that very low concentrations of dietary pigments inhibit the formation of tumors in mice.[10,11] The current evidence suggests a strong health-promoting potential of betalains.

Beet roots (*Beta vulgaris*) and the fruits of cacti belonging to the genus *Opuntia* are the best known edible sources of betalains among the Caryophyllales plants.[12,13] However, there are *Amaranthus* species and berries from *Rivina humilis* containing betalains that are consumed fresh.[14,15] The main source of betalains worldwide is beet; however, the extracts of this plant may have an unpleasant taste and odor due to the presence of pyrazines and high concentrations of nitrates.[16] *Chenopodium quinoa* Willd (quinoa) belongs to the family Amaranthaceae, subfamily Chenopodioideae, in the order Caryophyllales. It has recently been shown that varieties of the edible grains of this plant also contain betalains, both betacyanins and betaxanthins.[17−19] Quinoa was the traditional grain crop of the prehispanic civilizations in America, being used as a feedstock with an important economic and cultural background. It is a pseudocereal with high nutritional value, as it is rich in proteins, lipids, fiber, vitamins, and minerals, and has an extraordinary balance of essential amino acids.[20] Since quinoa was introduced as a gourmet grain in the international markets the grain exports have increased from 5000 to 40000 tons, mainly in the producing countries of the Andean region: Peru, Bolivia, and Ecuador.[21] However, the cultivation of quinoa is destined to the production of grain and not to obtaining the pigments. In addition, its agronomic management

Received: October 9, 2017
Revised: December 13, 2017
Accepted: December 14, 2017
Published: December 14, 2017

DOI: 10.1021/acs.jafc.7b04642
J. Agric. Food Chem. 2018, 66, 467−474

is not well established, which is reflected in the obtaining of low yields[22,23]. Furthermore, betalains, as most of secondary metabolites, represent a low content in the plant (<0.04%).[19]

The establishment of callus cultures of *Chenopodium quinoa* Willd is a production alternative that would allow one to obtain cell lines with the ability of yielding quinoa derived betalains. With the tools of plant tissue culture, production can be afforded by eliminating the disadvantages of seasonality and geographical and annual variations in the crop. Callus culture opens the possibility of establishing pigment-producing cell lines of a new species with nutritional and agronomic relevance. The present work is aimed to establish callus cultures of *Chenopodium quinoa* Willd and to characterize the production of pigments in relation to those found in grains and seedlings of quinoa.

MATERIALS AND METHODS

Chemicals. Chemicals and reagents were purchased from Sigma (St. Louis, MO, EEUU). Solvents were from Merck Chemicals Ltd. (Dorset, England). HPLC-grade acetonitrile and methanol were purchased from Labscan Ltd. (Dublin, Ireland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA).

Plant Material. *Chenopodium quinoa* Willd grains (quinoa) of the varieties BGQ-174, BGQ-77, POQ-132 and "Blanca de Junín" were obtained from the quinoa germplasm bank at the National Agricultural University La Molina (Lima, Peru). Codes used in this work correspond to the bank accession numbers except that corresponding to a known commercial variety named by its trivial name.

Establishment of Callus Cultures of Quinoa. The obtaining of seedlings of four different varieties of quinoa (BGQ-174, BGQ-77, POQ-132, and "Blanca de Junín") was as follows: sterile grains were transferred to glass Petri dishes (100 mm × 15 mm), which contained filter paper moistened with autoclaved distilled water. Environmental conditions consisted of an initial period of darkness for 8 days and temperatures of 23 °C (day) and 18 °C (night) for 16 and 8 h, respectively, afterward.

For obtaining callus culture, grains of the four different varieties of quinoa were surface sterilized in commercial liquid detergent 1% (composition: 15% anionic surfactants and below 5% nonionic surfactant) for 10 min, followed by 70% ethanol for 30 s, and by 0.6, 1.2, and 1.8% sodium hypoclorite solution containing 0.2% Tween-20 for 15 and 30 min. Grains were then washed three times with sterile water under aseptic conditions. Sterile grains were transferred to sterilized tubes (25 × 150 mm) containing 15 mL of semisolid 50% Murashige and Skoog medium (MS) and 2 g L^{-1} of the solidifying agent phytagel. Seedlings were grown up to 2 cm (8 days) and 3 cm (20 days), and then hypocotyls and young leaf segments (1 × 1.5 cm) were removed as explants source. Hypocotyls were considered as source of primary explant due to the presence of meristematic tissue containing endogenous levels of auxins and cytokinins.[24] The immature young leaves segments were also used as primary source of explant due to the endogenous presence of auxins and cytokinins.[25] BAP and 2,4-D as plant growth regulators were assayed in the ranges 0.00−8.88 and 0.00−9.05 μM, respectively.

Hypocotyls and segment leafs were transferred to individual Gerber flasks (66 mm × 59 mm), with 25 mL of 150% MS medium supplemented with 45 g L^{-1} of sucrose, phytagel (2 g L^{-1}), and different plant growth regulators (PGRs). Media were enriched with different concentrations and combinations of the PGRs 6-benzylaminopurine (BAP) (0.0, 2.22, 4.44, 6.66, 8.88 μM), and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.0, 2.26, 4.52, 6.79, 9.05 μM) in order to assess response to callus explant induction. All culture media were adjusted to pH 5.8 with 1 N NaOH and HCl and sterilized by autoclaving at 121 °C for 18 min. The cultures were incubated under a 16 h photoperiod under fluorescent light at photon flux density of 50 μmol m^2 s^{-1} and temperatures of 23 (day) and 18 °C (night). After selecting the most suitable PGRs concentrations for callus culture of *C. quinoa* derived from explants of leaf and hypocotyl (6.66 μM of BAP and 6.79 μM of 2,4-D), this combination was selected for propagation by subcultures on fresh medium every 30 days for 2 subculture cycles. Further propagation was done under the same conditions in fresh MS medium every 30 days for 5 subculture cycles. For pigment induction, the nitrogen source was decreased (3.09 mM NH_4NO_3 and 2.82 mM KNO_3), the 2,4-D concentration was kept constant (6.79 μM), and the BAP concentration was increased (8.88 μM) in the MS medium.

