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Evaluation of hydrogen sulfide supply to biostimulate the nutritive and phytochemical quality and the antioxidant capacity of Cabbage (*Brassica oleracea* L. 'Bronco')

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Summary

The potential effects of the hydrogen sulfide on shoot biomass, nutritional quality and antioxidant capacity of *Brassica oleracea,* were investigated through the application of increasing doses of NaHS (H_2S donor NaHS; 0.5, 1, 2.5, and 5 mM). The results showed that the 0.5 and 1 mM NaHS treatments increased biomass and the quality composition of 'Bronco' cabbage (i.e. chlorophylls, carotenoids, anthocyanins, flavonols, total phenolics and sinigrin). On the other hand, there was an increase in lipid peroxidation and hydrogen peroxide content with the application of doses higher than 2.5 mM NaHS. Therefore, we selected the 0.5 and 1 mM NaHS dosages as optimal for cabbage. The 2.5 and 5 mM NaHS produced an excessive lipid peroxidation, decreases in plant biomass and losses of chlorophylls, being all considered negative effects, and clear evidences of stressful situation for the plants. For practical purposes, this study suggested that exogenous application of H_2S donor NaHS at 0.5 and 1 mM may be useful as bio-stimulant to boost the yield and the health-promoting composition of 'Bronco' cabbage (*Brassica oleracea* L.).

Keywords
Hydrogen sulfide, health-promoting compounds, *Brassica oleracea*, antioxidant capacity.

Introduction

Sulfur is an essential mineral nutrient element, crucial for sulfur-amino acids (cysteine and methionine), natural antioxidants (reduced glutathione; GSH), co-enzymes, prosthetic groups, vitamins, secondary metabolites, phytochelatins (PCs) and lipids (Khan et al., 2014 a; Khan et al., 2014 b). With respect to the different forms of application to plants of this macronutrient in last few years there has been an increased interest in the study of hydrogen sulfide (H_2S) on plant physiology (Lisjak et al., 2013). High doses of H_2S in greenhouses caused leaf lesions, defoliation, reduced growth, and death of sensitive species such as alfalfa (*Medicago sativa* L.), lettuce (*Lactuca sativa*), sugar beet (*Beta vulgaris*) (Thompson and Kats, 1978). Although several works showed that this compound can adversely affects the growth and physiology of crops (Koch and Erskine, 2001), recent works suggests that H_2S is a more fundamental molecule which is produced by plants and used to control plant function (Zhang et al., 2010). So, H_2S has shown its action as a signal molecule and it has been used to promote the antioxidant enzyme activities and uptake of elements against abiotic stress in barley (Gadalla and Snyder, 2010; Dawood et al., 2012). Moreover, it was observed that H_2S involved in the antioxidant response against osmotic stresses (i.e. excessive boron and drought) in cucumber and sweet potato respectively (Zhang et al., 2009; Wan et al., 2010). Nonetheless, the application of H_2S increased the antioxidant capacity and quality parameters in fruits and berries (i.e., mulberry, kiwi, and strawberry) (Hu et al., 2012; Hu et al., 2014; Zhu et al., 2014).

Brassicaceae vegetables are economically relevant crops and important human foods worldwide, highly used in China, Japan, India, and European countries (Cartea et al., 2010). The popularity and consumption of Brassica is growing because of their renewed relevance on human health through the prevention of certain degenerative diseases (i.e. cardiovascular, cognitive, Alzheimer's and Parkinson's, etc.) and reduction in the risks of suffering from cancer (i.e., lung, breast, colon, prostate) (Cartea et al., 2010; Podsedek, 2007; Bazzano et al., 2002; Smith-Warner et al., 2003; Cho et al., 2004).

These health-promoting properties are attributed to their composition very rich in intrinsic and indirect antioxidants (Cartea et al., 2010). Natural antioxidants in Brassica, includes ascorbate, anthocyanins, phenolic compounds and carotenoids (Galati and O'Brien, 2004). Of particular relevance, ascorbate or vitamin C and phenolic compounds are highlighted, as well as the phenolic compounds, which has been shown to exert antioxidant, anticarcinogenic, antimicrobial, antiallergic, antimutagenic, and anti-inflammatory activities (Martínez-Valverde et al., 2002).

Moreover, essentially unique to cruciferous crops and foods, and more relevant in terms of health-promoting effects are the glucosinolates (GLSs). The GLSs are a heterogeneous family of molecules characterized by a similar basic structure containing a sulphur-linked β-D-glucopyranoside, a sulphonated oxime, and a side chain derived from different amino acids, which allow their classification in: aliphatic, aromatic, and indolic GLSs (Fahey et al., 2001). GLSs have gained growing attention for their potential health-promoting properties, as their hydrolysis products (isothiocyanates, ITCs) are able to induce phase 2 detoxification enzymes and protect mammals against chemically induced cancer (Zhang et al., 1992). So, the consumption of vegetables containing glucosinolates, such as *Brassica oleracea* varieties, may confer protection against different types of cancer (Cartea et al., 2010; Zhang et al., 1992).

