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Water stress modulates secondary metabolites in *Brassica oleracea* L. convar. *acephala* (DC) Alef, var. *sabellica* L.

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ABSTRACT

BACKGROUND

Nowadays the preference of the consumers turned towards the consumption of functional food and the reduction of chemical preservatives for food conservation. Additionally, the antimicrobial and human health promoting quality of plant secondary metabolites are well known. Moreover,

due to the forecasted climate changes and increasing population, the agricultural practices for saving water have become a concern. In the present study, the physiological responses of curly kale *Brassica oleracea* L. convar. *acephala* (DC) var. *sabellica* to water stress and the impact of water limitation on the concentration of selected secondary metabolites were investigated in laboratory-controlled conditions.

RESULTS

Results indicated that water stress increased the content of two volatile carbon compounds, namely trans-2-hexenal and phytol, as well as the total tocopherols, while decreasing chlorophyll content. In addition, the antioxidant capacity and the levels of total isothiocyanates (ITC) a by-products of glucosinolates, increased significantly in water-stressed plants. Moreover, water stress positively modulated the expression of *AOP* gene, involved in glucosinolate biosynthesis, and of three genes, namely TGG1, *TGGE* and *PEN2*, encoding for myrosinases, the enzymes involved in isothiocyanate synthesis. The role of the secondary metabolites is discussed both in term of account their function in plant stress response and their known bioactive effects for human health.

CONCLUSION

The present study demonstrates that water limitation during the growing phase might be exploited as a sustainable practice for producing curly kale with a higher concentration of nutritionally important health-promoting bioactive metabolites.

Keywords: curly kale; drought; phytol; trans-hexenal; tocopherols

INTRODUCTION

During the last decade, the preference of the market turned towards the production of functional food, which can offer nutrients specific to health-promoting functionality and the reduction of chemical preservatives for food conservation[1,2]. In this regards, worldwide researches have been encouraged on the use of plant secondary metabolites for their potentiality in favouring human health and also their potential use to extend the shelf-life of food products based on their natural preservative properties[3].

Generally, secondary metabolites play a crucial role in plant growth and development, and, under stress, they contribute to plant fitness helping plants to interact with their environment for adaptation and defence. Under biotic attack, such as pathogens and pest, secondary metabolites are known to act as antibiotic, antifungal, antiviral and signalling compounds[4]. Also abiotic stress, such as drought and salt, affect the synthesis of secondary metabolites, which play a role in the response of plants, mainly for their antioxidant properties[5] .

Water is the most critical resource for sustainable agricultural development in many areas of the world, including Mediterranean countries[6] and strategies for minimizing water use in agriculture are a worldwide concern since the beginning of 21st century[7] . Thus, to evaluate whether a water stress condition enhances the accumulation of target metabolites without causing losses in plant health may be of interest to indicate a sustainable agriculture strategy for saving water, increasing the economic value of the crop[8].

The plants belonging to Brassicaceae family are considered a healthy food, as they are rich of secondary metabolites which can be useful for preserving human health[9]. In the present paper, the effects of water stress on the concentration of target secondary metabolites, [ITC and volatile organic compounds], as well as the expression of some genes involved in ITC in curly kale, *Brassica olearacea*, covar *acephala*, var *sabellica* have been investigated.

MATERIAL AND METHODS

Plant material and water stress experiment

Ten seedlings of curly kale [*Brassica oleracea* L., convar. *acephala* (DC.) Alef. var. *sabellica* L.], purchased by a local nursery, were transplanted after six-weeks in pots (12 x 10 x 10 cm) containing universal soil, and acclimated in a growth chamber under controlled conditions (temperature 23 °C, photoperiod 16/8 hours light/dark, relative humidity 60-70%, light intensity of 500 µE m^{-2} s^{-1}). Plants were divided into two groups: half of them were maintained in well-watered conditions and the others were subjected to water stress. The pots were randomly rearranged fortnightly to minimize possible positional effects and physiological parameters were measured every two days. After 2 weeks from the beginning of water stress, when the stomatal conductance (g_s) dropped to about 40 % of the initial value, the plants were harvested and secondary metabolites were quantified.