Preparation of Extracts. Portions of callus samples (around 100 mg) and 8 day old seedlings (around 25 mg) of the different varieties of *Chenopodium quinoa* Willd were exactly weighted and manually homogenized in 500 μL of 20 mM acetate buffer pH 5.0 containing 10 mM ascorbic acid. The resulting homogenates were centrifuged at 14000*g* for 15 min. The supernatant of each sample was then used for HPLC analysis of betalamic pigments (n = 3). The whole process was carried out at 4 °C.

Standard Betalains. Known pigments extracted from characterized plant sources and semisynthetic molecules obtained by following a previously established method,[26] were used as standard betalains. Betanin was obtained from roots of *Beta vulgaris*, betanidin was obtained from violet flowers of *Lampranthus productus*,[27] amaranthin was extracted from *Celosia argentea*,[28] and phyllocactin was extracted from violet flowers of Christmas cactus (*Schlumbergera* × *buckleyi*).[29] Betaxanthins were obtained by a combined procedure of betalamic acid release and further condensation with amines and amino acids.[26] All compounds were characterized spectrophotometrically, chromatographically, and by electrospray ionization mass spectrometry (ESI-MS/MS).

Anion Exchange Chromatography. Anion exchange chromatography for the purification of betalains was performed in an Äkta purifier apparatus (Amersham Biosciences, Uppsala, Sweden) using a 5 mL Q-Sepharose Fast Flow column (cross-linked agarose with quaternary ammonium as exchanger group, 90 μm particle size). Solvents used were sodium phosphate 20 mM, pH 6.0 (solvent A), and 20 mM sodium phosphate, pH 6.0, with 2 M NaCl (solvent B). After sample injection, the elution process was as follows: 0% B from beginning to 20 mL, and then a linear gradient was developed from 0% B to 16,5% B in 50 mL. The flow rate was 2 mL min^{-1}, 3 mL fractions were collected, and injection volume was 5 mL. Elutions were followed by UV−vis detection at 280, 480, and 536 nm.

Solid Phase Extraction. One milliliter C-18 cartridges (Waters, Milford, MA) were conditioned with 5 mL of ethanol followed by 10 mL of purified water. Aqueous solutions of extracted pigments and semisynthetic betalains were injected and bound to the minicolumn. Salts and buffers were washed off by rinsing the column with purified water. Betalains were eluted with ethanol, and the resulting fraction was evaporated to dryness under reduced pressure at room temperature. The residue was redissolved in water for further use or stored at −80 °C.

UV−Vis Spectroscopy. A V-630 spectrometer (Jasco Corporation, Tokyo, Japan) attached to a Tectron thermostatic bath (JP Selecta, Barcelona, Spain) was used for UV−vis spectroscopy. For quantitation of betalains, pigment concentration was evaluated using molar extinction coefficients of ε = 48000 M^{-1} cm^{-1} at 480 nm for betaxantins, ε = 54000 M^{-1} cm^{-1} at 536 nm for betanidin, and ε = 65000 M^{-1} cm^{-1} at 536 nm for betanin, amaranthin, and phyllocactin. All measurements were performed in water at 25 °C.

HPLC-DAD Analysis. A Shimadzu LC-20AD apparatus (Kyoto, Japan) equipped with a SPD-M20A photodiode array detector (DAD) was used for analytical HPLC separations. Reversed phase chromatography was performed with a 250 mm × 4.6 mm i.d., 5 μm, Kromasil 100 C-18 column (Teknokroma, Barcelona, Spain) for identification of betalains.[30] Solvent A was water with 0.05% trifluoroacetic acid (TFA), and solvent B was composed of acetonitrile with 0.05% TFA. A linear gradient was performed for 35 min from 0% B to 35% B. The flow rate was 1 mL min^{-1}, operated at 25 °C. Elutions were followed at λ = 480 nm (betaxanthins) and 536 nm (betacyanins). Injection volume was 40 μL.

Quantitation of betalains was carried out by calibration curves performed with previously described pure betalain standards. All

