Basis for the new challenges of growing broccoli for health in hydroponics

Diego A Moreno,[1]* Carmen López-Berenguer,[1,2] M. Carmen Martínez-Ballesta,[2] Micaela Carvajal[2] and Cristina García-Viguera[1]

[1]*Department of Food Science and Technology, CEBAS-CSIC, Campus Universitario de Espinardo, PO Box 164, Espinardo 30100-Murcia, Spain*

[2]*Department of Plant Nutrition, CEBAS-CSIC, Campus Universitario de Espinardo, PO Box 164, Espinardo 30100-Murcia, Spain*

Abstract

BACKGROUND: Variations in the contents of phytochemicals with biological activity in broccoli could originate as a result of genetic and environmental factors. An understanding of the effects of growth conditions on the bioactive compounds in broccoli is essential for improving its quality and nutritive value. Using salinity (40 mmol L^{-1} NaCl), and foliar sprayed compounds (methionine, tryptophan and chitosan) as different stress conditions, broccoli developed in soilless culture in the greenhouse was analysed for biologically active phytochemicals (glucosinolates, caffeoyl-quinic, ferulic and sinapic derivatives and vitamin C).

RESULTS: The application of elicitors during head formation could be beneficial for the enrichment in phytochemicals in broccoli. Management practices for increasing a given phytochemical (e.g., glucoraphanin or glucobrassicin) may be related to a decreased level of natural antioxidants (hydroxycinnamic acids). Growing broccoli hydroponically in the greenhouse in winter (Mediterranean climate) needs the supporting treatment of abiotic stress during development (i.e., NaCl, elicitors).

CONCLUSION: The use of hydroponic growth conditions for broccoli and the application of stress factors (elicitors) at head induction and during development may serve the purpose of enhancing its nutritional quality to deliver a health-promoting food.

Keywords: *Brassica oleracea* var. *italica*; hydroxycinnamic acids; flavonoids; glucosinolates; vitamin C; soilless culture

INTRODUCTION

Today, consumers are more proactive and conscious in managing their health and the prevention of diet-related diseases. Many of these diseases are at epidemic levels, making the prevention of these lifestyle diseases attractive markets to exploit for both the food and pharmaceutical industries.

The consumption of diets containing five to ten servings of fruits and vegetables daily is the foundation for cancer prevention, and combinations of tomato and broccoli in the Dunning R3327-H prostate adenocarcinoma model was more effective at slowing tumour growth than either tomato or broccoli alone. This supports public health recommendations to increase the intake of a variety of health-giving fruits and vegetables.[1]

Broccoli, the worldwide-known immature flower vegetable of Brassicaceae (*Brassica oleracea* L. (Italica group)), is also well recognized as a health-promoting vegetable owing to its high content of beneficial biologically active compounds. Numerous epidemiological studies indicate that brassicas in general, and broccoli in particular, have potential for chemoprevention of degenerative diseases and certain types of cancer since they are rich sources of glucosinolates and dietary natural antioxidants: vitamins, flavonoids and hydroxycinnamic acids.[2,3]

Variations in the contents of phytochemicals with biological activity in broccoli could originate from genetic and environmental factors: variability of accessions, cultivars, organ, inflorescence development, temperature and radiation, growth system, fertilization practices, post-harvest storage and processing.[4–11] In this respect, water and dissolved salts are essential to plant growth, but water reuse and high evaporation rates in arid or semi-arid regions such as southeastern Spain (Murcia) concentrate the salts and salinization occurs. Broccoli is considered moderately sensitive/tolerant to salinity.[12] Looking into the glucosinolate composition of broccoli leaves and inflorescences, soilless-grown broccoli treated with

* Correspondence to: Diego A Moreno, Department of Food Science and Technology, CEBAS-CSIC, Campus Universitario de Espinardo, PO Box 164, Espinardo 30100-Murcia, Spain
E-mail: dmoreno@cebas.csic.es
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40 mmol NaCl in outdoor cultivation showed higher glucosinolate content than untreated plants.[13]

The lack of reproducibility of phytochemical analysis or activities is a major obstacle when plants are regrown in the field, resampled and re-extracted over the years. The biochemical profiles of plants harvested at different times and locations vary greatly and whole plant elicitation may also increases the amounts of natural products widely used in different areas of research. However, hydroponic cultivation is fast, simple and applicable to a great majority of plant species. Chemical composition and bioactivity could be readily reproduced if plants are regrown and re-elicited under standard greenhouse conditions.[13–15]

It is well known that different stresses, location climates, microenvironments and physical and chemical stimuli (often called elicitors) qualitatively and quantitatively alter the content of bioactive secondary metabolites, and whole-plant elicitation increases the amounts of bioactive compounds in foods of plant origin.[14–16] Clearly, genotypic variations observed in different plant species imposed both substantial variation and a genetic limit on the production of bioactive compounds. However, elicitation may be able to increase the production of some bioactive compounds up to the genetic limit.[15] To date, there are almost no reports on the effect of methionine or tryptophan elicitation or fertilization on glucosinolate-producing vegetable crops. Scheuner *et al*.[17] showed that methionine foliar fertilization increased the glucosinolate content in broccoli, but not in radish hypocotyls of greenhouse-grown plants. Chitosan has been reported as stimulating the growth and yield of soybean sprouts without adverse effects on vitamin C or their post-harvest characteristics.[18] Nonetheless, chitosan did not improve the production of glucotrapeolin hydrolysis products and the recorded levels were very close to control values in *Farsetia aegyptia* cultures.[19]

Studies on broccoli have been carried out either in open-air cultivation or in the growth chamber, but to date little is known about growing broccoli hydroponically in the greenhouse for the production of bioactive compounds of interest for human health.