Physiological parameters

Photosynthesis (A, µmol CO_2 m^{-2} s^{-1}), g_s, (mmol H_2O m^{-2} s^{-1}) and chlorophyll fluorescence were measured with a portable system equipped with a fluorimeter (LI-COR 6400, LI-COR Biosciences Inc., NE, USA). Leaves were clumped in the 2 cm^2 LI-COR cuvette and exposed to PPFD of 500 µmol photons $m^{-2}s^{-1}$, block temperature of 25°C, 400 ppm of CO_2, (achieved by fully scrubbing CO_2 from ambient air by soda lime and replacing it with the LI-COR CO_2 injector system), humidity (RH) ranging between 40–50%. Instantaneous photosynthesis (A) and stomatal conductance (g_s) were measured in the first fully expanded leaf of five plants for each treatment after adapting and reaching a steady state condition inside the cuvette. The maximum quantum efficiency of the photosystem II (F_v/F_m) was measured in dark-adapted leaves, whereas the non-

photochemical quenching (NPQ) was measured on the same leaf in light-adapted conditions according to Sharkey *et al.,* [10].

To measure the relative water content (RWC), fully expanded leaves were collected from well-watered and water-stressed plants between noon and 2 p.m. and immediately weighted (fresh mass, FM). Subsequently, the leaves were put in a jar at 4 °C in dark conditions, where they remained floating in distilled water for 24 hours. Afterwards, the leaves were gently wiped and weighted, in order to measure the turgid mass (TM), and placed in a pre-heated oven at 80 °C. After 72 hours, they were weighted again to measure the dry mass (DM). RWC was calculated using the following formula: RWC (%) = [(FM - DM)/(TM - DM)] * 100 according to Barrs and Weatherly [11].

Determination of proline and ITC concentration, and radical scavenging activity.

Extraction and determination of proline were performed according to the method of Bates *et al.*,[12]. Leaf samples (0.02g FM) were pulverized by liquid nitrogen and extracted with ethanol:water (70:30 v/v). Extracts were held for 20 min a 95 °C, with 1 ml ninhydrin reagent [1% ninhydrin (w/v) in 60% glacial acetic acid (v/v), 20% ethanol (v/v)]. Proline content was measured with a spectrophotometer EASYSPEC UV-Vis spectrophotometer (SAFAS, Monaco) at 520 nm and calculated against a proline standard curve (0.2-5 mM proline in 40:60 v/v ethanol:water). The proline concentrations was expressed as dry mass (DM) after normalization respect of the leaf RWC in well- watered and drought- stressed seedlings.

Total ITC content of extracts was measured at 365 nm using a colorimetric method described by Zhang *et al.*,[13] with slight modifications. Leaf samples (0.5g FM) were pulverized by liquid nitrogen and homogenized in 2 ml of methanol. Extracts (500 µl) were first evaporated to dryness and dissolved in 200 µl distilled water. Glucosinolates were quantitatively converted to ITC by

enzymatic treatment with 20 µl myrosinase (28 Uml^{-1}) for 1 hours at 37 °C. Successively, 100 µl of 80 mM 1,2-benzenedithiol were added to 900 µl methanol and 780 µl 0.1 M potassium phosphate buffer (pH8.5) and incubated at 65 °C for 1 hours. This step allowed the cyclocondensation of ITC with 1,2-benzenedithiol to generate 1,3-benzedithiol-2-thione. ITC concentration was calculated using the extinction coefficient of 1,3-benzedithiol-2-thione (ε = 23,000 M-1cm-1 at 365 nm).

The radical scavenging activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as described by Boudjou *et al.*,[14]. The absorbance was recorded at 517 nm and the antiradical activity (ARA) was expressed as percentage of DPPH inhibition using the following equation: ARA = [1 - (AS/AC)] x 100, where AS is the absorbance of the sample and AC is the absorbance of control. Trolox was used as antioxidant standard (Sigma-Aldrich, Italy).

