Effect of stress on germination of djulis (Chenopodium formosanum Koidz.) sprouts: a natural alternative to enhance the betacyanin and phenolic compounds

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Research Article

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Effect of stress on germination of djulis (*Chenopodium formosanum* Koidz.) sprouts: a natural alternative to enhance the betacyanin and phenolic compounds

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Abstract

BACKGROUND: Germination is regarded as a natural method for improving the bioavailability of seed nutrients against stress, which enhances the accumulation of bioactive compounds. The present study aimed to determine the effect of stress (H_2O_2, catechin, gallic acid, tyrosine, and NaCl) during germination of djulis (*Chenopodium formosanum* Koidz.) sprouts on betacyanin, phytochemicals, and antioxidant capacities.

RESULTS: The betacyanin and antioxidant activities of the djulis sprouts increased significantly compared to seeds. The lowest betacyanin was found in NaCl-stressed sprouts. The djulis sprouts reported the presence of celosianins I and II (50.72%), which was absent in seeds. Hydroxycinnamic acids accounted for > 60% of the total phenolic compounds in sprouts, whereas rutin predominated in the seeds.

CONCLUSION: Germination under stress may represent an effective natural method for improving the bioactive potential of sprouts, an alternative to use seeds, in the development of bioactive compounds-enriched healthy foods that are good for public health.

Supporting information may be found in the online version of this article.

Keywords: djulis; stress; germination; betacyanin; polyphenols; antioxidant activities

INTRODUCTION

Djulis (*Chenopodium formosanum* Koidz.) belongs to the genus *Chenopodium* and family Amaranthaceae, comprising a native dicotyledonous plant cultivated by aboriginals in mountain areas of eastern and southern Taiwan.[1] Generally, djulis is a shallow-rooted plant with spike-shaped inflorescence and is able to produce seeds at the axillary and main shoots. These seeds are similar to that of gramineous plants and thus djulis is classified as a pseudo-cereal crop[2] along with its close botanical relative of quinoa. Djulis seeds and leaves are considered as viable food options because of their high nutritional value, environmental importance, and economic advantages linked with tolerance to the unfavorable climatic conditions[3].

Djulis seeds are potential sources of high-quality proteins, essential amino acids, dietary fiber, starch, minerals, and vitamins.[2,3] They can be used to formulate gluten-free food products as an alternative to gluten-rich food products. In addition to their well-recognized nutrient profile, djulis seeds are promising sources of pigments and bioactive compounds with significant health-promoting properties. These bioactive compounds, including phenolics, triterpenes, phytosterols, and betacyanin, demonstrate antioxidant, anti-diabetic, anti-inflammation, and antiapoptotic activities.[4-6]

Seed germination, comprising the sprouting of a seed, has received increased interest with respect to improving the nutritional and bioactive properties of seeds, which in turn have an impact on the potential health effects of the final food products,[7] especially in cereals with high protein and carbohydrates. During seed germination, many biochemical changes have been documented, including degradation, accumulation, and remobilization in the nutrient and bioactive compounds within the same type of grain cultivars.[8] Both biotic and abiotic stress can cause changes in the biochemical content of seeds. Generally, plants produce bioactive molecules in response to

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biotic and abiotic stress,[9] which boost stress tolerance by altering key physiological and biochemical processes.

Stress is caused by extreme environmental factors, such as temperature, light, pH, salinity, chemicals, and soil moisture, which adversely affect the physiology and biochemistry of plants. For example, stress caused by sodium chloride (NaCl) and hydrogen peroxide (H_2O_2) was reported to activate the plant defense system by accumulating carotenoid and betanin contents in buckwheat and *Suaeda salsau* sprouts, respectively.[10,11] Likewise, Benincasa *et al.*[12] and Sogoni *et al.*[13] concluded that the different concentration of NaCl (0–200 mM) can improve the generation of secondary metabolites in maize sprouts and *Tetragonia decumbens*, respectively. Another study by Mendoza-Sánchez *et al.*[14] used chemical stress, such as salicylic acid (0.10 to 2 mM), chitosan (0.10 to 7 μM), and H_2O_2 (10 to 30 mM), in which salicylic acid at 1 and 2 mM, chitosan at 7 μM, and H_2O_2 at 30 mM exhibited an increase in phenolic compounds (1.80-fold), total flavonoids (3-fold), saponins (1.80-fold), and antioxidant capacity (37%) of *Phaseolus vulgaris* L. These studies showed that the application of abiotic stress at optimum concentration could be considered as a natural alternative for improving the nutritional and phytochemical composition during germination. Moreover, many studies have reported the enhanced bioactivity in sprouts, such as phenolics, glucosinolates, vitamins, and γ-aminobutyric acid, through elicitation.[15] Likewise, rapeseed, cabbage, and broccoli seed sprouts harvested from salt stressed plants have demonstrated a greater phenolic and bioactivities.[16] Generally, chemicals such as tyrosine may be involved in the formation of pigments and influence biochemical changes during germination. Thus, the selection of stress conditions and their concentration is a crucial factor for improving the nutritional, pigment, and phytochemical composition during germination.[17] Additionally, chemical elicitors (e.g. salts) would provide a multidisciplinary approach on the physiology of seed germination and related genetic implications,[16] such as a remarkable beneficial effect on the generation of bioactive compounds.

Until now, research has not yet investigated the influence of stress on djulis sprouts germination and therefore we hypothesized that the accumulation of bioactive compounds may be enhanced by stress factors during germination. Hence, the present study aimed to evaluate the effect of stress caused by H_2O_2, catechin, gallic acid, tyrosine, and NaCl during germination of djulis sprouts on betacyanin, phytochemicals, and antioxidant capacities.