The objective of this investigation was to determine the effects of different stress factors under soilless hydroponic growth conditions on the contents of glucosinolates, phenolic compounds (flavonoids and hydroxycinnamic acids) and vitamin C in broccoli. An understanding of the effects of growth conditions on bioactive compounds in broccoli is essential for improving its quality and nutritive value.

MATERIALS AND METHODS

Conditions of plant growth

The object of our investigations was 'Marathon' broccoli (*Brassica oleracea* (Plenck) Italica Group, cv. 'Marathon'). The plants were cultivated in the autumn–winter season (October 2006 to February 2007) in the greenhouse of the CEBAS-CSIC located in Espinardo (Murcia, Spain) under a semi-arid Mediterranean climate.

Broccoli and design of experiments

Broccoli seeds obtained from Ramiro Arnedo SA (Murcia, Spain), were pre-hydrated with aerated, deionized water for 2 h and germinated in vermiculite, at 28 °C in an incubator, for 2 days. They were then transferred to a controlled-environment growth chamber with a 16 h light–8 h dark cycle, and air temperatures of 25 and 20 °C, respectively. The relative humidity (RH) was 60% (day) and 80% (night) and photosynthetically active radiation (PAR) was 400 $\mu mol\, m^{-2}\, s^{-1}$, provided by a combination of fluorescent tubes (Philips TLD 36W/83, NY, USA and Sylvania F36W/GRO Munich, Germany); metal halide lamps (Osram HGI.T400W, Munich, Germany). After 5 days, the seedlings were placed in 15 L containers with continuously aerated Hoagland nutrient solution (published elsewhere), replaced completely every week.[12,13]

Broccoli (49-day-old) plants were transplanted to the greenhouse (day '0' after transplanting; 0 DAT). The experiments were conducted under a non-controlled environment in an aluminium-framed greenhouse with polycarbonate panels in a single gable structure. The humidity achieved in the greenhouse averaged 65%/85% (day/night) and the air temperature 16/9 °C (Table 1). The greenhouse was vented when the temperature exceeded the norm. Daily mean temperature and relative humidity were calculated from measurements taken every 10 min using dataloggers (AFORA SA, Barloworld Scientific, Murcia, Spain). A total of 25 'Marathon' broccoli plants were placed in a randomized design, using five plants per treatment, with each plant being grown in a perlite-filled 15 L container, spaced from each other, resulting in a density of 4 plants m^{-2}. All plants were grown under the same conditions and irrigated with complete Hoagland solution twice a week under natural light conditions, until 14 DAT (Table 2). At that moment, the application of 40 mmol NaCl in the nutrient solution started as treatment T1 (five plants), chosen because the previous studies, provided by López-Berenguer *et al*.,[12,13] had shown that 40 mmol NaCl increased glucosinolate levels in leaves (metabolic active leaves) and inflorescences (commercial size heads) of broccoli. The untreated control (five plants) and remaining plants did not show any symptom of deficiency or toxicity. Plants being treated with NaCl were also randomly subdivided into groups of five and subjected to the different stress conditions (also called elicitors) once approximately 30% of the plants reached head induction (0.3–0.5 cm head arc diameter; 52–56 DAT). The plants were sprayed with 40 mL of elicitor dissolved in 0.04% ethanol. A full description of the treatments is given in Table 2.

At harvest (75 DAT) all the plants were collected. Plants were separated into three parts: leaves

Table 1. Experimental conditions in the greenhouse (day/night) for growing 'Marathon' broccoli hydroponically

	Plant age (DAT)[a]						
	0	1–14	15–27	29–42	43–56	57–70	70–75
Air temperature (°C)[b]	16.3/11.7	15.4/9.4	13.4/7.5	16.7/8.7	15.7/9.0	15.8/9.1	21.6/12.4
Relative humidity (%)[b]	45.2/82.6	64.5/81.3	66.6/82.2	66.2/93.2	68.1/87.1	83.1/90.1	62.2/82.1

[a] Days after transplanting (DAT) to greenhouse; 7-week-old plants = 0 DAT.
[b] Average values of the time period.

Table 2. Description of treatments

Key	Treatment	DAT[a]	NaCl treated[b] (DAT)	Elicitor treated[c] (DAT)
Control	Complete Hoagland's nutrient solution	0–75		
T1	40 mmol NaCl added to nutrient solution	0–14	15–75	
T1 +E1	200 mmol DL-methionine (Alfa Aesar GmbH & Co. KG, Kralsruhe, Germany)	0–14	15–75	52–56
T1 +E2	200 mmol DL-tryptophan (Alfa Aesar GmbH & Co. KG, Kralsruhe, Germany)	0–14	15–75	52–56
T1 +E3	1 g L^{-1} chitosan (from crab shells; Sigma-Aldrich Química SA, Tres Cantos, Madrid)	0–14	15–75	52–56

[a] Days after transplanting (DAT) to greenhouse; 7-week-old plants = 0 DAT.
[b] 40 mmol NaCl added to Hoagland's complete nutrient solution.
[c] Broccoli plants were sprayed at 11:00 a.m. and at 15–20 cm distance, to minimize differences due to daily fluctuations (solution in 0.04% ethanol).