Chlorophyll and tocopherols analysis

Chlorophylls (*a* and *b*) and tocopherols (α, γ and δ) were determined by HPLC according to Döring *et al.,*[15]. Pulverized leaf material (0.05 g FM) was homogenized in 0.4 ml of 100% HPLC-grade methanol and incubated overnight at 4 °C in the dark. The supernatant was filtered through 0.2 µm Minisart SRT 15 aseptic filters and immediately analysed at room temperature with a reverse-phase Dionex column (Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter × 150 mm length). Chlorophylls and tocopherols were eluted at a flow rate of 1 ml min^{-1} using 100% solvent A (acetonitrile/methanol, 75/25, v/v) for the first 14 min followed by a 3 min linear gradient to 100% solvent B (methanol/ethylacetate, 68/32, v/v), 15 min with 100% solvent B. Chlorophylls and tocopherols were detected at 445 and 280 nm, respectively. Authentic standards (Sigma-Aldrich, Italy) were used to quantify the chlorophylls and tocopherols content of each sample.

Volatile organic compounds

Leaf samples (0.5 g FM) were extracted with 2.0 ml of heptane containing tridecane (internal standard); the sample was filtered and 1 µl volume was injected in the GC-chromatograph (GC) in splittles mode. A 7820 GC-chromatograph equipped with a 5977A MSD mass spectrometer with EI ionisation from Agilent Tech. (Palo Alto, AC, USA) was used for analysis. The chromatographic settings were as follows: injector set at 260 °C, J&W Innovax column (30 m, 0,25 mm i.d., 0.5 µm df); oven temperature program: initial temperature 40 °C for 1 min, then 5 °C min^{-1} until 200 °C, then 10 °C min^{-1} until 220 °C, then 30 °C min^{-1} until 260 °C, hold time 3 min. The mass spectrometer was operating with an electron ionisation of 70 eV, in scan mode in the m/z range 29-330, at three scans sec^{-1}. The deconvoluted peak spectra, obtained by Agilent Masshunter software, were matched against NIST 11 spectral library for tentative identification. Kovats' retention indices were calculated for further compound confirmation and compared with those reported in literature for the chromatographic column used. Standard curves for phytol and trans-2-Hexen-1-al (trans-hexenal) were constructed with standards (phytol: CRM40375; trans-2-Hexen-1-al:132659, Sigma Aldrich, Italy).

RNA extraction and semi-quantitative reverse transcription PCR analysis

Leaf materials (0.1 g FM) were crushed by liquid nitrogen and then suspended in 200 µl PBS. Total RNA extraction and cDNA synthesis were performed modifying the protocol of the Taqman Gene Expression Cells-to-CT TM Kit (4399002, Applied Biosystems, Italy) according to Del Carratore *et al.*,[16]. To detect *TGG1, TGG2*, *PEN2* and *AOP* genes, the primers reported by Yi *et al.*,[17] were used (Table 1). The following standard thermal profile was used for semi-quantitative reverse transcription PCR: 94 °C for 5 min; followed by 32 cycles (*TGG1* and *TGG2*), 33 cycles (*PEN2*), 38 cycles (*AOP*) and 34 cycles (*Actin*) at 94 °C for 30 s, 58 °C for 50 s, and 72 °C for 30 s followed by a final extension at 72 °C for 5 min. *Actin* was chosen as a reference gene. Three independent

experiments were performed. PCR products were separated by 1% agarose gel electrophoresis. The amplicons were excised from gels and purified using the Wizard SV Gel PCR Clean-Up System (a9281, Promega, Italy) following the manufacturer's protocol, and sequenced.

Statistical analysis

All the parameters were subjected to a Shapiro-Wilk W test to check the data normality distribution. The data were then analysed using Student's test, when normality conditions were not met according to variance check, data were analysed with Kruskal–Wallis test and Bonferroni pairwise comparison at a 95% confidence level. All analyses were performed with Prism 8 (GraphPad Software, USA).