One strategy to improve the nutritional characteristics of crops is the optimal application of different forms of S. The most widely used form of S for this purpose has been SO_4^-, favoring yield in number of fruits and the vitamin C content in strawberries (Eshghi and Jamali, 2014). Others authors also reported increased total phenolics and antioxidant capacity in mango fruits upon application of sodium bisulfate (Siddiq et al., 2013). In addition to SO_4^-, in the last few years it has been observed that the application of S in a more reduced form than SO_4^- such as H_2S could have a positive effect on the nutritional quality, but such treatments were performed during postharvest, for example in mulberry, after applying 0.8 mM of the H_2S donor NaHS, increased contents of ascorbate and soluble proteins, were found, maintaining the postharvest quality (Hu et al., 2014). Using the same

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fumigation (0.8 mM of the H_2S donor NaHS) resulted in increased firmness and colour, and delayed respiratory damage in strawberries, prolonging postharvest shelf life (HU et al., 2012). Nevertheless, there are very limited studies to examine the potential beneficial effects of H_2S on the nutritional quality, yield and phytochemical quality of leafy vegetables not affected by any abiotic or biotic stress, and therefore, the aim of this study was to determine the influence of H_2S application (as sodium hydrosulfide hydrate, NaHS + H_2O) on the biomass production and antioxidant capacity response and the nutritional and phytochemical quality of 'Bronco' cabbage (*Brassica oleracea* L.).

Material and methods

Plant material and treatments

Seeds of *B. oleracea* cv. Bronco (Saliplant S.L., Spain) were germinated and grown for 35 days in cell flats of 3 cm × 3 cm × 10 cm filled with a perlite mixture substratum. The flats were placed on benches in an experimental greenhouse located in Southern Spain (Saliplant S.L., Motril, Granada). After 35 days, the seedlings were transferred to a growth chamber under the following controlled environmental conditions: Relative humidity 50%; Day/night temperatures 25/18 °C; 16/8 h photoperiod at a photosynthetic photon flux density (PPFD) of 350 μmol $m^{-2}s^{-1}$ (measured at the top of the seedlings with a 190 SB quantum sensor, LI-CORInc., Lincoln, Nebraska, USA). Under these conditions the plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) of 3 L volume. Throughout the experiment the plants were treated with a growth solution made up of 4 mM KNO_3, 3 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2 mM $MgSO_4{\cdot}7H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4{\cdot}2H_2O$, 2 μM $MnCl_2{\cdot}4H_2O$, 10 μM $ZnSO_4{\cdot}7H_2O$, 0.25 μM $CuSO_4{\cdot}5H_2O$, 0.1 μM $Na_2MoO_4{\cdot}2H_2O$, 5 mg L^{-1} Fe-chelate (Sequestrene; 138 FeG100) and 10 μM H_3BO_3. This solution, with a pH of 5.5-6.0, was changed every three days.

Experimental design

The NaHS treatments were initiated 43 days after germination and were maintained for 30 days. To determine the concentrations of NaHS to apply, It was carried out a previous culture in which it was observed the response of *Brassica oleracea* L. 'Bronco' to a wide range of concentrations of NaHS, ranging from 0.01 mM to 6 mM in which was observed as the most appropriate doses to study the effects of fortification and toxicity in this species were: 0.5, 1, 2.5 and 5 mM of NaHS. For that reason the experiment consisted in a randomized complete block design with five treatments that were supplied with the irrigation solution (complete nutrient solution amended with different NaHS levels: 0 mM NaHS; and 0.5 mM, 1 mM, 2.5 mM and 5 mM NaHS), arranged in 8 plants per tray (In expanded polystyrene support) and three replicates per treatment.

Plant sampling

Plants of each treatment (73 days after germination) were divided into roots and leaves, washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of leaves from each treatment were frozen at -30 °C for further work and biochemical assays and the other half of the plant material was lyophilised for 48 h to obtain the dry weight (DW) and the subsequent analysis of phenolics and GLSs.

Determination of H_2S content

0.1 g of *Brassica oleracea* leaves were ground under liquid nitrogen and extracted by 1 mL phosphate buffered saline (50 mM, pH 6.8) containing 0.1 M EDTA and 0.2 M ascorbic acid. After centrifugation at 12000 rpm for 15 min at 4 ºC, 400 mL of the supernatant was injected to 200 mL 1% zinc acetate and 200 mL 1 N HCl. After 30 min reaction, 100 mL 5 mM dimethyl-p-phenylene-diamine dissolved in 7 mM HCl was added to the trap followed by the injection of 100 mL 50 mM ferric ammonium sulfate in 200 mM HCl. After 15 min incubation at room temperature, the amount of H_2S was determined at 667 nm. Solutions with different concentrations of Na_2S were used in a calibration curve (SEKIYA et al., 1982).

Chlorophyll concentration and lipid peroxidation

For the extraction of chlorophylls, chlorophyll a (Chla) and b (Chlb), 0.1 g of leaves were ground in semidarkness and resuspended in 10 mL of cold acetone at 80%. Immediately afterwards, the samples were centrifuged at 800 rpm and the absorbance of the supernatant was measured at 653 and 666. The concentrations of Chl a and Chl b were calculated (WELLBURN, 1994).

For the MDA assay, leaves were homogenized with 3 mL of 50 mM solution containing 0.07% $NaH_2PO_4{\cdot}2H_2O$ and 1.6% $Na_2HPO_4{\cdot}12\ H_2O$ and centrifuged at 15.000 rpm for 25 min in a refrigerated centrifuge. For measurement of MDA concentration 3 mL of 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) was added to a 1 mL aliquot of the supernatant. The mixture was heated at 95 ºC for 30 min, quickly cooled in an ice bath and then centrifuged at 10400 rpm for 10 min. The absorbance of the supernatant was read at A_{532} and A_{600} nm (HEATH and PACKER, 1968). The result of MDA was expressed as Abs g^{-1} FW.