(leaf blades and petioles), stalks/stems and heads (inflorescences). For analytical purposes, the sampled organs of each treatment were cut into pieces and mixed thoroughly, to be again separated into five well-mixed replicates per treatment. Fresh weight was determined. The plant material was then flash frozen using liquid nitrogen and kept at −80 °C and dried in a freeze-drier Alpha (Type 1–4, Christ, Osterode am Harz, Germany). Dry weight was then determined and plant material was ground to a fine powder and stored at −20 °C for further analysis.

Extraction and determination of phenolic compounds

Freeze-dried powder samples (1 g) were homogenized with 25 mL of 70% methanol three times. The homogenates were filtered through a cheesecloth and kept in ice. The homogenates were centrifuged (4000 × *g*, 5 min, 4 °C) and the supernatants were evaporated under vacuum at 30 °C to approximately 1 mL and diluted to 2 mL with water. The supernatants were filtered through a 0.45 µm Millex-HV filter (Millipore, Bedford, MA, USA). The extracted samples (20 µL) were analysed on a Waters high-performance liquid chromatography (HPLC) system (Waters Cromatografía SA, Barcelona, Spain) consisting of a W600E multi-solvent delivery system, inline degasser, W717plus autosampler and a W2996 photodiode array detector, using a Luna C18 column (25 × 0.46 cm, 5 µm particle size; Phenomenex, Macclesfield, UK) with a security guard C18-ODS (4 × 3.0 mm) cartridge system (Phenomenex). The mobile phase was a mixture of 1 mL L^{-1} TFA (A) and acetonitrile/TFA (99.9:0.1, v:v) (B). Phenolic compounds were eluted off the column in 35 min. The flow rate was 1 mL min^{-1} in a linear gradient starting with 0% B 0–5 min, reaching 17% B in 15 min, 17% B at 17 min, 25% B at 22 min, 35% B at 30 min and 50% B at 35 min. Chromatograms were recorded at 320 and 360 nm.[20,21] Caffeoyl-quinic derivatives were quantified as chlorogenic acid (5-caffeoyl-quinic acid, Sigma, St Louis, MO, USA), flavonoids as quercetin 3-rutinoside (Sigma) and sinapic acid and ferulic derivatives as sinapinic acid (Sigma).

Extraction and determination of vitamin C

Ascorbic (AA) and dehydroascorbic (DHAA) acid contents were determined as described by Vallejo *et al*.[10,21] Briefly, 200 mg of freeze-dried sample was homogenized in a vortex stirrer for 20 s with 10 mL of extractant solution consisting of MeOH–H_2O (5:95) plus citric acid 2.1%, ethylenediaminetetraacetic acid (EDTA) 0.05% and NaF 0.01%; the homogenate was filtered through a cheesecloth and the pH adjusted to 2.2–2.4 by addition of 6 mol HCl. The extract was centrifuged (3.600 × g for 15 min at 4 °C) and the supernatant was recovered, filtered through a C18 Sep-Pack cartridge (Waters, Milford, MA, USA), previously activated with 10 mL of methanol followed by the same volume of water and then the same volume of air, and filtered through a 0.45 µm polyethersulfone filter (Millex-HV, Millipore). HPLC analysis of vitamin C (AA + DHAA) was achieved after derivatization of DHAA into the fluorophore 3-(1,2-dihydroxyethyl)furo(3,4-b) quinoxaline-1-one (DFQ) with fresh daily prepared 1,2-ortophenylenediamine (OPDA). OPDA solution was added to the water-soluble fraction eluted from a C18 solid-phase extraction cartridge Sep-Pak (1:3, v:v). Samples were incubated for 37 min at room

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temperature in the dark, and 20 μL analysed with a Merck-Hitachi (Tokyo, Japan) HPLC, equipped with an L-4000 UV detector and an L-6000 pump. Separations of DFQ and AA were achieved on a Kromasil 100 C18 column (25 × 0.4 cm; 5 μm particle size; Tecnokroma, Barcelona, Spain). The mobile phase was methanol–water (5:95, v:v) containing 5 mmol cetrimide and 50 mmol potassium dihydrogen phosphate at pH 4.5. The flow rate was 0.9 mL min^{-1}; the detector wavelength was initially set at 348 nm, and after elution of DFQ was manually shifted to 261 nm for AA detection.

Extraction and determination of glucosinolates

We followed the procedure as fully described in Martínez-Sánchez *et al*.[22] for extraction of intact glucosinolates.[3] Briefly, freeze-dried samples (50 mg) were extracted with 1.5 mL of 70% MeOH, and placed in a sonicator bath for 10 min to improve the methanol extraction. The mixture was heated at 70 °C for 30 min with a heating bath and shaking every 5 min with a vortex stirrer, and centrifuged (30 min, 17 500 × *g*, 4 °C). Supernatants were collected and methanol completely removed using a rotary evaporator; the obtained dry material was redissolved in 1 mL ultrapure water and filtrated through 0.45 μm Millex-HV filter. Each sample (20 μL) was analysed in a Waters HPLC system (Waters Cromatografía SA, Barcelona, Spain) under the same conditions mentioned above for polyphenolic compounds. Chromatograms were recorded at 227 nm. Samples were identified using the previously described intact glucosinolate LC-MS method and quantified by HPLC–diode array detection (HPLC-DAD) using sinigrin (sinigrin monohydrate from *Sinapis nigra*, Phytoplan Diehm & Neuberger GmbH, Heidelberg, Germany) as standard.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using Statgraphics version 7.0 software (Manugistics, Inc.). The data shown are mean values and the significance of the differences was compared using a multiple comparison test at LSD $P < 0.05$ probability level (Duncan's multiple range test).