RESULTS

Physiological parameters, proline concentration and antiradical activity

The physiological parameters in the leaves of well-watered and water-stressed plants are reported in Table 2. Water stress affected photosynthesis (P_n) and stomatal conductance (g_s) which decreased about of -42 and -54% in water-stressed plants in comparison to control ones, respectively. The values of NPQ increased significantly in water stressed plants compared to well-watered ones, whereas, the maximal quantum efficiency of PSII ($F_v/F_{m)}$ was not sensitive to water stress, as for both treatments the value was around 0.8 as reported for healthy plants by Björkman and Demming[18]. A reduction of 20% of the leaf RWC values was observed in water stressed plants.

PLEASE INSERT TABLE 2

Water stress increased proline concentration about 10-fold respect to the value measured in well-watered leaves (Figure 1a). The radical scavenging capacity (2.5-fold higher compared to well-watered leaves), was increased as demonstrated by the increased values of ARA from 4% to 10% (Figure 1b).

PLEASE INSERT FIGURE 1

Chlorophyll, tocopherol and volatile compound content.

Water stress decreased the concentration of total chlorophylls, while an opposite trend was observed regarding the total concentration of tocopherols (Figure 2a and b) as well as the amounts of two organic volatile compounds, namely phytol and trans-hexenal (raised up to 50% in stressed leaves; Figure 2c and d).

PLEASE INSERT FIGURE 2

Expression of genes involved in glucosinolate synthesis and degradation and total ITC concentration.

Total isothiocyanates increased from 0.7 to 2.5 mg g^{-1} DM) in the water-stressed curly kale seedlings (Figure 3a). Moreover water stress triggered the expression of three myrosinase encoding genes *TGG1*, *TGG2* and *PEN2* involved in the glucosinolate degradation increased in water stressed plants (Figure 3b) and the expression of the gene *AOP2* which belongs to the aliphatic glucosinolate biosynthesis pathway (Figure 3b).

PLEASE INSERT FIGURE 3

DISCUSSION

The promotion of sustainable agriculture and nutritional food quality are two of the objectives indicated in Goal 2 of the 2030 Agenda for Sustainable Development of the United Nations[19]. Furthermore, consumers are pushing towards production of health-promoting functionality, desirable sensory attribute and less chemical preservatives[1,2]. Abiotic stress are known to enhance the accumulation of secondary metabolites and, since the end of last century, several secondary metabolites have a recognized active role as functional products for human health [20]. In this contest, the aim of this work was to provide information about water stress impact on the amount of selected secondary metabolites, which have a potential bioactive role on human health and/or antimicrobial properties, in curly kale a variety of *Brassica*, largely used in Mediterranean countries.

First, physiological parameters were measured as markers of stress level. The reduction of 20% of leaf RWC indicates the expected diminished leaf hydration in drought stressed plants[21]. The decrease of photosynthesis and stomatal conductance observed in water-stressed cabbage is in accordance reported by Chaves *et al.,*[6] Despite the F_v/F_m ratio was not sensitive to water stress, in accordance to Pavlovic et al.[22], the excess excitation energy due to photosynthesis imbalance was probably dissipated as heat, as suggested by the increase in NPQ[23]. Proline increased under water stress acting as osmolyte for osmotic adjustment[24]. The higher antioxidant capacity observed in the water-stressed leaves of curly kale compared to control ones is in accordance with the increasing of antioxidant capacity found in *Rehmannia glutinosa* [25] and *Vitis vinifera*[26] under drought stress as part of the response to putative increased production of reactive oxygen species[27]. Overall, the evaluation of the physiological parameters, proline concentration and antiradical activity confirmed the impact of water stress in curly kale and suggests the attempt of the plants to overcome the stress effects[28,29].

The main output of our work is the observed increase of two volatile compounds, trans-hexenal and phytol under water stress, as by our knowledge no other reports on the behaviour of such

metabolites in *Brassica* under stress are available. Trans-hexenal is a volatile metabolite whose strong antimicrobial properties[30] might make this compound useful for extending the post-harvest shelf-life of curly kale[1,3,31]. Moreover such metabolite is believed to be beneficial for human health due to its potential role in blood pressure regulation[32].