The H_2O_2 content of leaf samples was colorimetrically measured (MUKHERJEE and CHOUDHURI, 1983). Leaf samples were extracted with cold acetone to determine the H_2O_2 levels. An aliquot (1 mL) of the extracted solution was mixed with 200 mL of 0.1% titanium dioxide at 20% (v/v) H_2SO_4 and the mixture was then centrifuged at 8000 rpm for 15 min. The intensity of yellow colour of the supernatant was measured at 415 nm. The result of H_2O_2 concentration was expressed as mg g^{-1} FW.

Antioxidant activity test

In the free radical scavenging effect (DPPH) assay, antioxidants reduce the free radical 2, 2-diphenyl-1-picrylhydrazyl, which has an absorption maximum at A_{515}. To measure the DPPH test, the absorbance of the reaction mixture at A_{517} was read with a spectrophotometer. Methanol (0.5 mL), replacing the extract, was used as the blank. The percentage of free-radical scavenging effect was calculated as follows: scavenging effect (% g^{-1}) = [1 – (A_{517} sample/A_{517} blank)] × 100 [29].

To measure the reducing power test, 300 μL of leaves extract (0.1 g per mL methanol 80%), phosphate buffer (0.2 M, pH 6.6, 0.5 mL) and $K_3Fe(CN)_6$ (1% v/w, 2.5 mL, Fluka, Steinheim, Germany) was placed in a ependrof tube and allowed to react for 20 min at 50 ºC. The tube was immediately cooled over crushed ice, and then 150 μL Cl_3CCOOH (10%, Fluka) was added. After centrifugation at 5000 rpm for 10 min, an aliquot of 300 μl supernatant was mixed with 300 μL distilled water and 40 μL $FeCl_3$ (0.1%).Then, the absorbance at 700 nm was measured with a spectrophotometer. Increased absorbance of the reaction mixture indicated greater reducing power (HSU et al., 2003).

Antioxidant compounds

For the extraction of total carotenoids, 0.1 g of leaves were ground in semidarkness and resuspended in 1 mL of cold acetone at 80%. Immediately afterwards, the samples were centrifuged at 6.000 rpm and the absorbance of the supernatant was measured at 470 nm. To calculate the concentrations of total carotenoids and anthocyanins,

The edible leaves were homogenized in propanol: HCl:H_2O (18:1:81) and further extracted in boiling water for 3 min. After centrifugation at 7.400 rpm for 40 min at 4 ºC, the absorbance of the supernatant was measured at 535 and 650 nm (WELLBURN, 1982). The absorbance due to anthocyanins was calculated as $A = A_{535}-A_{650}$ (LANGE, SHROPSHIRE and MOHR, 1971). Finally the determination of reduced ascorbate (AsA) in leaf extracts was following the method based on the reduction of Fe^{3+} to Fe^{2+} by AsA in acid solution. Leaves material were homogenized in liquid N_2 with metaphosphoric acid at 5 % (w/v) and centrifuged at 4 ºC for 15 min. Absorbance was measured at A_{525} nm against a standard AsA curve that followed the same procedure as above. The results of reduced AsA were expressed as μg g^{-1} FW (LAW et al., 1983).

Extraction and determination of phenolic compounds and glucosinolates

Lyophilised samples (50 mg) were extracted with 1 mL of methanol 70 % V/V in a vortex for 1min, then heated at 70 ºC for 30 min in a heating bath, with shaking every 5 min using a vortex stirrer, and centrifuged (12000 × g, 10 min, 4 ºC). The supernatants were collected and methanol was completely removed using a rotary evaporator. The dry material obtained was re-dissolved in 1 mL of ultrapure water and filtered through a 0.22 μm Millex-HV13 filter (Millipore, Billerica, MA, USA).

HPLC-DAD-ESI-MS^n qualitative and quantitative analysis

Glucosinolates and phenolic compounds were determined using a LC-MS multipurpose method that simultaneously separates intact glucosinolates and phenolics (FRANCISCO et al., 2009), with slight modifications. Firstly, the GLSs were identified from the extracted samples following their MS^2 [M-H] fragmentations in HPLC-DAD-ESI-MS^n, carried out on a Luna C18 100A column (250 × 4.6 mm, 5 μm particle size; Phenomenex, Macclesfield, UK). Water:formic acid (99:1, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 800 μL/min. The linear gradient started with 1 % of solvent B, reaching 17 % solvent B at 15 min up to 17 min, 25 % at 22, 35 % at 30, 50 % at 35, which was maintained up to 45 min. The injection volume was 5 μL. The HPLC-DAD-ESI/MS^n analyses were carried out in an Agilent HPLC 1200 (Agilent Technologies, Waldbronn, Germany) and coupled to a mass detector in series. The HPLC system consisted of a binary capillary pump (model G1376A), an autosampler (model G1377A), a degasser (model G1379B), a sample cooler (model G1330B), and a photodiode array detector (model G1315D), and controlled by Chem Station software (v.B.0103-SR2). The mass detector was a Bruker, model Ultra HCT (Bremen, Germany) ion trap spectrometer equipped with an electrospray ionization interface (ESI) and controlled by Bruker Daltonic Esquire software (v.6.1). The ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 60.0 psi and 11 L/min, respectively. The full-scan mass covered the range from *m/z* 50 up to *m/z* 1000. Collision induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionization mode for glucosinolates. MS^n was carried out in the automatic mode on the more abundant fragment ion in $MS^{(n-1)}$. Chromatograms were recorded at 227 nm for glucosinolates and 330 nm for phenolic compounds. Sinigrin was used as aliphatic glucosinolate and glucobrassicin as indolicglucosinolate external standards (Phytoplan, Heidelberg, Germany). Caffeoylquinic acid derivatives were quantified as chlorogenic acid (5-caffeolylquinic acid, Sigma-AldrichChemie GmbH, Steinheim, Germany), flavonols (mainly quercetin and kaempferol derivatives) as quercetin-3-rutinoside (Merck, Darmstadt, Germany), and sinapic acid derivatives as sinapinic acid (Sigma).