RESULTS AND DISCUSSION

Broccoli parameters at harvest

The harvested inflorescences (commercial size heads) were significantly heavier in the T1 + E2 treatment, surpassing the inflorescences of the control plants by 25% in fresh mass (Table 3). The T1 and T1 + E1 were intermediate treatments between T1 + E2 and the control, only 12% and 8% higher, respectively. The T1 + T3 inflorescences did not differ from the untreated control. The fresh weight of the young fully expanded leaves (metabolically active leaves) showed a similar result, with T1 + E2 and T1 + E1 being 49% and 32% higher than the control, respectively (Table 3).

Table 3. Biomass parameters (g per plant) of greenhouse-grown 'Marathon' broccoli at harvest

Treatment	Broccoli head	Leaves
Control	165.79b[a]	324.37c
T1	188.70ab	352.14bc
T1 +E1	183.33ab	427.36ab
T1 +E2	211.77a	483.48a
T1 +E3	168.89b	341.11bc
ANOVA *P*-value	$P < 0.05$	$P < 0.05$
LSD ($P < 0.05$)	26.05	101.57

[a] Means ($n = 5$) within a column followed by the same lower-case letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Taking into account that the collected inflorescences were all of a very similar size (120–135 mm diameter in average), changes in fresh mass could be related more to density of the inflorescences. The application of abiotic stress through 40 mmol L^{-1} NaCl in the nutrient solution (T1) and the additional application of methionine (T1 + E1) or tryptophan (T1 + T2) would then prove positive in some way for the inflorescence biomass of broccoli grown hydroponically; this fact would be of interest in the event that a higher content of phytochemicals could be found in such inflorescences. On the contrary, the application of Chitosan (T1 + E3), showed values similar to the control.

Hydroxycinnamic acid derivatives and flavonoids

Hydroxycinnamoyl derivatives were identified by their chromatographic behaviour and UV spectra, using HPLC-MS and chromatographic comparisons with authentic markers.[20,21] The pattern found in broccoli was similar to that previously described by other authors.[8] A number of individual flavonoids (10–15, depending on the treatment) were detected but not fully identified, mainly quercetin and kaempferol glycosides, in agreement with previous reports on broccoli.[20,21]

The total flavonoid contents in broccoli inflorescences were significantly affected by the treatments (Table 4), T1 + E3 being the highest (by 52%) against the control. The caffeoyl-quinic derivatives were also higher in T1 + E3 than in the control, with intermediate values for the rest of the treatments. The same was found for the sinapic and ferulic derivatives, with the majority of the compounds also being higher in T1 + T3 and T1 + E1 than in the control. The 1,2-diferuloylgentiobiose (3) was similar in the control and T1 + E1, and higher than the remaining treatments. On average, total phenolic contents reflected the results of the individual compounds (Fig. 1), and in T1 + E3 (chitosan-sprayed salt-stressed broccoli) the total phenolic content was improved by 44%, followed by T1 + E1 (methionine-sprayed NaCl-treated plants), a 39% higher than the inflorescences in the

Table 4. Total flavonoids, caffeoyl-quinic derivatives, and individual sinapic and ferulic derivative levels (mg 100 g^{-1} fresh weight) in the inflorescences of greenhouse-grown 'Marathon' broccoli

		Caffeoyl-quinic derivatives[a]		Sinapic and ferulic derivatives[a]							
Treatment	Total flavonoids	C1	C2	1	2	3	4	5	6	7	8
Control	24.97c[b]	1.91b	1.07c	2.22c	2.80a	0.64a	0.24c	1.81b	1.41b	0.29ab	0.05c
T1	33.86b	2.61a	1.23c	2.45b	2.94a	0.54b	1.39a	1.84b	1.23c	0.23bc	0.28ab
T1 +E1	36.26ab	2.59a	1.74b	2.62ab	3.03a	0.58ab	0.79b	2.28a	1.68a	0.33a	0.26ab
T1 +E2	32.44b	2.04b	1.59b	1.86d	2.18b	0.40c	0.28c	1.53c	1.07c	0.18c	0.19b
T1 +E3	37.89a	2.62a	2.11a	2.82a	3.06a	0.54b	0.18c	2.30a	1.77a	0.33a	0.32a
ANOVA *P*-value	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.01$	$P < 0.001$
LSD ($P < 0.05$)	3.96	0.22	0.29	0.21	0.39	0.09	0.25	0.20	0.16	0.07	0.09