MATERIALS AND METHODS

Chemicals

Iron (II) chloride, butylated hydroxyanisole, ethylene diaminetetraacetic acid, 1-diphenyl-2-picryl-hydrazyl (DPPH), ABTS [2, 2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)], Folin–Ciocalteu's reagent, gallic acid, catechin, rutin, chlorogenic acid, caffeic acid, epicatechin, coumaric acid, quercetin, ferulic acid, ascorbic acid, tyrosine, potassium persulfate ($K_2S_2O_8$), and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were purchased from Sigma-Aldrich (St Louis, MO, USA). Formic acid (98–100%) was obtained from Merck KGaA (Darmstadt, Germany). All reagents and chemicals used were of high purity and were used as obtained without any further purification. All standard solutions were prepared with chromatographic grade water, Milli-Q water (Milli-DI® Water Purification System; MerckMillipore, Darmstadt, Germany).

Material collection and germination process

Djulis (*C. formosanum* Koidz.; red cultivar) seeds were procured from the aboriginal village of Pingtung County in Taiwan. Good quality seeds (see Supporting information, Fig. S1A) were selected based on visual observation without any physical and microbial contamination on the seed surface and were stored at −20 °C until the germination process was performed. The germination was performed by sprouting seeds (16.50 g) in a nursery pot (60 × 31 × 4 cm) with a well-drained cultivated soil (soil temperature 20–30 °C). The well-drained cultivated soil allows water to percolate through it reasonably quickly. The different stress materials, including H_2O_2 (0–588 mM), catechin (0–1 mM), gallic acid (0–1 mM), tyrosine (0–1 mM), and NaCl (0–342 mM) were imposed for 5 days (Fig. 1). Then, the concentration of stress materials was optimized based on the betacyanin content of the djulis sprouts. The optimized concentration of stress materials, including H_2O_2 (97 mM), catechin (0.50 mM), gallic acid (0.30 mM), tyrosine (0.28 mM), and NaCl (85.50 mM) were used for germination over 0, 5, 7, 9, 11, and 13 days (see Supporting information, Fig. S1B) to determine the betacyanin, phenolic compounds, and antioxidant capacities, respectively. The optimum humidity of 98% was

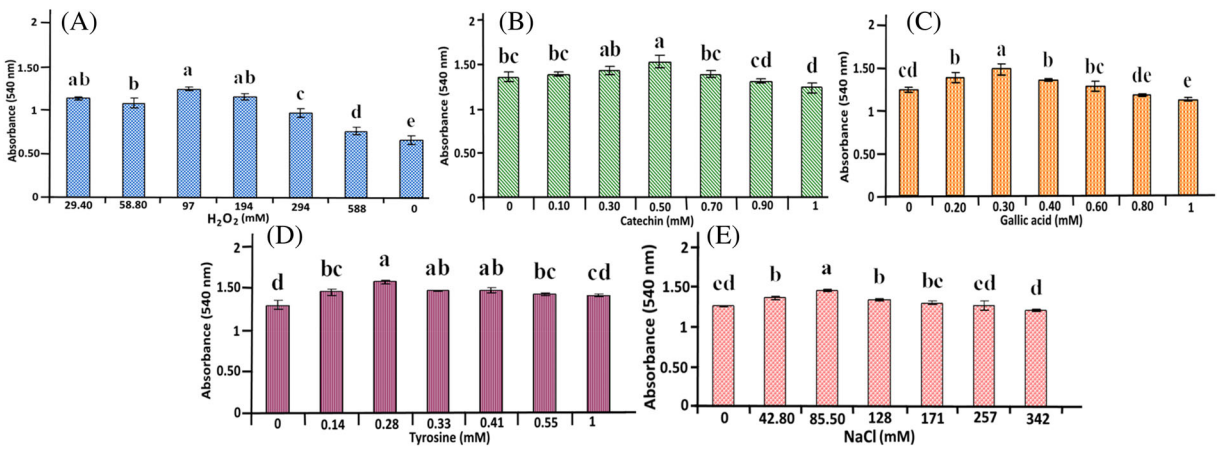

Figure 1. Effect of different stress concentrations on the accumulation of betacyanin in djulis sprouts 5-day old. (A) H_2O_2, (B) catechin, (C) gallic acid, (D) tyrosine, and (E) NaCl. Values are the mean of three independent replicate determinations ($n = 3$) along with error bars that show the SEM. Mean values with different lowercase letters represent a significant difference within each stress concentration ($P < 0.05$) based on Duncan's multiple range test.

maintained to prevent the drying of the sprouts by periodic watering.

Extraction

The harvested djulis sprouts were frozen for 12 h at −80 °C and then dried at −50 °C in a freeze dryer (FreeZone Plus 6; Labconco Corp., Kansas City, MO, USA). Using a laboratory grinder (Yu Chi Machinery Co., Ltd., Taipei, Taiwan), the dried samples were ground to a fine powder and placed in airtight plastic zip-lock reclosable packaging bags (130 × 150 mm) for further analysis. For extraction, sample (3 g) was mixed with distilled water (150 mL) at 4 °C for 24 h. Then, the sample was centrifuged (High-speed Micro Centrifuge, CF15RX2; Hitachi, Tokyo, Japan) at 9300 × *g* for 30 min and 4 °C, and filtered (90 mm; Advantec Toyo Kaisha, Ltd, Tokyo, Japan). The filtered solution was then stored in amber colored scintillation vials at −21 °C until further experimental analysis.