Statistical analysis

For statistical analysis, data compiled were submitted to ANOVA, and differences between the means were compared with Fisher's least significant difference (LSD; $P<0.05$).

Results

Effects of NaHS on shoot biomass and H_2S foliar concentration

The greatest increase in shoot biomass was observed after the application of the dose 0.5 and 1 mM of NaHS, while higher doses 5 mM resulted in a significant decrease of biomass respect to the control (Fig. 1). The content of H_2S was found at a moderate increase at 0.5 and 1 mM NaHS, and a higher increase was observed at 2.5 and 5 mM NaHS (Fig. 2).

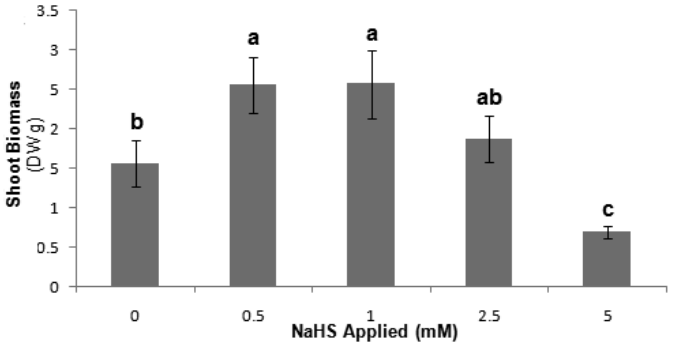


Fig. 1: Effects of different doses of NaHS on shoot biomass. Columns are mean ± S.E. (*n = 9*). Different letters indicate significant difference between values.

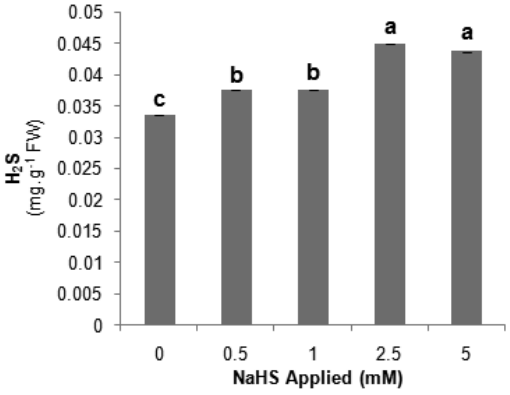


Fig. 2: Effects of different doses of NaHS on H_2S shoot concentration. Columns are mean ± S.E. (*n = 9*). Different letters indicate significant difference between values.

Effects of NaHS on chlorophylls concentration and lipid peroxidation

Tab. 1 shown Chlorophylls (Chl a and Chl b), Malonyldialdehyde (MDA) and H_2O_2 contents in 'Bronco' cabbage plants treated with NaHS. The Chl a concentration increased slightly and significantly at 0.5 mM NaHS, but decreased at 5 mM NaHS respect to control plants (Tab. 1). The treatments of 1 and 2.5 mM NaHS did not show any significant difference respect to the untreated control (Tab. 1). The concentration of Chl b only showed a significant increase in

Tab. 1: Effects of different doses of NaHS on chlorophylls, lipid peroxidation and hydrogen peroxide concentration.

Treatments	Chlorophyll a ($mg \cdot g^{-1}FW$)	Chlorophyll b ($mg \cdot g^{-1}FW$)	MDA ($Abs \cdot g^{-1}FW$)	H_2O_2 ($mg \cdot g^{-1}FW$)
Control	0.149 ± 0.003 b	0.069 ± 0.002 b	0.608 ± 0.004 d	0.429 ± 0.008 c
0.5 mM NaHS	0.162 ± 0.004 a	0.073 ± 0.001 a	0.656 ± 0.009 c	0.471 ± 0.007 b
1.0 mM NaHS	0.145 ± 0.002 b	0.071 ± 0.000 ab	0.733 ± 0.011 b	0.466 ± 0.010 b
2.5 mM NaHS	0.152 ± 0.002 b	0.072 ± 0.001 ab	0.715 ± 0.013 b	0.448 ± 0.002 ab
5.0 mM NaHS	0.119 ± 0.001 c	0.060 ± 0.001 ab	1.017 ± 0.015 a	0.533 ± 0.008 a
p-Value	***	*	***	***
$LSD_{0.05}$	0.008	0.004	0.033	0.028

Values are mean ± S.E. (n=9).
LSD, least significant difference; ns, not significant
* $P<0.05$; ** $P<0.01$; *** $P<0.001$

0.5 mM dose, whereas there were no significant differences in other treatments respect to the control plants (Tab. 1). A higher MDA concentration was observed at 1, 2.5 and 5 mM of NaHS (Tab. 1). Finally, the concentration of H_2O_2 increased moderately at 0.5, 1, and 2.5 mM NaHS and showed an acute increase at 5 mM (Tab. 1).