DOI: 10.1021/acs.jafc.7b04642
J. Agric. Food Chem. 2018, 66, 467−474

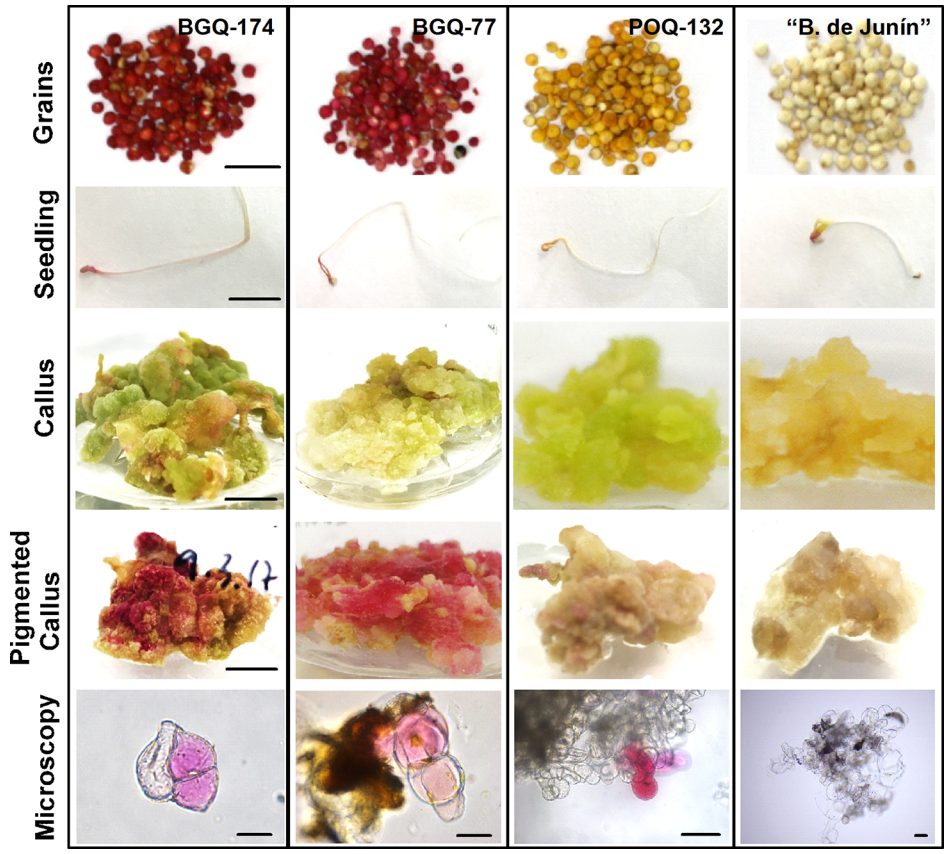

Figure 1. Development of plant cultures for quinoa varieties BGQ-174, BGQ-77, POQ-132 and "Blanca de Junín". Macroscopic images represent grains, seedlings, and callus lines of all the different varieties of *Chenopodium quinoa* Willd considered in this study. Scale bar for macroscopic images is 1 cm. Microscopy images show brightfield images of callus cells after pigment induction. In this case, scale bars represent 50 μm.

experiments were performed in triplicate (n = 3), and the results were expressed as mean values and standard deviations (SD). Data analysis was carried out by linear regression adjustment by Sigma Plot Scientific Graphing for Windows version 10.0 (2006; Systat Sofware, San Jose, CA).

Electrospray Ionization Mass Spectrometry. A VL 1100 apparatus with LC/MSD Trap (Agilent Technologies, Palo Alto, CA) was used for HPLC-ESI-MS/MS analyses. Elution conditions were as described above using the same column with a flow rate of 0.8 mL min^{-1}. Vaporizer temperature was 350 °C, and voltage was maintained at 3.5 kV. The sheath gas was nitrogen, operated at a pressure of 45 psi. Samples were ionized in positive mode. Ion monitoring mode was full scan in the range m/z 50−1200. The electron multiplier voltage for detection was 1350 V.

Microscopy. Brightfield and fluorescence microscopy were performed in a Leica DM 2500 LED microscope fitted with a Leica DFC550 camera (Leica Microsystems, Wetzlar, Germany) with incident light beam. The fluorescent dye DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) was used as nuclear marker. In the case of fluorescence imaging, the filtercube A (Leica Microsystems) was used limiting excitation to the range λ = 360−340 nm and emission to λ = 425 nm.

RESULTS AND DISCUSSION

Selection of Grain Varieties of *Chenopodium quinoa* Willd. Grains of the varieties BGQ-174, BGQ-77, POQ-132 and "Blanca de Junín" were selected from the quinoa germplasm bank of Peru. They represent all the possible variety of colors and pigment content in quinoa grains as recently characterized.[19] Purple quinoa grains mainly contain betacyanins and the variety BGQ-77 was selected as it showed the most violet hue in CIELAB color space analyses among all the quinoa grains studied and contained the highest amount of the betacyanin pigment amaranthin. Red quinoa grain varieties show this color due to the coexistence of betaxanthins and betacyanins. The variety BGQ-174 was selected because it contains a high amount of the major betacyanin amaranthin, the highest amount of the betaxanthin dopaxanthin, and the highest antioxidant capacity of all quinoa grains studied. Yellow quinoa grain varieties mainly contain betaxanthins. POQ-132 was selected because it was the yellow variety with the highest content of dopaxanthin and it did not contain any betacyanin. Among the most common white varieties, "Blanca de Junín" was selected because it did not present betalamic pigments and it is a commercial variety.[19] Absorbance spectra for aqueous extracts of all the selected varieties are shown in Supporting Information Figure 1.

Table 1. Disinfection Treatments Evaluated in the Sterilization of *C. quinoa* Grains

	conditions			
	ethanol 70%	NaClO (+0.2% Tween-20)		
treatment	time (s)	%	time (min)	contamination (%)
1	30	0.6%	15	70
2	30	0.6%	30	60
3	30	1.2%	15	40
4	30	1.2%	30	20
5	30	1.8%	15	15
6	30	1.8%	30	0

DOI: 10.1021/acs.jafc.7b04642
J. Agric. Food Chem. 2018, 66, 467−474

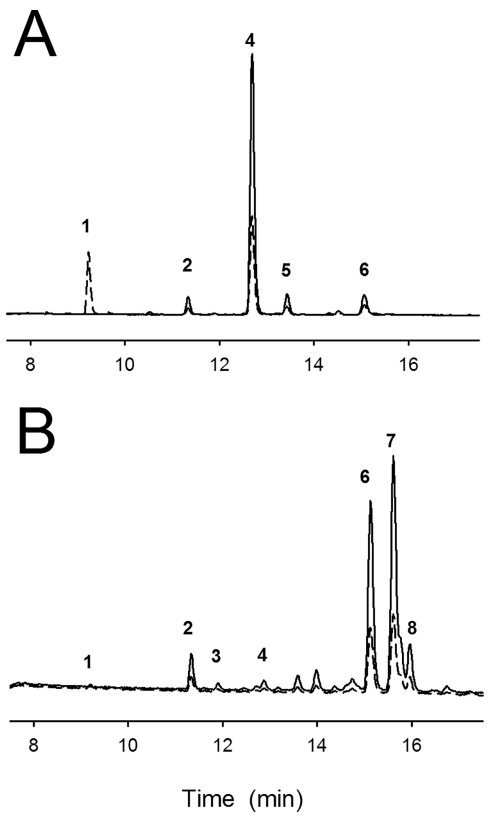

Figure 2. HPLC recordings for the aqueous extracts of callus cell line (A) and seedling (**B**) of the quinoa variety BGQ-174. Continuous line shows the signal registered at λ = 536 nm, dashed line at λ = 480 nm. Full scale is 150 mAU for panel (A) and 30 mAU for (B). Injection volume was 40 μL. Pigments shown are vulgaxanthin I (**1**), amaranthin (**2**), iso-amaranthin (**3**), betanin (**4**), iso-betanin (**5**), betanidin (**6**), phyllocactin (7), and iso-betanidin (**8**).