Color

The color of samples was determined using a Hunter colorimeter (ZE 2000; Nippon Deshoku Industries Co., Ltd, Tokyo, Japan) and examined according to the CIELAB color scale: *L* (lightness), *a* (greenness/redness), and *b* (blueness/yellowness) values (*L*: 0–100, *a*: −*a* to +*a*, and *b*: −*b* to +*b*). Chroma (C_H) and hue angle (θ_H) of samples were calculated using:

$$C_H = \left[\left(a^2 + b^2\right)^{\frac{1}{2}}\right] \quad (1)$$

$$\theta_H = \left[\tan^{-1}\left(\frac{b}{a}\right)\right] \quad (2)$$

Total phenolic content (TPC)

TPC of samples was determined colorimetrically following the Folin–Ciocalteu method as described by Singleton and Rossi[18] with minor modifications. The extracts (0.10 mL) were mixed with Folin–Ciocalteu's reagent (50%, 0.10 mL) and sodium carbonate (2%, 2 mL). The reaction mixture was incubated for 30 min under dark at room temperature and the absorbance was measured using a spectrophotometer (Model U-2001; Hitachi) at 760 nm. The results were reported as gallic acid equivalents (GAE) in mg 100 g^{-1} dried weight of the sample (mg GAE 100 g^{-1} DW) using gallic acid solutions as a reference standard.

Antioxidant activities

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured according to the method of Raungrusmee and Anal.[19] Briefly, extracts (0.10 mL) were added to the methanolic solution of DPPH (0.004%; 3 mL) and incubated for 30 min at room temperature in the dark. The absorbance of extracts was measured using the spectrophotometer at 517 nm. The percentage inhibition activity was calculated using:

$$\%\ \text{inhibition} = \left[\left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100\right] \quad (3)$$

where A_{control} = absorbance of control and A_{sample} = absorbance of the sample.

Ferric reducing antioxidant power (FRAP)

FRAP antioxidant activity of extracts was determined according to the method described by Tsai *et al*.[20] Briefly, 1.20 mL of freshly prepared FRAP reagent (10:1:1 of sodium acetate buffer (300 mM, pH 3.60), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$, respectively) was mixed with 0.12 mL distilled water and 0.04 mL of extract, and incubated at 37 °C for 30 min. The absorbance was measured spectrophotometrically at 593 nm against a blank (FRAP reagent + water). The FRAP values were calculated from the $FeSO_4$ standard solution and the reducing power was expressed as μM $FeSO_4$ equivalent L^{-1} extract.

Betacyanin estimation and quantitation by reverse phase-high-performance liquid chromatography (RP-HPLC)

The concentration of total betacyanin was determined using the extinction coefficient (ε) as $\lambda_{max} = 6.16 \times 10^4 \times$ concentration (M) according to Tsai *et al*.[21] Betacyanin quantitation was performed on a RP-HPLC system (Hitachi) coupled to Hitachi UV-visible detector according to the method described by Tsai *et al*.[21] Briefly, sample (20 μL) was injected onto a LiChrosorb RP-18 column (250 × 4.60 mm) (MerckMillipore) at room temperature with a flow rate of 1 mL min^{-1}. The gradient mobile phases were used including 5% formic acid (solvent A) and 60% acetonitrile (solvent B). The elution was conducted with a 97% to 80% A (0–30 min), 80% to 50% A (30–40 min), and 50% A (40–60 min). Sample extracts and mobile phase solvents were filtered via a disposable polytetrafluoroethylene (0.45 μm; Merck KGaA) filter and injected at a volume of 20 μL. The betacyanin content was measured at 530 nm, calculated, and compared to external standards based on their retention time.

Identification of phenolic and flavonoid compounds by HPLC

Phenolic and flavonoid compounds were quantified by an HPLC system (L-7100; Hitachi) equipped with a UV detector as described by Dobrinčić *et al*.[22] Chromatographic analysis was performed on a Mightysil RP-18 column (250 × 4.6 mm, 5 μm) (Kanto Corp., Tokyo, Japan) at 25 °C. The mobile phases were 5% acetic acid (solvent A) and 100% acetonitrile (solvent B) with a flow rate of 1 mL min^{-1}. The elution was conducted with 100 to 87% A (0–20 min), 87% to 76% A (20–45 min), and 76% to 72% A (45–60 min). The injection volume was 20 μL and the temperature was controlled at 25 °C. The detection wavelengths for all phenolic compounds were determined based on their UV-visible absorption spectra (280 nm) and compared with external standards. All phenolic and flavonoid compounds were expressed as milligrams per 100 g DW of the sample (mg 100 g^{-1} DW).

Liquid chromatography-mass spectrometry (LC-MS) analysis

The Thermo Finnigan LCQ Mass Spectrometer System (Thermo Fisher Scientific GmbH, Dreieich, Germany) was used to determine the betalain composition of extracts using an electrospray ionisation interface. The elution conditions were the same as in the RP-HPLC analysis. The flow rate of the sheath and the auxiliary gas (mixture of helium and nitrogen) was set at 50 and 10 arbitrary units, respectively. The other optimal conditions were set at 300 °C for the capillary temperature, capillary voltage of 4.60 kV, and scan range of 400–1500 aum. Positive selective ion monitoring mode was used to record all of the spectra.