Antioxidant capacity tests

DPPH and the reducing power antioxidant capacity tests are presented in Fig. 3. The DPPH assay results showed a significant increase only at the 5 mM NaHS dose. The reducing power test showed a significant increase at the 2.5 and 5 mM NaHS treatments, compared to the control.

Natural Antioxidants

With respect to the carotenoids there was a significant increase with the application of all doses of NaHS respect to the control plants (0 mM NaHS) (Tab. 2). The concentration of anthocyanins significantly increased with the increasing NaHS dosages (Tab. 2). Vitamin C (reduced AsA) concentration increased at 2.5 and 5 mM NaHS treatment and of the levels at 0.5 and 1 mM of NaHS were in the same range of the control (Tab. 2).

Phenolic compounds and glucosinolates

Tab. 3 shows the different types of phenolics quantified in the samples. The application of the 0.5 mM NaHS dose exerted a significant effect on the content, while at 5 mM NaHS there was a significant decrease respect to the untreated control (Tab. 3). On the other hand, we did not observe any statistically significant difference in the contents of caffeic and sinapic acid derivatives upon the treatments, only and slight increase in sinapic acid derivatives at 0.5 mM NaHS not statistically significant (Tab. 3). Then the addition of total phenolics, following response of the major components in the samples (flavonols and sinapic acid derivatives), showed increased content at 0.5 mM NaHS, and a significantly reduced content at 5 mM respect to the control (Tab. 3). The analysis of intact glucosinolates in 'Bronco' cabbage (Tab. 4), revealed only slight and significant increases at 1 mM for sinigrin, and for 4-hydroxyglucobrassicin at 2.5 and 5 mM.

The concentrations of glucoberin and glucobrassicin, as well as the addition of aliphatic or indolic GLSs varied only slightly according to the treatments, and at the end, the total GLSs remained unchanged (Tab. 4). It is worth mentioning that the glucoiverberin, a common glucosinolate in this species was absent in the studied samples (Data not shown).

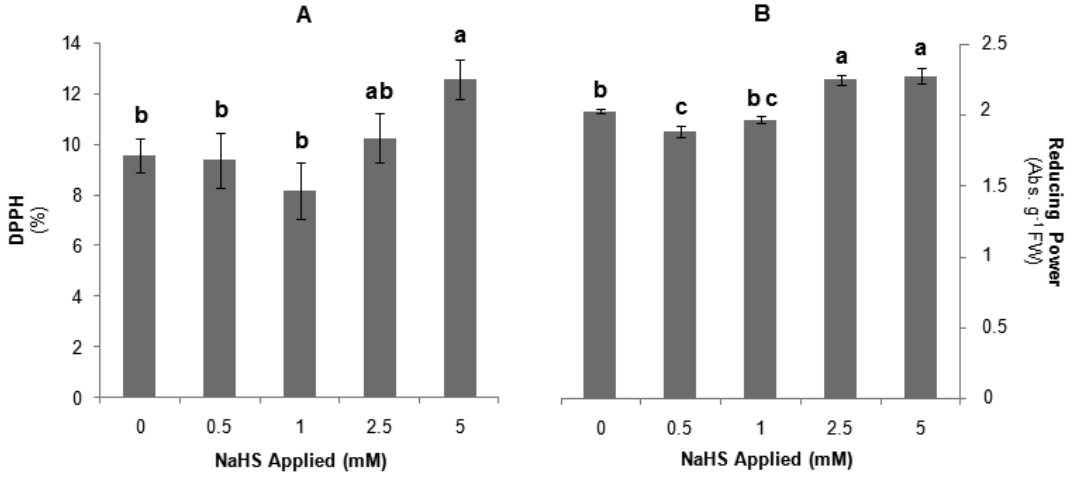


Fig. 3: Effects of different doses of NaHS on DPPH (A) and reducing power (B) antioxidant tests. Columns are mean ± S.E. ($n = 9$). Different letters indicate significant difference between values.

Tab. 2: Effects of different doses of NaHS on carotenoids, anthocyanin and reduced AsA concentration.

Treatments	Carotenoids ($mg \cdot g^{-1}FW$)	Anthocyanin ($A_{535} \cdot g^{-1}$ FW)	Reduced AsA ($\mu g\ g^{-1}$ FW)
Control	0.023 ± 0.0003 c	0.780 ± 0.005 d	674.62 ± 36.57 c
0.5 mM NaHS	0.028 ± 0.0008 a	0.875 ± 0.008 c	713.63 ± 12.13 c
1.0 mM NaHS	0.025 ± 0.0004 b	0.884 ± 0.007 c	674.09 ± 09.56 c
2.5 mM NaHS	0.026 ± 0.0002 ab	1.137 ± 0.056 b	818.59 ± 12.72 b
5.0 mM NaHS	0.025 ± 0.0002 b	1.270 ± 0.011 a	880.90 ± 10.84 a
p-Value	***	***	***
$LSD_{0.05}$	0.001	0.074	55.06

Values are mean ± S.E. (n=9).
LSD, least significant difference; ns, not significant
* $P<0.05$; ** $P<0.01$; *** $P<0.001$
Different letters indicate significant difference between values.