Establishment of Aseptic Conditions. To the establishment of aseptic cultures, six disinfection treatments were carried out on quinoa grains by varying the sodium hypochlorite concentration and the time of that stage of the process. Results are shown in Table 1, and it can be seen that the quinoa grains treated with 1% commercial liquid detergent for 10 min, followed by 70% ethanol for 30 s and by a solution of 1.8% sodium hypochlorite and 0.2% Tween-20 for 30 min did not present any contamination. Treatments with reduced time of exposition to the hypochlorite solution resulted in different grades of contamination. The grains of quinoa are fruits and they possess an outer covering which comprises a pericarp in addition to a seed coat. These different layers may explain the difficulties in obtaining sterile plant material. The hardest treatment was then established for subsequent disinfection protocols of quinoa grains used to obtain explants for each experimental trial. Each treatment was evaluated with lots of five units in triplicate.

Development of *Chenopodium quinoa* Willd Callus Lines. The treatment containing 6.66 μM BAP and 6.79 μM 2,4-D showed the highest cell growth, with clear green calli obtained from the pigmented grain varieties BGQ-174, BGQ-77, and POQ-132. White calli were produced from the white variety "Blanca de Junín" (Figure 1), both from hypocotyl and leaf explants. Although the colorations obtained did not indicate the presence of betalains, this medium was selected for propagation of the callus lines due to cell growth and

Table 2. Pigments Identified in the Different Varieties of *Chenopodium quinoa* Willd Callus Cell Lines and Seedlings Considered in This Study[a]

					callus lines				seedlings		
trivial name	peak	R_t (min)	ESI-MS (m/z)	λ_{max} (nm)	BGQ-174 (μg g^{-1})	BGQ-77 (μg g^{-1})	POQ-132 (μg g^{-1})	"B. de Junín" (μg g^{-1})	BGQ-174 (μg g^{-1})	BGQ-77 (μg g^{-1})	POQ-132 (μg g^{-1})	"B. de Junín" (μg g^{-1})
vulgaxanthin I	1	9.20	340.2	470	0.98 ± 0.25	0.07 ± 0.03	0.27 ± 0.16	nd[b]	0.18 ± 0.02	0.25 ± 0.03	0.34 ± 0.08	0.30 ± 0.06
amaranthin	2	11.30	727.0	535	1.15 ± 0.04	0.33 ± 0.1	0.26 ± 0.09	nd	1.46 ± 0.14	2.09 ± 0.10	1.63 ± 0.06	2.00 ± 0.36
iso-amaranthin	3	11.80	727.0	535	0.42 ± 0.09	nd	nd	nd	1.16 ± 0.36	3.81 ± 0.80	2.08 ± 0.45	1.80 ± 0.24
betanin	4	12.68	551.0	535	9.55 ± 0.97	2.80 ± 0.44	2.11 ± 0.18	nd	0.76 ± 0.06	1.10 ± 0.11	1.16 ± 0.27	1.09 ± 0.13
iso-betanin	5	13.40	551.0	535	0.88 ± 0.19	0.47 ± 0.15	0.47 ± 0.06	nd	0.99 ± 0.15	0.32 ± 0.04	0.27 ± 0.01	0.27 ± 0.01
betanidin	6	15.10	389.0	542	0.79 ± 0.08	0.40 ± 0.14	0.50 ± 0.07	nd	2.33 ± 0.89	2.83 ± 0,86	1.41 ± 0.17	2.25 ± 0.20
phyllocactin	7	15.50	637.0	536	0.45 ± 0.04	0.29 ± 0.09	0.16 ± 0.02	nd	6.79 ± 0.07	10.40 ± 1.62	4.33 ± 1.72	5.09 ± 1.13
iso-betanidin	8	15.90	389.0	542	nd	0.18 ± 0.01	0.33 ± 0.00	nd	0.99 ± 0.15	2.83 ± 0.86	1.11 ± 0.24	1.14 ± 0.32

[a]HPLC retention times, molecular ions as determined by ESI-MS, and DAD maximum wavelengths are shown together with the concentrations determined for each betalain. [b]nd: not detected.

DOI: 10.1021/acs.jafc.7b04642
J. Agric. Food Chem. 2018, 66, 467−474

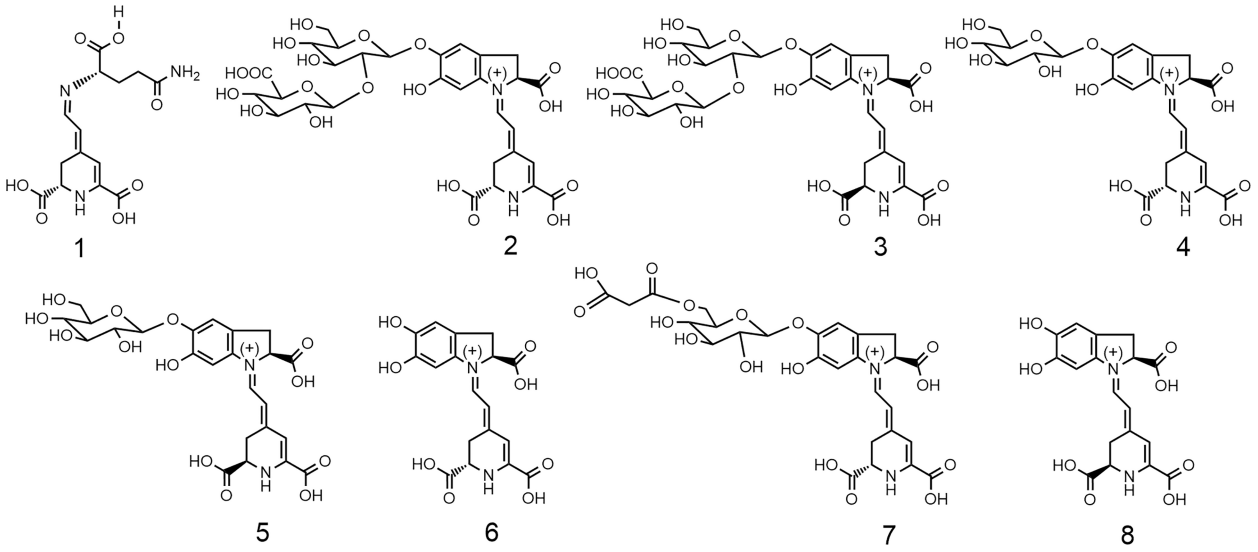

Figure 3. Betalains structures identified in the cell lines and seedlings obtained from colored quinoa grains: vulgaxanthin I (**1**), amaranthin (**2**), isoamaranthin (**3**), betanin (**4**), iso-betanin (**5**), betanidin (**6**), phyllocactin (**7**), and iso-betanidin (**8**).