[a] C1 (neochlorogenic acid) and C2 (chlorogenic acid); sinapic and ferulic derivatives: 1 (1,2-disinapoylgentiobiose); 2 (1-sinapoyl-2-feruloylgentiobiose); 3 (1,2-diferuloylgentiobiose); 4 (1,2,2′-trisinapoylgentiobiose); 5 (1,2′-disinapoyl-2-feruloylgentiobiose); 6 (1-Sinapoyl-2,2′-diferuloylgentiobiose); 7 (1,2,2′-trisinapoylgentiobiose); 8 (1,2,2′-triferuloylgentiobiose); compound identification according to HPLC-DAD-MS analysis.[21]
[b] Means ($n = 5$) within a column followed by the same lower-case letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

control. The results of polyphenolics in broccoli were probably not related to a dilution effect, even though T1 + E3 inflorescences were smaller (Table 3). The correlation coefficients between individual and total phenolics and the weight of inflorescences were not significant ($P > 0.1$; data not shown). Thus, the application of elicitors (methionine and chitosan) during head formation could be beneficial for the enrichment in phytochemicals in broccoli grown hydroponically, and the effect was additional to the salt-induced stress in these plants, since T1 + E1, T1 + E2, and T1 + T3 were all significantly higher (total phenols; Fig. 1) than T1 and the untreated control.

When looking at the effects on leaves – the metabolic factory of the plant, but in terms of phytochemical farming, treated as a byproduct of the broccoli agri-food activity – the hydroxycinnamic acids in young fully expanded leaves (Table 5) were also significantly affected by treatments. The total content of flavonoids was much higher than in the inflorescences (Table 4), but in this case the control, salt-stressed T1 and elicited T1 + E1 leaves were similar in content while the leaves of T1 + E2- and T1 + E3-sprayed plants were surpassed by the control. The trend was very similar for the caffeoyl-quinic derivatives and sinapic and ferulic derivatives (Table 5). Figure 2 shows the total content of phenolics in broccoli leaves, where T1 + E2 and T1 + T3 were surpassed by the control by 41% and 25%, respectively.

The flavonoids in greenhouse-grown broccoli are stated to be present at lower levels than in field cultures.[23] Broccoli produced in late summer and early autumn field crops in different parts of Europe presented different flavonol contents, ranging from 1.5–6.5 mg 100 g^{-1} fresh weight[8] to 5.7–9.6 mg 100 g^{-1} fresh weight ('Marathon' broccoli).[4] Growing broccoli hydroponically in the greenhouse during the autumn/winter in southeastern Spain, we found that total flavonoid contents were higher than previously

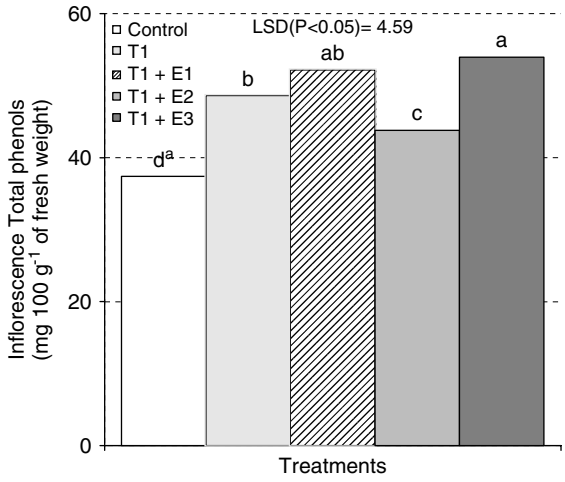

Figure 1. Total phenolics (mg 100 g^{-1} of fresh weight) in the inflorescences of greenhouse-grown 'Marathon' broccoli. [a]Means ($n = 5$; $P < 0.001$) with the same lower-case letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

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Table 5. Total flavonoids, caffeoyl-quinic derivatives, and individual sinapic and ferulic derivative levels (mg 100 g^{-1} fresh weight) in young fully expanded leaves of greenhouse-grown 'Marathon' broccoli

		Caffeoyl-quinic derivatives[a]		Sinapic and ferulic derivatives[a]							
Treatment	Total flavonoids	C1	C2	1	2	3	4	5	6	7	8
Control	51.15a[b]	5.16ab	5.03a	5.06ab	4.25a	0.05ab	0.35bc	1.35b	1.32bc	0.27a	0.05b
T1	55.14a	6.05a	4.39a	5.26a	4.38a	0.04b	0.43ab	2.03a	1.64a	0.34a	0.07ab
T1 +E1	49.65a	5.22ab	4.65a	5.65a	3.99a	0.06a	0.44a	2.00a	1.54ab	0.38a	0.09a
T1 +E2	34.32b	4.25c	3.62b	3.66c	2.96b	0.05ab	0.25d	1.58b	1.27c	0.27a	0.06b
T1 +E3	40.35b	4.48bc	3.53b	4.35bc	3.25b	0.04b	0.32cd	1.51b	1.15c	0.23a	0.05b
ANOVA *P*-value	$P < 0.001$	$P < 0.01$	$P < 0.001$	$P < 0.01$	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.01$	$P > 0.05$	$P < 0.05$
LSD ($P < 0.05$)	8.30	0.91	0.67	0.90	0.73	0.01	0.08	0.28	0.24	0.19	0.03

[a] C1 (neochlorogenic acid) and C2 (chlorogenic acid); sinapic and ferulic derivatives: 1 (1,2-disinapoylgentiobiose); 2 (1-sinapoyl-2-feruloylgentiobiose); 3 (1,2-diferuloylgentiobiose); 4 (1,2,2′-trisinapoylgentiobiose); 5 (1,2′-disinapoyl-2-feruloylgentiobiose); 6 (1-sinapoyl-2,2′-diferuloylgentiobiose); 7 (1,2,2′-trisinapoylgentiobiose); 8 (1,2,2′-triferuloylgentiobiose); compound identification according to HPLC-DAD-MS analysis.[21]