Tab. 3: Effects of different doses of NaHS on phenolic compounds.

Phenolic compounds ($mg\ g^{-1}DW$)				
	Flavonols	Caffeic acid derivatives	Sinapic acid derivatives	Total Phenolics
Control	7.88 ± 0.05 b	1.36 ± 0.04 a	39.93 ± 1.28 a	49.27 ± 1.63 b
0.5 mM NaHS	10.53 ± 0.21 a	1.44 ± 0.17 a	47.68 ± 4.70 a	64.40 ± 1.44 a
1.0 mM NaHS	7.95 ± 0.20 b	1.20 ± 0.28 a	39.07 ± 0.36 a	49.56 ± 1.36 b
2.5 mM NaHS	6.51 ± 0.09 b	1.41 ± 0.18 a	38.07 ± 3.95 a	46.01 ± 4.59 bc
5.0 mM NaHS	4.47 ± 0.01 c	1.01 ± 0.03 a	34.98 ± 1.79 a	40.56 ± 2.56 c
p-Value	***	n.s	n.s	***
$LSD_{0.05}$	1.46	0.54	9.22	8.25

Values are mean ± S.E. (n=9).
LSD, least significant difference; ns, not significant
* $P<0.05$; ** $P<0.01$; *** $P<0.001$
Different letters indicate significant difference between values.

Tab. 4: Effects of different doses of NaHS on glucosinolate concentrations.

Glucosinolates ($mg\ g^{-1}DW$)							
	Glucoiberin	Sinigrin	4-Hydroxy-glucobrassicin	Glucobrassicin	Total aliphatics	Total indolics	Total GLSs
Control	1.84 ± 0.05 a	0.74 ± 0.04 b	0.02 ± 0.01 b	0.76 ± 0.05 a	2.59 ± 0.08 a	0.78 ± 0.02 a	3.70 ± 0.05 a
0.5 mM NaHS	1.49 ± 0.21 a	0.63 ± 0.09 b	0.01 ± 0.00 b	0.47 ± 0.05 a	2.12 ± 0.30 a	0.49 ± 0.08 b	2.61 ± 0.05 a
1.0 mM NaHS	1.47 ± 0.20 a	1.01 ± 0.06 a	0.02 ± 0.00 b	0.50 ± 0.05 a	2.49 ± 0.27 a	0.53 ± 0.01 b	3.02 ± 0.05 a
2.5 mM NaHS	0.80 ± 0.09 b	0.39 ± 0.02 c	0.03 ± 0.00 ab	N.D	1.18 ± 0.07 b	0.03 ± 0.00 c	1.21 ± 0.05 a
5.0 mM NaHS	0.31 ± 0.01 c	0.22 ± 0.01 c	0.04 ± 0.03 a	N.D	0.53 ± 0.53 c	0.04 ± 0.00 c	0.57 ± 0.05 a
p-Value	***	***	*	n.s	***	***	n.s
$LSD_{0.05}$	0.44	0.17	0.016	0.11	0.6	0.7	8.25

Values are mean ± S.E. (n=9).
LSD, least significant difference; ns, not significant
* $P<0.05$; ** $P<0.01$; *** $P<0.001$
Different letters indicate significant difference between values.

Discussion

Previous studies found that applications of low concentrations of H_2S at 100 mg/L, could significantly increase the growth of alfalfa, lettuce and sugar beets (THOMPSON et al., 1979). Under the 0.5 and 1 mM NaHS treatments the Cabbage (*Brassica oleracea* L. 'Bronco') shoots biomass improved in final result by 64 and 65% respectively if compared to the untreated plants (Fig. 1). In leaves of *Brassica*

napus the application of H_2S at 0.2 mM increased the growth in terms of plant height by 5% (Basharat Ali, 2014). On the other hand in the 5 mM NaHS treatment a significant decrease in shoots biomass was found (Fig. 1). The application of NaHS at elevated concentrations may exert a toxic effect to the *B. oleracea*a indicated by the decreased biomass (Koch and Erskine, 2001; Lamers et al., 2013). Supported by the results, the effect of NaHS on the shoots biomass is positive, at low dosages (0.5 and 1 mM) while the high levels (5 mM) applied are negative for the plant growth and biomass with significant losses of 55% (Fig. 1). Although, the physiological mechanisms involved in the effects of the H_2S are unknown, it is clear that the intervention of secondary metabolites and proteins (i.e. enzymes) and alterations in cell membranes be involved and probably not as simple disturbance in the sulphur balance in the plant (Kuramata et al., 2009).

On the other hand the phytotoxicity of H_2S appears to result from its high affinity to the metallo-groups of proteins (Beauchamp et al., 1984). Both, damage to cell membranes and the interaction of H_2S with cell enzymes, could explain the reduction of biomass that occurs at 2.5 and 5 mM of NaHS. These results are correlated with the foliar levels of H_2S found in the samples (Fig. 2). These data are consistent with previous works where an increased concentration of H_2S after the supply of 200 mg H_2S Kg^{-1} to wheat plants (Khan et al., 2015). Others reports also showed increased H_2S concentrations in mulberry after application of 0.8 mM of NaHS (Hu et al., 2014).