Determination of total free amino acids

The total free amino acid composition was determined by the method modified from Chen *et al.*[23] Briefly, the sample (1 mL) was thoroughly mixed with sodium-phosphate buffer (0.50 mL). Then, ninhydrin (0.50 mL) was added to the reaction mixture during boiling for 15 min and allowed to cool to room temperature. The absorbance was measured using spectrophotometer at 570 nm. The total free amino acids were calculated using an aspartic acid calibration curve and expressed as milligram of aspartic acid per 100 g DW of the sample (mg 100 g^{-1} DW).

Determination of individual free amino acids by HPLC

The individual free amino acid composition of djulis sprouts (after the end of germination period) and seeds was determined by FLD-HPLC (ELITE LaChrom L-2485; Hitachi), consisting of an autosampler with a fluorescence detector set at excitation and emission wavelengths of 338 and 450 nm, respectively. Separation was carried out on chemical Mightysil RP-18 GP column (250 × 4.60 mm; 5 μm) (Kanto Corp.) at room temperature. The gradient elution was accomplished using ultrapure water (solvent A), 100% acetonitrile (solvent B), 100% methanol (solvent C), and 0.04 M sodium hydrogen phosphate at pH 7.80 (solvent D) with a flow rate of 1 mL min^{-1} as shown in Table 1.[24] The sample derivatization was performed by mixing 2 μL sample solution with 10 μL sodium borate buffer (0.10 M; pH 9.90). Then, it was mixed with 6 μL of *O*-phthaldialdehyde, 9-fluorenylmethyl chloroformate (5 mg mL^{-1}), and 128 μL distilled water. Finally, the reaction mixture (20 μL) was injected into a column.[24] The quantification of amino acid composition was performed using absorbance measurements at 262 and 338 nm, as well as retention times, which were compared to external standards. All of the solvents and samples were filtered through a 0.45 μm disposable polytetrafluoroethylene filter. The results were expressed as milligrams per 100 g of dried weight of the sample (mg 100 g^{-1} DW).

Statistical analysis

All the experiments were performed in triplicate and the results were expressed as the mean ± SD. Data were subjected to one-way analysis of variance (one-way ANOVA) using SAS, version 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the mean differences among the samples were analyzed by Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

Table 1. The gradient elution program for the determination of individual free amino acids by HPLC (high-performance liquid chromatography)

Time (min)	A (%)	B (%)	C (%)	D (%)
0	–	–	–	100
3.80	–	–	–	100
36.20	5.70	25.70	25.70	42.90
37.20	10	45	45	–
44.60	10	45	45	–
46.60	5.70	25.70	25.70	42.90
52.60	–	–	–	100

The composition of mobile phases: ultrapure water (solvent A), 100% acetonitrile (solvent B), 100% methanol (solvent C), and 0.04 M sodium hydrogen phosphate at pH 7.80 (solvent D).

RESULTS AND DISCUSSION

Stress concentration and the accumulation of betacyanin

The effect of stresses, including H_2O_2 (0–588 mM), catechin (0–1 mM), gallic acid (0–1 mM), tyrosine (0–1 mM), and NaCl (0–342 mM) on the accumulation of betacyanin in djulis sprouts is illustrated in Fig. 1. The stress concentration was optimized based on the accumulation of betacyanin in djulis sprouts. On day 5, stress-treated samples exhibited a higher betacyanin content than the control (data not shown). The stress concentration of 97 mM for H_2O_2, 0.50 mM for catechin, 0.30 mM for gallic acid, 0.28 mM for tyrosine, and 85.50 mM for NaCl showed a significantly ($P < 0.05$) higher absorbance at 540 nm compared to other stress concentrations, indicating that these stress concentrations might have induced the accumulation of betacyanin in djulis sprouts (Fig. 1). At the optimum concentration for each stress factor, samples showed almost similar absorbance at 540 nm (1.30–1.60). It is assumed that the djulis sprouts had capability to synthesize betacyanin in response to stress caused by the H_2O_2, catechin, gallic acid, tyrosine, and NaCl at specific concentrations. However, the high concentration of stresses may result in destruction with respect to pigment synthesis, which was indicated by the decrease in absorbance of stressed samples at a higher concentration. These findings were consistent with previous results for maize, wheat,[25,26] and peanut[27] plants, where the optimum concentration of NaCl (50–100 mM) and tyrosine (100 mg L^{-1}) exhibited an increase in photosynthetic pigments. Likewise, these observations accordingly suggested that the optimized concentrations of H_2O_2 (97 mM), catechin (0.50 mM), gallic acid (0.30 mM), tyrosine (0.28 mM), and NaCl (85.50 mM) may play a significant role in increasing the betacyanin content in djulis sprouts during germination. Therefore, we further used the same concentration to understand the effect of stress on betacyanin, phenolic compounds, and antioxidant capacities.