[b] Means ($n = 5$) within a column followed by the same lower-case letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

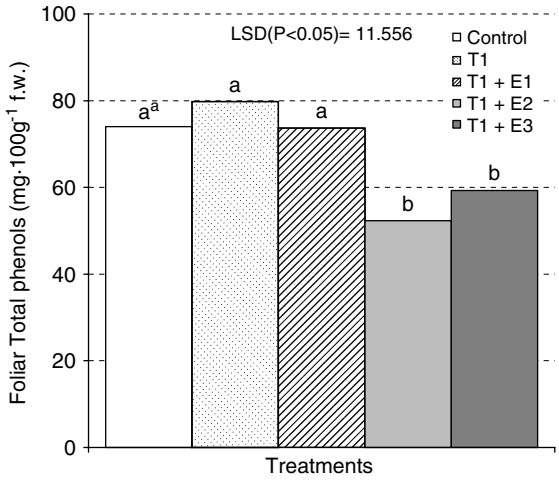

Figure 2. Total phenolics (mg 100 g^{-1} of fresh weight) in young fully expanded leaves of greenhouse-grown 'Marathon' broccoli. [a]Means ($n = 5$; $P < 0.001$) with the same lower-case letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

reported. The agronomic conditions (growing in the greenhouse during winter) should be taken into consideration if we are intending to obtain broccoli for raw material/ingredients for the development of functional foods or phytochemically rich vegetables, growing the plants under the controlled greenhouse environment and with the putative use of stress factors (elicitors), increasing the content of phytochemicals in broccoli.

The results of the phenolic contents in the leaves could be not be explained by a possible 'dilution' of the phytochemicals with development, because of the absence of correlation between these parameters ($P > 0.05$; data not shown). Instead, we could find at least part of the explanation in the physiological function of the leaves in a stressed plant, and the source–sink relationships for the biosynthesis and translocation of different phytochemicals to the inflorescences, to maximize the resource-use efficiency of the plant.[24]

Vitamin C

The AA and the dehydroascorbic acid DHAA in the broccoli inflorescences were affected by the treatments, but not the total content of vitamin C (Fig. 3). Our 'Marathon' inflorescences were less rich in vitamin C than the field-produced inflorescences (74–107 mg 100 g^{-1} fresh weight),[20,21] although the content could be considered as normal (25–80 mg 100 g^{-1} fresh weight).[25] The remarkable data of the T1 + E3 (chitosan) inducing the significant highest and lowest AA and DHAA levels, respectively, confirmed the effects of this elicitor with no negative effect on ascorbate,[18] but the general trend is the absence of effects on the total content of vitamin C, which is also a positive outcome. The effects on young fully expanded leaves were quite different (Fig. 4), because the higher content of vitamin C in leaves than in the inflorescences was owed to the high to high levels of DHAA,

and in this case the T1 + E3 treatment induced the lowest content of vitamin C in leaves. The highest vitamin C content was found in the control plants, not different from T1 + E1 or T1 + E2. A possible explanation of these results may be related to the source/sink trends between leaves and inflorescences in broccoli (weak positive correlation coefficients of $r^2 < 0.4$; data not shown). From these results, it looks as though the changes in vitamin C and its components (AA and DHAA) could be more related to the environmental growth conditions (Table 1) than to the imposed stress factor treatments (Table 2, Fig. 4). Ascorbic acid declined under full sunlight conditions in mustard greens, and supplementing natural light with blue or sodium vapour light increased ascorbic acid concentrations in broccoli. Rainy, cloudy, cool conditions also decrease ascorbic acid.[26] In such conditions, similar to our experimental setup, the relationships between size and phytonutrient concentrations may or may not always be linear, and may not always be negative for ascorbic acid.

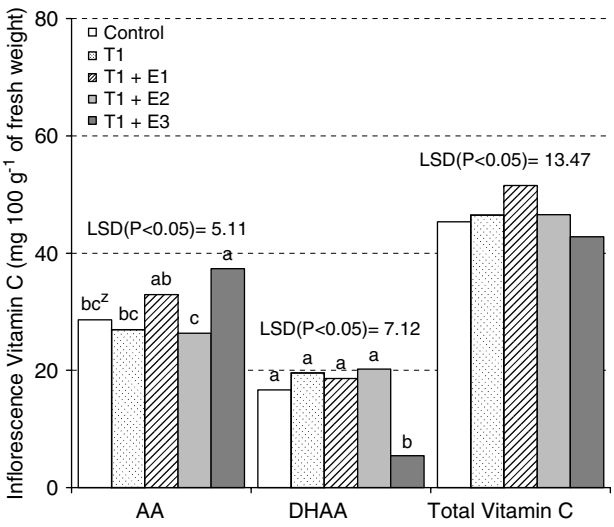

Figure 3. Ascorbic acid (AA), dehydroascorbic acid (DHAA), and total vitamin C (mg100 g^{-1} of fresh weight), in the inflorescences of greenhouse-grown 'Marathon' broccoli. [a]Means ($n = 5$; AA $P < 0.01$; DHA $P < 0.05$) with the same lower-case letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Glucosinolates