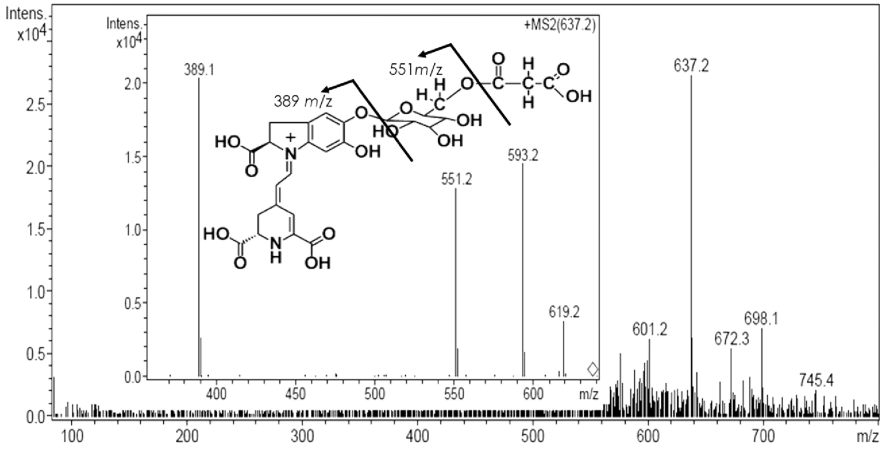

Figure 4. Fragmentation pattern obtained by ESI-MS/MS of phyllocactin main ion *m*/*z* 637 (inset) and identification of derived daughter ions.

friability. Subcultures were performed on fresh medium after 30 days for two subculture cycles. The composition of the medium was then changed in an attempt to promote the accumulation of pigments. The nitrogen source in the medium was decreased by 10 times, reducing the NH_4NO_3 concentration from 30.9 mM to 3.09 mM and the KNO_3 concentration was lowered from 28.2 to 2.82 mM. In addition, the concentration of BAP was increased from 6.66 to 8.88 μM. Both changes were done with the aim of causing stress in the cells of the different callus lines and activate the biosynthesis of secondary metabolites.[31]

Twenty days after the decrease of nitrogen and the increase in BAP concentration, cultures of the callus lines of quinoa varieties BGQ-174, BGQ-77 and POQ-132 showed the presence of pink pigmentation in the tissue (Figure 1). Thus, the same conditions were used for additional subcultures every 30 days for 5 cycles in fresh medium. The novel conditions made possible to establish pink callus lines of quinoa varieties BGQ-174, BGQ-77, and POQ-132. The cell phenotype suggested the presence of betalains, which coincides with that observed in plant cell cultures of *Suaeda salsa*,[32] *Beta vulgaris*,[33] *Celosia cristata*,[34] and *Celosia argentea*.[28] The callus line derived from the white variety "Blanca de Junín" and cultured under the same conditions did not show any changes in its pigmentation (Figure 1). As a result of this process stable and colored lines of *Chenopodium quinoa* Willd of the varieties BGQ-174, BGQ-77, and POQ-132 were stablished.

The resulting callus lines were observed under brightfield microscopy. The pink callus lines of the quinoa varieties BGQ-174, BGQ-77, and POQ-132 presented a morphology with elongated and round cells with a major presence of the former. This phenomenon might be in relation with the cohesive properties of the cell wall reported to occur during plant cell division.[35] Pink pigmentation was observed within the callus line compatible with the presence of betacyanins. The callus line derived from "Blanca de Junín" showed cells without color (Figure 1). The fluorescent dye DAPI was used to visualize the nucleus of the cells through fluorescence microscopy and to identify the individual cells (Supporting Information Figure 2).

Pigment Identification and Quantitation in Callus Lines and Seedlings. Samples of the different varieties of quinoa seedlings and callus lines were obtained and analyzed by HPLC-DAD in terms of the presence of the bioactive pigments betalains. Peaks detected in chromatograms (Figure 2) indicated the presence of colored molecules with retention times and spectra compatible with betalains. Mass spectrometry analyzes were performed with electrospray ionization (HPLC-