Color

Among the color parameters, chroma (C_H) is the perceived strength of a surface colour. A high C_H represents the presence of more colored compounds. Figure 2(A) shows the significant ($P < 0.05$) variations in C_H values of djulis sprouts treated with stress during germination. On day 5, all samples exhibited increased C_H values (35 to 56) compared to the control (C_H value of 11), which increased to C_H values of 55–75 on day 11 and then declined to C_H values of 45–75 by the end of germination process on day 13. Among the different stresses, samples treated with gallic acid showed the highest C_H value (45–72), whereas NaCl treated samples reported the lowest C_H values (35–69). On the other hand, hue angle (θ_H) is the important parameter in determination of color change, which indicates the difference of color with reference to a grey color. The samples treated with stress showed the θ_H values between 331.64° and 358.44° (data not shown). In particular, a change in θ_H from 344.99–358.44° (day 5) to 332.83–336.79° (day 11) demonstrated the color change from red to purple red in djulis sprouts during the germination. The variations in C_H and θ_H may be attributed to the stress induced in djulis sprouts in response to H_2O_2, catechin, gallic acid, tyrosine, and NaCl stresses. The decrease in color values after 11 days could be ascribed to the inhibitory efficacy of the stress concentration on the accumulation of pigments by the plant defense system against reactive oxygen species. Another possible explanation for this could be related to destruction and instability of colored pigments under a high concentration of stresses. Islam *et al.*[26]

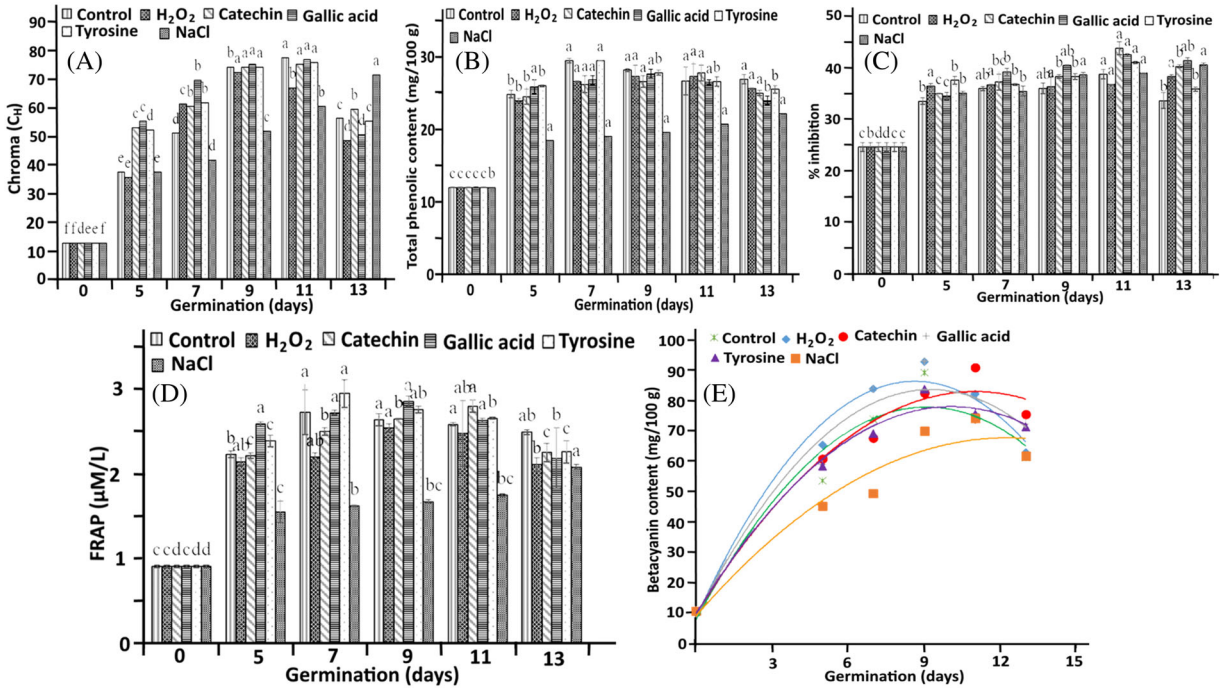

Figure 2. Effect of stress on physical and biochemical changes in djulis sprouts during germination. (A) Chroma, (B) total phenolic content, (C) DPPH scavenging activity, (D) FRAP, and (E) betacyanin content. Values are the mean of three independent replicate determinations ($n = 3$) along with error bars that show the SEM. Mean values with different lowercase letters represent a significant difference ($P < 0.05$) within germination days based on Duncan's multiple range test. In (E), different colored lines indicate non-linear regression.

reported significant increase in color values (C_H and θ_H) for NaCl stressed (0–100 mM) wheat microgreen extract, in which the stress concentration of 12.50 mM resulted in the high color values (C_H and θ_H). Similarly, He *et al.*[11] and Wang *et al.*[10] concluded that there was an accumulation of colored pigments (carotenoid and betanin) when buckwheat and *S. salsau* sprouts, respectively, were stressed with NaCl and H_2O_2, indicating a relative change in color attributes under stresses. Therefore, our results indicated that the stressed samples promoted the formation of colored pigments but appeared to have negative effects at higher concentrations. Hence, djulis sprouts with appropriate H_2O_2, catechin, gallic acid, tyrosine, and NaCl concentrations may exert a positive role with improved color characteristics.

Total phenolic content and antioxidant capacities

The TPC and antioxidant capacities of samples under stress are illustrated in Fig. 2(B–D). The samples exposed to stress had the highest polyphenol content (18–28 mg 100 g^{-1}) compared to the control samples (12 mg 100 g^{-1}). The highest amount of polyphenol content was found on days 9 and 11, which ranged from 26 to 28 mg 100 g^{-1}, except for the NaCl stressed samples (19.57 mg 100 g^{-1}), as shown in Fig. 2(B). The NaCl treatment showed an insignificant effect ($P > 0.05$) on total polyphenol content during germination from days 5 to 13, demonstrating that the NaCl stress induced no polyphenol content formation during the germination process. For DPPH scavenging activity, all the stress treated samples showed a significantly ($P < 0.05$) increased level of percentage inhibition during germination (Fig. 2C). The percentage inhibition increased from 33% to 43% and was found to be the highest on day 11 with the inhibition capacity of 38.73%, 36.75%, 43.76%, 42.47%, 41.03%, and 38.93% for H_2O_2, catechin, gallic acid, tyrosine, and NaCl treated samples, respectively. Likewise, a similar tendency in antioxidant activity was evident by the FRAP activity (Fig. 2D), where stressed samples exhibited the higher FRAP values during germination on day 11. For both DPPH and FRAP on day 11, NaCl stressed samples reported significantly ($P < 0.05$) lower antioxidant activities, as exemplified by the lower FRAP value (1.55 mM L^{-1}) for NaCl treated samples compared to catechin stressed samples with a FRAP value of 2.80 mM L^{-1}. It was obvious that the presence of phenolic compounds further contributed to antioxidant activities.