The broccoli inflorescences (Table 6) contained the aliphatic glucosinolates glucoiberin (GI), glucoraphanin (GR) and glucoerucin (GE), as well as the indole glucosinolates 4-hydroxy-glucobrassicin (HGB), glucobrassicin (GB), 4-methoxyglucobrassicin (MGB) and neoglucobrassicin (NGB). The main glucosinolates were glucoraphanin and glucobrassicin, but other glucosinolates found in very small amounts (glucoalyssin, gluconapin, etc.) were taking into account only for the total content of glucosinolates (Table 6). Both aliphatic and indole glucosinolates were affected by the treatments, and T1 + E1 induced significantly higher amounts of the aliphatic glucosinolates GI (by 47%) and GR (by 21%) than the control. T1 + E2 also surpassed the control by 24% for GE. The indole glucosinolates were similar between the control, T1 (40 mmol NaCl) and T1 + E1, with significantly higher amounts of HGB, GB, MGB and NGB than T1 + E2, and with T1 + E3, the treatment with the lowest content in total glucosinolates.

GR concentration in the broccoli inflorescences was significantly increased compared to the control when methionine (T1 + E1) was applied at the time of head formation. In previous reports of greenhouse-grown broccoli under controlled conditions and fertilized with methionine, the same kind of response was found.[17] In any case, differences in total contents were not relevant if compared to the untreated control.

The metabolically active leaves showed that GR was highest in the T1 + E1 treatment (Table 7), surpassing the control by 78%, whereas GI and GE were significantly the lowest in T1 + E3. The indole glucosinolates showed a similar variation between treatments, with HGB, GB, MGB and NGB being higher in control, T1, T1 + E1 and T1 + E2, and lowest in T1 + E3, as repeated in the total content of glucosinolates, and similar to what was found for the inflorescences (Table 5).

Table 6. Aliphatic, indole and total glucosinolates (mg 100 g^{-1} fresh weight) in the inflorescences (broccoli heads) of greenhouse-grown 'Marathon' broccoli

	Aliphatic glucosinolates[a]			Indole glucosinolates[a]				
Treatment	GI	GR	GE	HGB	GB	MGB	NGB	Total glucosinolates
Control	14.62b[b]	38.12bc	15.72b	22.10abc	68.36a	17.10a	58.47a	260.94a
T1	14.61b	42.73ab	15.50b	25.55ab	59.07b	16.68a	38.82b	235.39a
T1 +E1	21.55a	46.12a	16.37b	25.37a	52.49c	14.17b	36.52b	234.58a
T1 +E2	15.63b	33.01c	19.56a	20.44bc	43.40d	12.61b	25.36c	201.82b
T1 +E3	13.88b	33.62c	10.56c	17.86c	49.68c	9.928c	23.03c	178.45b
ANOVA *P*-value	$P < 0.01$	$P < 0.01$	$P < 0.001$	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
LSD ($P < 0.05$)	4.26	6.33	2.95	5.12	6.08	2.35	6.23	29.46

[a] GI: 3-methylslfinylpropyl-glucosinolate; GR: 4-methylsulfinylbutyl-glucosinolate; GE: 4-methylthiobutyl-glucosinolate; HGB: 4-hydroxyindol-3-ylmethyl-gls; GB: 3-indolylmethyl-gls; MGB: 4-methoxy-3-indolylmethyl-gls; NGB: *N*-methoxy-3-indolylmethyl-gls.

[b] Means ($n = 5$) within a column followed by the same lower-case letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

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Table 7. Aliphatic, indole and total glucosinolates (mg 100 g^{-1} fresh weight) in the young fully expanded leaves of greenhouse-grown 'Marathon' broccoli

	Aliphatic glucosinolates[a]			Indole glucosinolates[a]				
Treatment	GI	GR	GE	HGB	GB	MGB	NGB	Total glucosinolates
Control	24.56a[b]	17.93b	18.01a	18.21a	26.90bc	27.91a	15.97cd	208.95a
T1	17.81b	16.64b	18.73a	10.27bc	35.26bc	26.79a	21.91ab	221.89a
T1 +E1	15.80b	31.98a	16.17a	12.27b	48.78a	25.61a	18.97bc	207.83a
T1 +E2	26.54a	15.42b	15.39ab	12.44b	36.09b	22.86ab	23.24a	206.66a
T1 +E3	10.09c	10.32c	12.54b	7.67c	26.24c	18.72b	14.68d	140.77b
ANOVA *P*-value	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.01$	$P < 0.001$
LSD ($P < 0.05$)	5.78	5.10	3.51	2.818	9.62	5.41	4.17	21.69

[a] GI: 3-methylslfinylpropyl-glucosinolate; GR: 4-methylsulfinylbutyl-glucosinolate; GE: 4-methylthiobutyl-glucosinolate; HGB: 4-hydroxyindol-3-ylmethyl-gls; GB: 3-indolylmethyl-gls; MGB: 4-methoxy-3-indolylmethyl-gls; NGB: *N*-methoxy-3-indolylmethyl-gls.
[b] Means ($n = 5$) within a column followed by the same lower-case letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