DOI: 10.1021/acs.jafc.7b04642
J. Agric. Food Chem. 2018, 66, 467−474

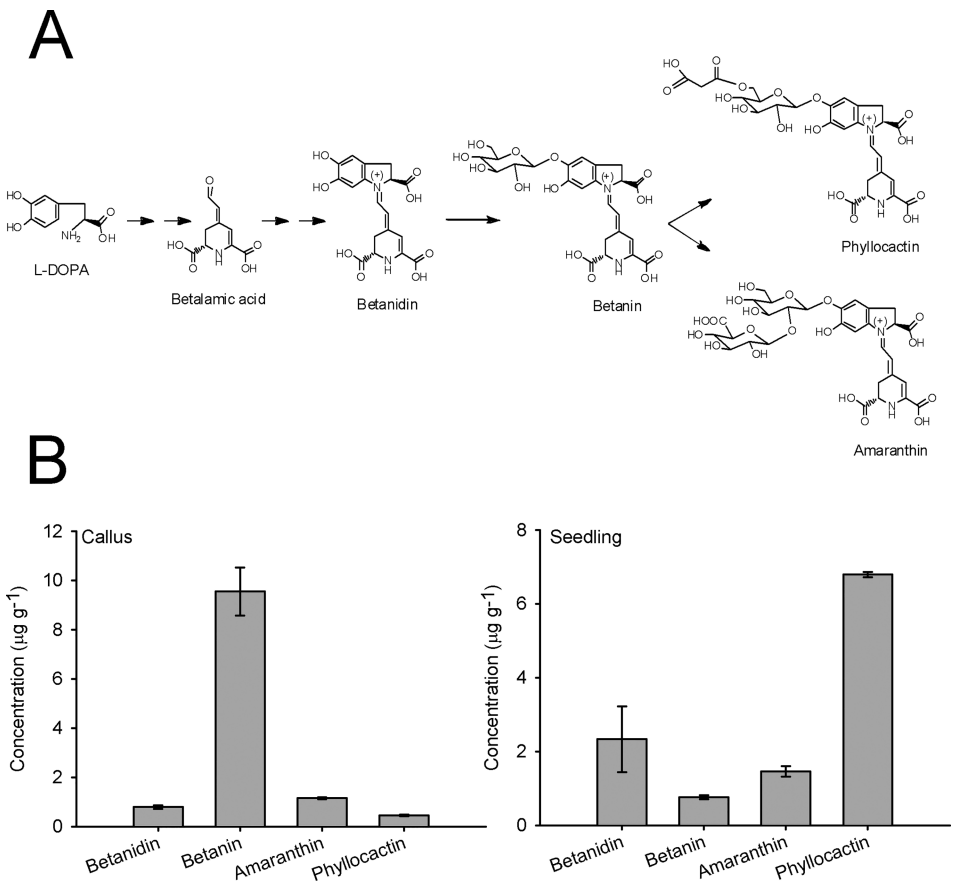

Figure 5. (A) Biosynthetic pathway of betalains proposed to occur in seedlings and pink cell lines of *Chenopodium quinoa* Willd. (B) Comparison of betalains content in seedling and callus of BGQ-174 quinoa variety.

ESI-MS/MS), to provide compounds mass/charge (m/z) ratio and fragmentation. Real betalain standards compatible with retention times and masses were extracted and purified from natural sources or obtained by a semisynthetic procedure.[26]

All pigments were identified by comparison with real standards in terms of HPLC retention times, DAD spectra, and electrospray ionization mass spectrometry (ESI-MS/MS) analyses (Table 2). The mass values determined for the parent ions of all compounds were as expected for the corresponding protonated molecular ions $[M + H]^+$ of the betalains, thus confirming the proposed structures. For pigments containing a quaternary ammonium substructure, the mass obtained corresponds to the charged form, as drawn in Figure 3. Betanin (R_t = 12.68 min) yielded a molecular ion m/z 551 and the main daughter ion was m/z 389, corresponding to the mass determined for betanidin (R_t = 15.10 min) obtained through cleavage of the glucose unit at the level of the *O*-glucosidic bond (m/z $[M + H]^+ - 162$). Amaranthin (R_t = 11.30 min) yielded a molecular ion of m/z 727 that was fragmented into m/z 551, corresponding to the loss of a glucuronic acid moiety m/z $[M + H]^+ - 176$ (betanin) and into m/z 389, corresponding to an additional loss of glucose m/z $[M + H]^+ - 176-162$ (betanidin). Phyllocactin (R_t = 15.5 min) yielded a molecular ion m/z 637 that was fragmented into m/z 593, corresponding to the decarboxylation of the phyllocactin and a molecular ion m/z 551 corresponding to betanin due to another decarboxylation and finally a ion of m/z 389, corresponding to the pigment aglycone (Figure 4). A betaxanthin derived from the condensation of betalamic acid with glutamine, known by the trivial name vulgaxanthin I (R_t = 9.2 min), was also found and yielded a molecular ion of m/z 340 and a daughter ion of m/z 323.

Thus, by HPLC-DAD and ESI-MS/MS analysis and standards comparison, the nature of the pigments present in quinoa seedlings and callus was determined for the first time. In the pink callus lines, the betacyanin content was high, with betanin determined as the major pigment in the varieties BGQ-174, BGQ-77, and POQ-132. The line derived from the red BGQ-174 variety stands out because of the high concentration of the pigment (9.55 μg g^{-1} fresh weight) (Table 2). Among the betaxanthins only vulgaxanthin I was detected (0.98 μg g^{-1}). No pigment was detected in the "Blanca de Junín" variety by HPLC, in accordance with the appreciable lack of color of the callus line.

In the case of quinoa seedlings, samples of all varieties grown for 8 days showed violet coloration, mainly on stem and cotyledons (Figure 1). They were analyzed for the presence of pigments in the developing plant. In all cases the major pigment was the betacyanin phyllocactin, with BGQ-77 variety showing the highest concentration of this pigment (10.40 μg g^{-1}) (Table 2). In the biosynthetic pathway of betalains,[1] after the condensation of *cyclo*-DOPA with betalamic acid, betanidin is formed, which is then glycosylated by the enzyme betanidin-*O*-glucosyltransferase to form betanin (betanidin 5-*O*-β-glucoside), a more stable compound than the aglyca. The presence of phyllocactin, detected here in *C. quinoa* for the first time, suggests that in quinoa developing plants, the biosynthetic pathway continues with the addition of malonic acid for the

DOI: 10.1021/acs.jafc.7b04642
J. Agric. Food Chem. 2018, 66, 467−474

formation of betanidin 5-*O*-(6′-*O*-malonyl)-*β*-glucoside (phyllocactin) (Figure 5).[36] However, the pink callus lines derived from the same plants show a greater amount of betanin and a minor content of phyllocactin (Table 2), thus indicating that the betalain biosynthetic pathway has been altered with the main accumulation of the intermediate instead of the final compound (Figure 5).

The variety BGQ-174 contains the highest amount of betalain pigments (betacyanins and betaxanthins) both in the grains and in the derived callus line. At the same time the variety "Blanca de Junín" lacks the pigments in the grains, as previously reported[19] and no pigment could be detected in the derived callus lines under the same experimental conditions than the colored ones. The varieties BGQ-77 and POQ-132 present betalains both in grains and derived callus lines. Grains of the yellow variety POQ-132 only present betaxanthins,[19] but seedling and cell lines present both betaxanthins and betacyanins. Another aspect to emphasize is that the main pigment in red and purple quinoa grains is amaranthin while in the yellow varieties it is dopaxanthin. In contrast, seedlings present phyllocactin as the main pigment regardless the variety considered. Phyllocactin has not previously been detected in *C. quinoa*. Both seedlings and callus lines presented vulgaxanthin I as the only betaxanthin detected, which was not detected in grains and previously not described in quinoa.