The betacyanin content showed a similar tendency as the color C_H value, where gallic acid treated samples showed a higher betacyanin content than NaCl treated samples. After 9 days of germination, betacyanin content was found to be 32.40, 33.74, 29.98, 33.82, 30.45, and 25.44 mg 100 g^{-1}, with non-linear progression in all samples (Fig. 2E). This suggested that the stress might induced the formation of betacyanin in djulis sprouts, which was in accordance with the pigment induced by the salt, light, and temperature of *S. salsau* sprouts.[10]

These findings are in agreement with previous studies, which showed a significant increase in phenolic content and antioxidant activities of maize sprouts, *T. decumbens*, and *Phaseolus vularis* L under different chemical stresses.[12-14] A similar trend was also reported by Nurnaeimah *et al.*[28] regarding the effects of chemical stress on the biochemical characteristics of *Ficus deltoidei*, where the total phenolic content and antioxidant activity in H_2O_2 treated *F. deltoidei* was significantly higher than the control. The increase in phenolic content and antioxidant activities could be associated with the plant defense mechanism through enhanced activities of phenylalanine ammonia lyase, a key enzyme in the polyphenol synthesis pathway.[26] The lower antioxidant activities in NaCl treated samples were attributed to a lower phenolic content and other biochemical changes in sprout metabolism, such as the

uptake of phosphorus and potassium that might be influenced by the accumulation of phenolic compounds and the release of aglycones from conjugated glycosides.[9,26] Overall, these findings demonstrate the role of stress, which positively influenced the accumulation of phenolic compounds or the synthesis of new bioactive compounds with respect to the reduction of biological radicals and the formulation of polyphenol with enhanced healthy attributes.

Identification of betacyanin

Identification of betacyanin in djulis sprouts under stress and seeds is performed by HPLC and the HPLC profile of betacyanin in the djulis seeds and sprouts is shown in Fig. 3(A,B). The major pigment composition of djulis seeds was characterized by the presence of amaranthine, isoamaranthine, betanin, and isobetanin with a retention time at 4.95, 7.07, 8.30, and 11.50 min, respectively (Fig. 3A). However, stressed djulis sprouts exhibited a high amount of amaranthine with a retention time similar to that for amaranthine present in djulis seeds. In addition, djulis sprouts under stress showed two unknown compounds at a retention time of 19.74 and 21.47 min (Fig. 3B). It was apparent that unknown compounds should be to expected in stressed djulis sprouts during germination, which could be associated with the synthesis of new bioactive compounds by the activity of self-sustaining enzymes manufactured during germination[29] in response to stress. Further identification of two unknown compounds was performed by LC-MS and these were tentatively identified as celosianin I with *m/z* 873.23 (Fig. 3C) and celosianin II with *m/z* 903.44 (Fig. 3D). According to Cai *et al.*,[30] celosianins belong to amaranthine-type (substituted at C-5 of betanidin) betacyanins and are exclusively found in *Celosia argentea* var. *cristata* (cockscomb). Another study by Srivarathan *et al.*[31] identified the presence of celosianin II in *Tecticornia* sp. (samphire) with *m/z* 903.2321 $[M + H]^+$ and a fragmented protonated ion at 389.0984, suggesting the presence of betanidin. Generally, amaranthine-type betacyanins, including amaranthine, celosianins, and iresinins, are the well-known highly abundant pigments in the *Amaranthaceae* family along with their isoforms.[32] The structures of celosianins and iresinins are formed by the addition of an acyl group to the amaranthine. In our findings, we assume that the stressed djulis sprouts promoted the accumulation of *p*-coumaroyl and feruloyl groups, which added to the amaranthine for the formation of celosianins I and II, respectively.[30] In agreement with our findings, previous studies demonstrated the presence of amaranthine, isoamaranthine, betanin, and isobetanin in djulis seeds.[21,33] Furthermore, the present study is one of the first investigations to document the presence of celosianins I and II as the major pigments in stressed djulis sprouts. In brief, stressed djulis sprouts provide naturally synthesized pigments, especially amaranthine-type betacyanins, which should be considered for scaling-up as a result of their promising health benefits.