The main indicators of the presence or absence of stress in plants, in addition to the production of biomass, are Chls levels and lipid peroxidation (Van Kooten and Snel, 1990; Ashrafi et al., 2015). In our study Chl a and Chl b significantly increased at 0.5 mM NaHS (Tab. 1). These results agreed with those obtained in *Spinacia oleracea* after applying 0.1 mM NaHS resulting in a significant increase in plant biomass, chlorophyll content and the number of grana lamellae in chloroplasts (Chen et al., 2011), and with the observation of an increased chlorophyll content when elemental sulfur was applied to wheat (*Triticum aestivum*) (Khan et al., 2015). On the contrary, in our study we observed a decrease in Chl when applying the 5 mM treatments, and it is well known that the reduction in leaf pigments is a typical oxidative stress indicator, which might be attributed to pigment photo-oxidation, chlorophyll degradation and/or chlorophyll synthesis deficiency (Ahmed et al., 2009).

With regard to the H_2O_2, the higher dose of NaHS (5 mM) gave rise to the highest levels (Tab. 1), and could be related to a decline in biomass (Fig. 1). So in this case may the overproduction of radicals, result in severe cellular damages or trigger a genetically controlled cell death program (Van Breusegem et al., 2001). On the other hand MDA content presents a very charged increase in treatment 5 mM (67%) relative to the control (Tab. 1), which confirms that a type oxidative stress is occurring. The increases in MDA and H_2O_2 levels and the degradation of chlorophyll were observed in *Brassica oleracea* exposed to the highest dose (5 mM) (Tab. 1), must be, directly or indirectly, attributable to the high dose of NaHS-induced oxidative damage. However we observed a small increase in 0.5 and 1 mM treatments, which does not correspond to a decrease in biomass (Tab. 1 and Fig. 1). This could be explained because hydrogen peroxide production could act as a signal to trigger mechanisms involved in the antioxidant response in plants (Wimalasekera et al., 2011).

The toxic or beneficial effects a priori produced by NaHS treatments could affect the nutritional characteristics of plants (Hu et al., 2014). Therefore it is appropriate to measure the antioxidant capacity of fruits and vegetables to get an index of its health benefits (Molyneux et al., 2004). There are a number of tests that can be used to measure the antioxidant capacity, among them are reducing power test, significant indicator of potential antioxidant activity and DPPH free-radical scavenging test, based on the removal of 1,1-diphenyl-2-picryl-hydrazyl radical. In our study we observed an increase in both test with the doses 2.5 and 5 mM (Fig. 3). These results corroborate the obtained in others works that found an increase in the DPPH and reducing test after application of H_2S in Cucumber and lotus roots (Yu et al., 2013; Sun et al., 2015). Correlating the results obtained in the antioxidant test, exogenous toxic NaHS application could be enhancing the antioxidant response through the increase in the synthesis of antioxidant compounds in treatments 2.5 and 5 mM with respect to control plant (Fig. 3).

One of these group of compounds are carotenoids that in our study showed a significant increase (by 22%) when plants were subjected to 1 mM of NaHS as compared with the control (Tab. 2). These results are consistent with those obtained by other researchers after applying H_2S to broccoli plants, and finding an increased content of carotenoids (Li et al., 2014). With respect to the anthocyanins concentration, we observed a concomitant increase in the content of anthocyanins to the applied dose of NaHS, presenting the highest concentrations at 5mM (Tab. 2). Finally, we found an increase in vitamin C content at the 2.5 and 5 mM of NaHS, by 21 and 30% respectively, and non-significant differences in the other treatments, with respect to the control (Tab. 2). This increase may be resulting from the promoted ascorbate homeostasis in response to high dose of NaHS. According to this, increased contents of AsA were observed with the application of NaHS to mulberry fruits and broccoli plants (Hu et al., 2014).

The highest dosages of NaHS (2.5 and 5 mM) in our study promoted higher contents of natural antioxidants, which could be part of a stress response mechanism. However the application of beneficial dosages in our work as 0.5 and 1 mM of NaHS potentiate the increase in concentrations of antioxidant compounds like carotenoids, several of them are precursors of vitamin A (i.e. β-carotene, γ-carotene, and β -cryptoxanthin), and due to conjugated double bonds they are both radical scavengers and quenchers of singlet oxygen. Lower serum β-carotene levels have been linked to higher rates of cancer and cardiovascular diseases, as well as to increased risk of myocardial infarction among smokers (Rice-Evans et al., *1*997). Another important antioxidant compound which are increased in our study after application of NaHS are anthocyanins. These compounds are present in various fruits and vegetables provide the natural pigmentation and exhibit a wide range of antioxidant protection and therapeutic benefits including the integrity of genomic DNA, potent cardioprotective, neuroprotective, antiinflammatory, and anticarcinogenic propertie (Juranić and Zizak, 2005).

Finally, more than 85% of vitamin C in human diets is supplied by fruits and vegetables (Lee and Kader, 2001). In fact Vitamin C has many biological activities in human body and has been found that reduce levels of C-reactive protein (CRP), a marker of inflammation and possibly a predictor of heart disease (Block et al., 2004). Therefore the increase in concentration of these compounds in leafly vegetables like *Brassica oleracea* by the application of adequate doses of H_2S as 0.5 and 1 mM can increase the production of biomass and its nutritional quality making their consumption may be beneficial to human health.