The amount and variety of betalains present in the callus cultures indicates that the presence of 6.79 μM 2,4-D and 8.88 μM BAP, coupled with the decrease of nitrogen in the culture medium, promotes the biosynthesis of different betalains compared to the starting plant material in *C. quinoa* cell cultures on solid media. The increase of the BAP hormone and the decrease of nitrogen in the culture medium is key to activate the biosynthetic pathway of betalains. In vitro culture offers numerous utilities such as micropropagation of plants, obtaining organs and tissues. They are, therefore, a way to maintain a reservoir of plants due to the minimum growing space required under totally controlled and aseptic conditions, of interest for germplasm banks. The described work reports the first obtaining of quinoa callus lines producing betalain pigments. It opens up the possibility of using cell cultures derived from colored quinoa varieties for the production of natural bioactive pigments and might be of interest for the food, pharmaceutical, and cosmetic industries.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b04642.

Absorption spectra of quinoa extracts and microscopy images of betalain producing cells under the brightfield and fluorescence techniques (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +34 868 889592. Fax: +34 868 884147. E-mail: fgandia@um.es.

ORCID

Fernando Gandía-Herrero: 0000-0003-4389-3454

Author Contributions

§P.H.-E. and B.G.-F. contributed equally to this work.

Funding

This work was supported by "Ministerio de Economía y Competitividad" (MEC, FSE, Spain) (Project AGL2014-57431) and by "Programa de Ayudas a Grupos de Excelencia de la Región de Murcia, Fundación Séneca, Agencia de Ciencia y Tecnología de la Región de Murcia" (Project 19893/GERM/15). B.G.-F. holds a postdoctoral fellowship from "Consejo Nacional de Ciencia y Tecnología" (CONACYT, Mexico), M.A.G.-R. holds a contract financed by MEC-FSE (Spain), and P.H.-E. holds a fellowship "Ayudas de Iniciación a la Investigación" from the University of Murcia (Spain).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful to Dr. José Rodriguez (SAI, University of Murcia) for skillful technical assistance in ESI-MS/MS experiments.

■ REFERENCES

(1) Gandía-Herrero, F.; García-Carmona, F. Biosynthesis of betalains: yellow and violet plant pigments. *Trends Plant Sci.* **2013**, *18*, 334−343.

(2) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. Structural implications on color, fluorescence, and antiradical activity in betalains. *Planta* **2010**, *232*, 449−460.

(3) Brockington, S. F.; Yang, Y.; Gandía-Herrero, F.; Covshoff, S.; Hibberd, J. M.; Sage, R. F.; Smith, S. A. Lineage-specific gene radiations underlie the evolution of novel betalain pigmentation in Caryophyllales. *New Phytol.* **2015**, *207*, 1170−1180.

(4) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. Biological activities of plant pigments betalains. *Crit. Rev. Food Sci. Nutr.* **2016**, *56*, 937−945.

(5) Escribano, J.; Pedreño, M. A.; García-Carmona, F.; Muñoz, R. Characterization of the antiradical activity of betalains from *Beta vulgaris L.* roots. *Phytochem. Anal.* **1998**, *9* (3), 124−127.

(6) Gliszczyńska-Świgło, A.; Szymusiak, H.; Malinowska, P. Betanin, the main pigment of red beet: Molecular origin of its exceptionally high free radical-scavenging activity. *Food Addit. Contam.* **2006**, *23*, 1079−1087.

(7) Nowacki, L.; Vigneron, P.; Rotellini, L.; Cazzola, H.; Merlier, F.; Prost, E.; Ralanairina, R.; Gadonna, J. P.; Rossi, C.; Vayssade, M. Betanin-enriched red beetroot (*Beta vulgaris* L.) extract induces apoptosis and autophagic cell death in mcf-7 cells. *Phytother. Res.* **2015**, *29*, 1964−1973.

(8) Wu, L.-C.; Hsu, H.-W.; Chen, Y.-C.; Chiu, C.-C.; Lin, Y.-I.; Ho, J.-A. A. Antioxidant and antiproliferative activities of red pitaya. *Food Chem.* **2006**, *95*, 319−327.

(9) Sreekanth, D.; Arunasree, M. K.; Roy, K. R.; Reddy, T. C.; Reddy, G. V.; Reddanna, P. Betanin, a betacyanin pigment purified from fruits of *Opuntia ficus-indica* induces apoptosis in human chronic myeloid leukemia cell line-K562. *Phytomedicine* **2007**, *14*, 739−746.

(10) Kapadia, G. J.; Azuine, M. A.; Sridhar, R.; Okuda, Y.; Tsuruta, A.; Ichiishi, E.; Mukainake, T.; Takasaki, M.; Konoshima, T.; Nishino, H.; Tokuda, H. Chemoprevention of DMBA-induced UV-B promoted, NOR-1-induced TPA promoted skin carcinogenesis, and DEN-induced phenobarbital promoted liver tumors in mice by extract of beetroot. *Pharmacol. Res.* **2003**, *47*, 141−148.

(11) Lechner, J. F.; Wang, L. S.; Rocha, C. M.; Larue, B.; Henry, C.; McIntyre, C. M.; Riedl, K. M.; Schwartz, S. J.; Stoner, G. D. Drinking water with red beetroot food color antagonizes esophageal carcinogenesis in N-nitrosomethylbenzylamine-treated rats. *J. Med. Food* **2010**, *13*, 733−739.

(12) Hempel, J.; Böhm, H. Betaxanthin pattern of hairy roots from *Beta vulgaris* var. *lutea* and its alteration by feeding of amino acids. *Phytochemistry* **1997**, *44*, 847−852.