Identification of individual phenolic compounds

As noted, total phenolic compounds increased with the progression of germination time, followed by a decrease over the end of germination time by day 13. Moreover, different phenolic compounds were detected in different amounts for the different stress-treated djulis sprouts. The content of phenolic compounds and their derivatives were grouped into categories, such as catechin and epicatechin grouped as flavanols, whereas chlorogenic acid, caffeic acid, coumaric acid, and ferulic acid were grouped as hydroxycinnamic acids, and their changes were investigated

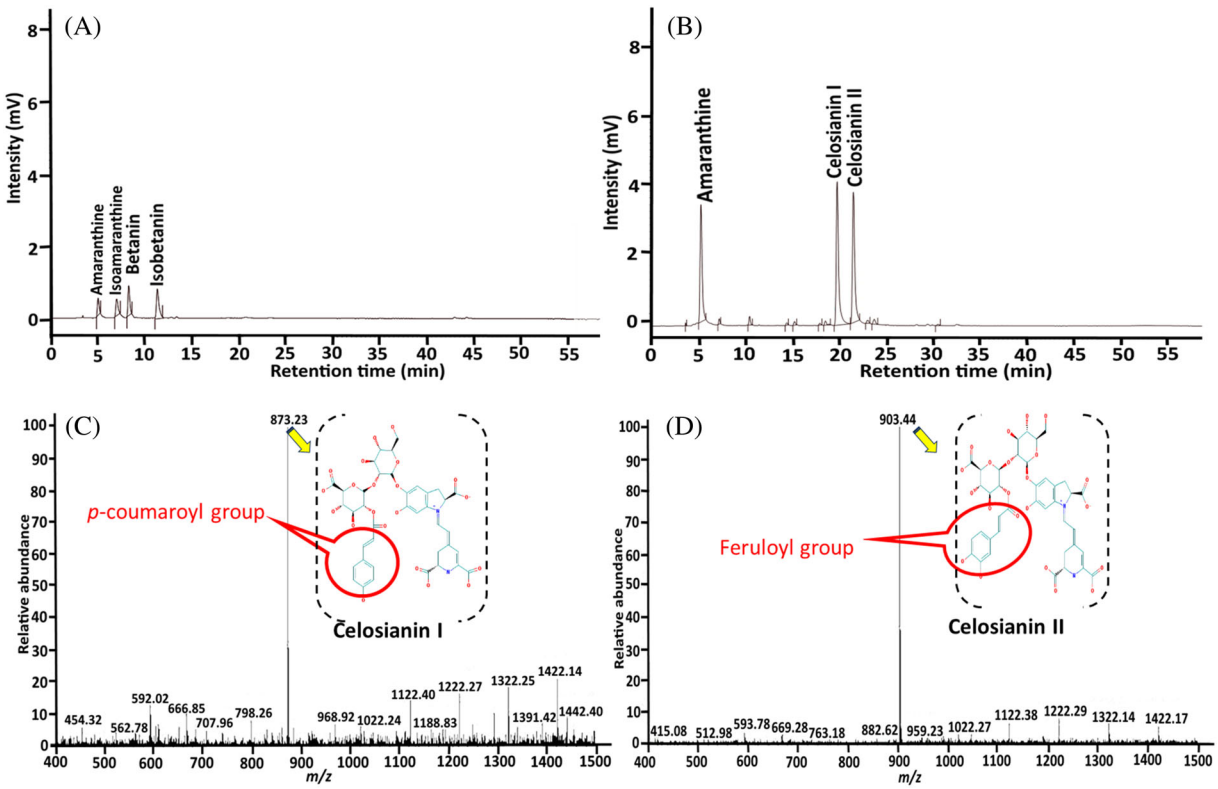

Figure 3. HPLC profile of (A) djulis seeds and (B) sprouts under stress along with LC-MS spectrum of two unknow compounds: (C) celosianin I and (D) celosianin II.

Table 2. Changes in individual phenolic composition of djulis sprouts during germination

Phenolic compounds (mg 100 g^{-1})	Germination (days)					
	0	5	7	9	11	13
Rutin	$37.32 \pm 0.05^{a,A}$	$26.30 \pm 0.09^{c,B}$	$10.92 \pm 0.10^{c,C}$	$7.06 \pm 0.22^{c,D}$	$3.63 \pm 0.08^{d,E}$	$4.21 \pm 0.40^{c,E}$
Quercetin	$0.16 \pm 0.02^{e,B}$	$0.34 \pm 0.02^{e,B}$	$0.42 \pm 0.12^{d,B}$	$0.80 \pm 0.17^{d,B}$	$3.20 \pm 0.04^{d,A}$	$4.38 \pm 0.14^{c,A}$
Flavanols	$23.84 \pm 0.07^{b,C}$	$40.14 \pm 0.27^{b,A}$	$38.41 \pm 0.06^{b,A}$	$32.81 \pm 0.26^{b,B}$	$25.90 \pm 0.05^{b,C}$	$14.54 \pm 0.22^{b,D}$
Gallic acid	$4.75 \pm 0.05^{d,E}$	$17.88 \pm 0.14^{d,C}$	$41.50 \pm 0.21^{b,A}$	$28.94 \pm 0.11^{b,B}$	$29.64 \pm 0.15^{b,B}$	$11 \pm 0.65^{b,D}$
Hydroxycinnamic acids	$14.47 \pm 0.02^{c,E}$	$71.12 \pm 0.24^{a,D}$	$174.06 \pm 0.08^{a,A}$	$153.26 \pm 0.57^{a,B}$	$124.33 \pm 0.31^{a,C}$	$133.05 \pm 0.06^{a,C}$

Results are presented as the mean ± SD ($n = 3$). Mean values with different lowercase letters within a column and uppercase letters within a row represent a significant difference ($P < 0.05$) based on Duncan's multiple range test.