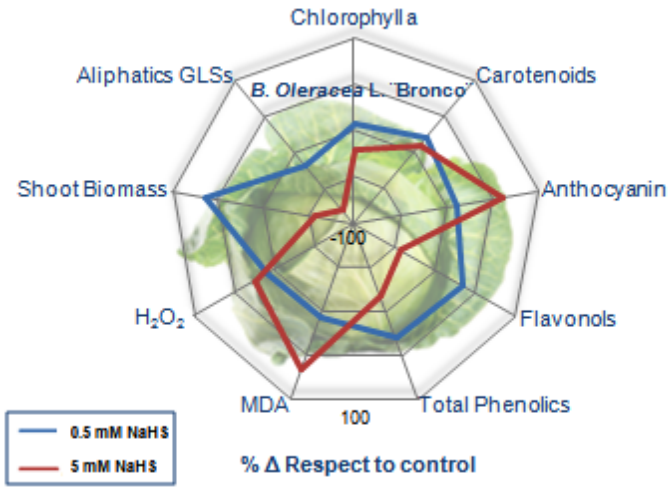
In addition to the antioxidant compounds that we have just described, in the present study we observed an increase in the concentration of flavonols and total phenolics with application of 0.5 mM of NaHS (Tab. 3). Recent works showed an increase in phenol content after the application of 15 µl L^{-1} of H_2S in Lotus roots (Sun et al., 2015). The health benefits of phenolic compounds have been widely studied in recent years, including cardioprotective, anti-inflammatory, anticarcinogenic, and antimicrobial activities, mainly attributed to their antioxidant and radical scavenging properties, and their metal chelation ability (Del Rio et al., 2013). So the increase in flavonols concentration and total phenols with the application of 0.5 mM of NaHS increases the nutritional quality of *Brassica oleracea*. On the

other hand the flavonols and total phenols concentration decreased in 5 mM NaHS treatments since this high dose could be causing a stress in the plants of *Brassica oleracea* that inhibits the formation of these compounds (Tab. 3), similar results obtain others authors when after applying 2.4 mM H_2S donor NaHS in broccoli plants produced a decrease in the content of total phenols (Li et al., 2014). In terms of the caffeic acid derivatives and sinapic acid derivatives content not showed significant difference in any of the treatments with respect to the control.

In addition to antioxidant vitamins, carotenoids, and polyphenols, Brassica vegetables provide a large group of GLSs (Plumb et al., 1996). These compounds possess rather low antioxidant activity, but the products of their hydrolysis can protect against cancer (Keum et al., 2004). Some authors observed in their studies that the GLSs content was not affected by H_2S and SO_2 exposure, demonstrating that these sulfur compounds did not form a sink for excessive atmospheric sulfur supplied (Westerman et al., 2001; Aghajanzadeh et al., 2014). However in our study only the aliphatic sinigrin increased at 1 mM NaHS but the 4-hidroxiglucobrasicin was slightly higher at 2.5 and 5 mM dose (Tab. 4). This increase in sinigrin with the treatment 1 mM of NaHS is interesting in terms of improving the nutritional quality because hydrolysis products from sinigrin (i.e. Allyl isothiocyanate), is biologically active against cancer (Park et al., 2013).

It is also interesting that indolic glucosinolates (i.e. glucobrassicin) responded to the highest dosages, selected as toxic or dangerous for these plants, and could be also a sign of stress in the plant, since the indolic glucosinolates always respond to the stress conditions and the aliphatic glucosinolates are more influenced by the variety than to the environment (Fahey et al., 2001; Charron et al., 2005). Besides, the higher sinigrin content can have a significant impact on the organoleptic parameters (aroma and taste) of the cabbage (Banerjee et al., 2014). A decrease in the content of indolic and aliphatic glucosinolates witch 2.5 and 5 mM treatments was observed in our study and this decrease in the content of these bioactive compounds could have a negative effect on the nutritional quality of *Brassica oleracea*. Finally the application of certain doses of H_2S as we have seen is an effective strategy for modulate glucosinolates profile and thus improve the nutraceutical value of Brassica vegetables (Banerjee et al., 2014).

In view of the results obtained, we can conclude that the effect of the application of NaHS in plants of *Brassica oleracea* L. 'Bronco' is dose-dependent (Scheme 1). In the present study the application in the range of 0.5 to 1 mM of NaHS to a crop of leafy vegetables like *Brassica oleracea* seems to increase the production of antioxidant compounds such, carotenoids, anthocyanins, total phenols, flavonols, and the glucosinolate. Together with an increase in the biomass doses of 0.5 to 1 mM of NaHS can be defined as 'optimal' to improve the yield and nutritional quality of *Brassica oleracea* var. 'Bronco'. On the other hand, the application of higher dosages (i.e. 2.5 mM of NaHS) could be considered as 'toxic' according to generating an excess of lipid peroxidation, decreases in shoot biomass and losses of chlorophyll as well as reduction in bioactive compounds for the nutritional and phytochemical quality of Brassica vegetables phenolics and glucosinolates, showing clear evidences distress.



Scheme 1: Percent variation in some indicative quality and stress parameters after applying low dose (0.5 mM) and high dose (5 mM) of NaHS relative to control plants.

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