DOI: 10.1021/acs.jafc.7b04642
J. Agric. Food Chem. 2018, 66, 467−474

(13) Osorio-Esquivel, O.; Alicia-Ortiz-Moreno; Álvarez, V. B.; Dorantes-Álvarez, L.; Giusti, M. M. Phenolics, betacyanins and antioxidant activity in *Opuntia joconostle* fruits. *Food Res. Int.* **2011**, *44*, 2160−2168.
(14) Amin, I.; Norazaidah, Y.; Hainida, K. I. E. Antioxidant activity and phenolic content of raw and blanched *Amaranthus* species. *Food Chem.* **2006**, *94*, 47−52.
(15) Khan, M. I.; Sri Harsha, P. S. C.; Giridhar, P.; Ravishankar, G. A. Pigment identification, nutritional composition, bioactivity, and *in vitro* cancer cell cytotoxicity of *Rivina humilis* L. berries, potential source of betalains. *LWT - Food Sci. Technol.* **2012**, *47*, 315−323.
(16) Pavokovic, D.; Krsnik-Rasol, M. Complex biochemistry and biotechnological production of betalains. *Food Technol. Biotechnol.* **2011**, *49*, 145−155.
(17) Tang, Y.; Li, X.; Zhang, B.; Chen, P. X.; Liu, R.; Tsao, R. Characterisation of phenolics, betanins and antioxidant activities in seeds of three *Chenopodium quinoa* Willd. genotypes. *Food Chem.* **2015**, *166*, 380−388.
(18) Abderrahim, F.; Huanatico, E.; Segura, R.; Arribas, S.; Gonzalez, M. C.; Condezo-Hoyos, L. Physical features, phenolic compounds, betalains and total antioxidant capacity of coloured quinoa seeds (*Chenopodium quinoa* Willd) from Peruvian Altiplano. *Food Chem.* **2015**, *183*, 83−90.
(19) Escribano, J.; Cabanes, J.; Jiménez-Atiénzar, M.; Ibañez-Tremolada, M.; Gómez-Pando, L. R.; García-Carmona, F.; Gandía-Herrero, F. Characterization of betalains, saponins and antioxidant power in differently colored quinoa (*Chenopodium quinoa*) varieties. *Food Chem.* **2017**, *234*, 285−294.
(20) Fuentes, F.; Paredes-Gonzales, X. Nutraceutical perspectives of quinoa: biological properties and functional applications. In *State of the Art Report of Quinoa in the World in 2013*; FAO & CIRAD: Rome, 2015; pp 286−299, Chapter 3.5.
(21) FAO-ALADI. Tendencias y perspectivas del comercio internacional de quinua. FAO-ALADI joint report; 2014.
(22) Jancurová, M.; Minarovičová, L.; Dandár, A. Quinoa—a review. *Czech J. Food Sci.* **2009**, *27*, 71−79.
(23) Gómez-Pando, L. R.; Álvarez-Castro, R.; Eguiluz-de la Barra, A. Effect of salt stress on Peruvian germplasm of *Chenopodium quinoa* Willd.: A Promising Crop. *J. Agron. Crop Sci.* **2010**, *196*, 391−396.
(24) Chapman, E. J.; Greenham, K.; Castillejo, C.; Sartor, R.; Bialy, A.; Sun, T.; Estelle, M. Hypocotyl transcriptome reveals auxin regulation of growth-promoting genes through GA-dependent and -independent pathways. *PLoS One* **2012**, *7*, e36210.
(25) Yusnita; Hapsoro, D. *In vitro* callus induction and embryogenesis of oil palm (*Elaeis guineensis Jacq.*) from leaf explants. *HAYATI J. Biosci.* **2011**, *18*, 61−65.
(26) Gandía-Herrero, F.; García-Carmona, F.; Escribano, J. Development of a protocol for the semi-synthesis and purification of betaxanthins. *Phytochem. Anal.* **2006**, *17*, 262−269.
(27) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. Characterization of the activity of tyrosinase on betanidin. *J. Agric. Food Chem.* **2007**, *55*, 1546−1551.
(28) Guadarrama-Flores, B.; Rodríguez-Monroy, M.; Cruz-Sosa, F.; García-Carmona, F.; Gandía-Herrero, F. Production of dihydroxylated betalains and dopamine in cell suspension cultures of *Celosia argentea* var. *plumosa*. *J. Agric. Food Chem.* **2015**, *63*, 2741−2749.
(29) Kobayashi, N.; Schmidt, J.; Nimtz, M.; Wray, V.; Schliemann, W. Betalains from Christmas cactus. *Phytochemistry* **2000**, *54*, 419−426.
(30) Gandía-Herrero, F.; García-Carmona, F.; Escribano, J. A novel method using high-performance liquid chromatography with fluorescence detection for the determination of betaxanthins. *J. Chromatogr. A* **2005**, *1078*, 83−89.
(31) Bourgaud, F.; Gravot, A.; Milesi, S.; Gontier, E. Production of plant secondary metabolites: a historical perspective. *Plant Sci.* **2001**, *161*, 839−851.
(32) Zhao, S. Z.; Sun, H. Z.; Gao, Y.; Sui, N.; Wang, B. S. Growth regulator-induced betacyanin accumulation and dopa-4,5-dioxygenase (DODA) gene expression in euhalophyte *Suaeda salsa* calli. *In Vitro Cell. Dev. Biol.: Plant* **2011**, *47*, 391−398.
(33) Akita, T.; Hina, Y.; Nishi, T.). Production of betacyanins by a cell suspension culture of table beet (*Beta vulgaris L.*). *Biosci., Biotechnol., Biochem.* **2000**, *64*, 1807−1812.
(34) Taha, R. M.; Wafa, S. N. Plant regeneration and cellular behaviour studies in *Celosia cristata* grown *in vivo* and *in vitro*. *Sci. World J.* **2012**, *2012*, 359413.
(35) Trejo-Tapia, G.; Rodríguez-Monroy, M. Cellular aggregation in secondary metabolite production in *in vitro* plant cell cultures. *Interciencia* **2007**, *32*, 669−674.
(36) Herbach, K. M.; Stintzing, F. C.; Carle, R. Identification of heat-induced degradation products from purified betanin, phyllocactin and hylocerenin by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2603−2616.

DOI: 10.1021/acs.jafc.7b04642
J. Agric. Food Chem. 2018, 66, 467−474