Table 3. Comparison of free amino acid composition of djulis seeds and sprouts (control)

Free amino acid composition (mg 100 g^{-1})	Seeds	Sprouts
Aspartic acid	59.34 ± 0.54^{a}	61.18 ± 0.60^{b}
Glutamic acid	48.44 ± 0.25^{a}	144.58 ± 0.29^{b}
Asparagine	15.97 ± 0.28^{a}	81.71 ± 0.11^{b}
Serine	17.16 ± 0.29^{a}	76 ± 0.22^{b}
Glutamine	56.74 ± 0.02^{a}	614.91 ± 0.65^{b}
Histidine	20.18 ± 0.40^{a}	99.46 ± 0.21^{b}
Threonine	61.62 ± 0.61^{a}	249.63 ± 0.37^{b}
Arginine	24.67 ± 0.54^{a}	143.96 ± 0.04^{b}
Alanine	2.55 ± 0.05^{a}	222.85 ± 0.36^{b}
Tyrosine	26.69 ± 0.18^{a}	93.63 ± 0.13^{b}
γ-aminobutyric acid	12.36 ± 0.27^{a}	266.35 ± 0.23^{b}
Valine	6.32 ± 0.02^{a}	99.31 ± 0.11^{b}
Methionine	52.17 ± 0.14^{a}	82.77 ± 0.19^{b}
Tryptophan	50.79 ± 0.22^{a}	78.65 ± 0.22^{b}
Phenylalanine	11.44 ± 0.20^{b}	ND^{a}
Isoleucine	8.81 ± 0.03^{a}	94.77 ± 0.97^{b}
Leucine	13.52 ± 0.02^{a}	104.48 ± 1.54^{b}
Lysine	17.69 ± 0.09^{a}	101.52 ± 0.21^{b}

Results are presented as the mean ± SD ($n = 3$). Mean values with different superscript lowercase letters within a row represent a significant difference ($P < 0.05$) based on a paired t-test.
Abbreviation: ND, not detected.

during germination. HPLC data showed the presence of hydroxycinnamic acids in stressed djulis sprouts, which accounted for 68.77% (Table 2) compared to djulis seeds, where rutin (46.33%) was the predominant phenolic compound. By the end of germination process, rutin was found to be < 10%, indicating the transformation of rutin from glycosides to aglycones[34] by the action of hydrolysis caused by stress, which might have triggered the synthesis of hydrolytic enzymes. This was further indicated by increased quercetin as a result of the cleavage of rutin. Flavonols also followed a decreased trend with the progression of the germination period. Thus, the predominant phenolic compounds in djulis seeds were flavonoids and phenolic acids in stressed djulis sprouts. These findings were consistent with the results reported by Al-Qabba *et al.*[29] using *Chenopodium* quinoa sprouts. Because the germination cycle is a complex biological process, the accumulation of phenolic compounds may depend on many factors, including species, germination conditions, and stage of growth.[29] For example, in contrast to our findings, a significant increase in flavonoids was reported in lupin during germination.[35] The variations in phenolic compounds over the germination process could be related to decomposition of phenolic compounds, other biochemical changes in sprout metabolism, an alteration in production of self-sustaining enzymes (e.g., phenylalanine ammonia lyase), and the release of aglycones from conjugated glycosides, leading to the transformation and synthesis of new phenolic compounds in response to biological radicals caused by stresses.[9,26,29] Accordingly, these findings suggested that the appropriate stress and germination process may probably enhance the accumulation of bioactive compounds.

Total free amino acid composition

The total free amino acid composition was determined for both seeds and sprouts (control) and the results are presented in Table 3. The total free amino acid composition in sprouts (2518.06 mg 100 g^{-1}) was significantly ($P < 0.05$) increased compared to that of seeds (656.30 mg 100 g^{-1}), which was 3.87-fold higher than the total free amino acid composition in seeds. Among the total free amino acid composition, glutamine (614.91 mg 100 g^{-1}) was found to be the highest, followed by γ-aminobutyric acid (266.35 mg 100 g^{-1}), which increased by approximately 10- and 20-fold compared to that of seeds, respectively. Branched-chain amino acids (BCAAs), such as valine, leucine, and isoleucine, also increased significantly ($P < 0.05$) from 22.84 mg 100 g^{-1} to 305.31 mg 100 g^{-1} by the end of germination process. This clearly indicated the accumulation of free amino acids during germination by the hydrolysis of stored proteins in seeds, which further supports protein synthesis in endosperm and embryo for the germination of seeds.[36] Likewise, these findings were in line with a study by Huang and Jander[37] reporting the accumulation of BCAAs by the protein degradation to overcome the external stress conditions in *Arabidopsis thaliana*. Similar findings were also highlighted in a study conducted by Batista-Silva *et al.*,[38] where there was increased amino acid composition as a result of amino acid metabolism in stressed *A. thaliana*. The same study also showed the accumulation of amino acid derived secondary metabolites in stressed *A. thaliana*. Based on the above findings, it appears that protein degradation and amino acid metabolism are redirected towards an enhanced free amino acid composition in djulis sprouts, which can be considered as a good source of human nutrition.

CONCLUSIONS

The results obtained in the present study demonstrate the variations in biochemical changes for stressed djulis sprouts. The stress conditions enhanced the accumulation of betacyanin, phenolic compounds, and free amino acids with high antioxidant activities. The stressed sprouts exhibited mainly celosianins I and II, as well as phenolic acids, as the major biochemical compounds in stressed djulis sprouts. Therefore, germination and stress can be considered as a convenient and efficient natural method for enhancing the levels of bioactive compounds and the antioxidant activity of djulis sprouts over seeds. The findings of the present study enhance the application of djulis sprouts with accumulated bioactive compounds as ready-to-eat foods or as a functional ingredient in the development of bioactive compound-enriched healthy foods for public health and health-related benefits.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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