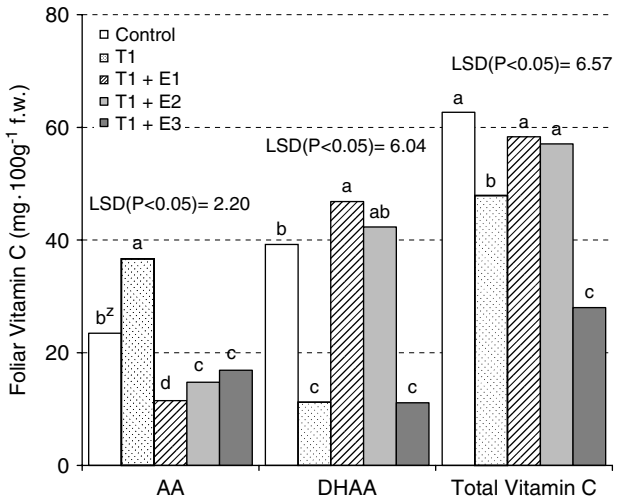

Figure 4. Ascorbic acid (AA), dehydroascorbic acid (DHAA), and total vitamin C (mg 100 g^{-1} of fresh weight), in young fully expanded leaves of greenhouse-grown 'Marathon' broccoli. [a]Means ($n = 5$; $P < 0.001$) with the same lower-case letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Numerous studies on single vegetables and phytochemicals have demonstrated that pre-harvest variables are factors that have the potential to influence the phytochemical content in the final produce.[5,11,27,28] In using immature broccoli flower crops for the production of glucosinolate-enriched raw plant material, for functional foods or supplements, cultivation of its cultivars (e.g. 'Marathon', 'Shogun') in seasons with relatively low daily mean temperatures (about 14 °C; springtime in Germany) has been recommended, combined with rising daily mean irradiation up to 450 µmol m^{-2} s^{-1} of the photosynthetic photon flux density.[28] Broccoli could be produced in Murcia (Spain) in the fall/winter and early spring seasons,[8,11,20] at temperatures within that range (Table 1). The effects of treatments on individual and total glucosinolates of the inflorescences and leaves in this experiment (greenhouse soilless culture) could be due to the changing source–sink relationship of photoassimilates, and glucosinolate exchange between the individual plant organs is also possible, since the reason for the different responses amongst the glucosinolate groups could be because the various enzymes involved in the synthesis of each glucosinolate are affected differently depending upon treatment and environmental conditions,[7] helping to clarify why the control, T1, T1 + E1 and T1 + E2 presented similar glucosinolate contents (individual and total glucosinolates in the inflorescences and leaves).

In most markets broccoli is sold on a per head basis, not by weight. Total and individual glucosinolates per head may be an essential criterion in considering enhancement of phytochemicals (i.e., glucosinolates) in certain broccoli genotypes.[27] In our work, there is no significant correlation between individual or total glucosinolate content and head weight, and there has been no indication of a dilution effect, only a weak correlation between GE and head weight ($r^2 = 0.439$, $P < 0.05$) or between leaf weight and total glucosinolates ($r^2 = 0.415$, $P < 0.05$).

Management practices such as nutrient supply have been investigated for their specific influences on the contents of glucosinolates and aroma volatiles by using broccoli and radish as examples.[8,11,29] An increased level of mineral nutrients (i.e., nitrogen, mineral and organic fertilization) in a field experiment with broccoli decreased the content of aliphatic glucosinolates. Using Hoagland's solution for broccoli may have supplied enough nutrients to maintain a high level of glucosinolates in control and treated plants (T1, T1 + E1, T1 + E2), but not in T1 + E3, where the influence of chitosan could account for the effect on glucosinolates and phenolics more importantly than the plant nutrient status.

Significant effects of the treatments on phytochemical content in broccoli indicate that the management practices for increasing one given phytochemical (i.e., glucoraphanin and glucobrassicin for chemoprevention) may be related to a decreased level of natural antioxidants (i.e., hydroxycinnamic acids). The response in the inflorescences for total glucosinolates (lowest in T1 + E3) and total phenolics (highest in T1 + E3) was somehow corroborated or supported by

the correlation between total glucosinolates and the total flavonoids ($r^2 = 0.518$; $P < 0.01$) and phenolics ($r^2 = 0.418$; $P < 0.05$) as a negative relationship. For the leaves, phenolics and glucosinolates were not significantly related ($r^2 < 0.1$). Obtaining broccoli that delivers high amounts of bioactive phytochemicals and chemoprotective potency as a feasible goal therefore needs the consideration of all the factors (i.e., environmental conditions, abiotic stress treatments, elicitors) responsible for the wide variation in phytochemical contents at harvest.[27,28]

CONCLUSIONS

To satisfy the increasing health consciousness of consumers worldwide, the demand for broccoli enriched with phytochemicals – available as fresh produce or raw material for functional foods and supplements – would need the integration of total quality management strategy with respect to the genetic and environmental effects on the formation of bioactive compounds, selecting the correct time of planting and harvesting as well as the use of abiotic stress (NaCl), during the vegetative period and additional factors during head induction and development (i.e., sprayed elicitors), for the induction of phytochemical biosynthesis, to manipulate the response of plants to different environmental factors, and to enhance the amount of dietary antioxidants and phytonutrients (i.e., human wellness compounds) which along with consumption of five or more servings per day of fruits and vegetables will make for a healthier population. From our experience at this point, growing broccoli hydroponically in the greenhouse as a winter crop in Spain (Mediterranean climate) needs the supporting treatment of abiotic stress during development (i.e., NaCl). Additionally, the use of stress factors at head induction and development (i.e., elicitors) may serve the purpose of enhancing the nutritional quality to deliver a health-promoting food.

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