Chlorophyll is the most abundant photosynthetic pigment in plants. During senescence, or abiotic stress, a large fraction of chlorophyll is broken down, giving rise to the accumulation of high amounts of free phytol[33]. Phytol is suggested to have a positive role in human health, minimizing metabolic disorders disease[34], having anticancer activities[35], playing a positive role in the treatment of type 2 diabetes[36]. Moreover, in plants phytol is incorporated into tocopherols[37], which are potent antioxidants protecting the photosynthetic membranes from oxidation caused by reactive oxygen species derived from photosynthesis [38]. In human, the antioxidant properties of natural tocopherols rather than the assumption of synthetic vitamin E[39] have been found to play a vital role in reducing risk of neurodegenerative disease[40,41,42].

Plants of the Brassicaceae family are known to be rich in glucosinolates, chemically stable compounds under normal conditions [43]. However, when plant tissues and cells are damaged, they are hydrolysed by the enzyme myrosinases, resulting in several degradation products, including ITC which defend plants against predators [44]. In the present work, the observed increased expression of *AOP2*, one of the gene involved in the glucosinolate synthesis is in accordance with previous observations in several *B. oleracea* varieties, such as *capitata*, and *italica* [45,46]. Moreover, the higher expression of three myrosinase encoding genes *TGG1*, *TGG2* and *PEN2* in water–stressed curly kale, respect to the values measured in well-watered ones, suggests the degradation of glucosinolates into their bioactive by-products and supports the higher values of ITC found in water-stressed curly kale. Sulforaphane and other ITC exhibit powerful biological functions in fighting cancers, as well as cardiovascular and neurodegenerative diseases[47,48,49].

CONCLUSION

In conclusion, despite records on the response of secondary metabolites in several varieties of *B. oleracea* under abiotic stress are available in literature, this work reports information on trans-hexenal, phytol and tocopherols levels in curly kale, for the first time. Overall, our results underline that increasing curly kale bioactive compounds as well as reducing water supply might provide a more attractive product for the market while decreasing production cost.

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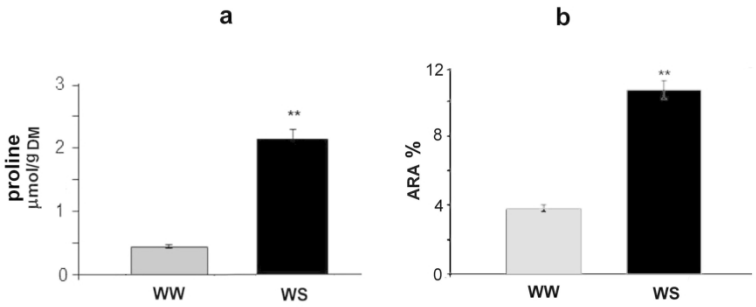
Figure legends

Figure 1. Proline concentration (a) and antiradical activity (b) in the leaves of well-watered (WW) or water-stressed (WS) curly kale seedlings. Data are the means of three leaves from five plants (±SD) for each experimental condition. Statistical analysis were carried out by Student's test. ** P<0.01. DM, dry mass.

Figure 2. Concentration of total isothiocyanate (a). A representative experiment of gene expression pattern of AOP2 and myrosinase encoding genes in the leaves of well-watered (WW) or water-stressed (WS) curly kale seedlings (b). The constitutively expressed actin was used as reference gene. Values of the signal intensity of each sample respect to actin expression are reported in the table (c) (mean of at least four separate experiments). Data are the means of three leaves from five plants (±SD) for each experimental condition. Statistical analysis was carried out by Student's test ** P≤0.01. DM, dry mass.

Figure 3. Total chlorophyll (a), tocopherol (b), trans-hexenal (c) and phytol (d) content in the leaves of well-watered (WW) or water stressed (WS) curly kale seedlings. Data are the means of three leaves from five plants (±SD) for each experimental condition. Statistical analysis was carried out by the Kruskal–Wallis test and Bonferroni pairwise. * P ≤ 0.05, DM, dry mass.



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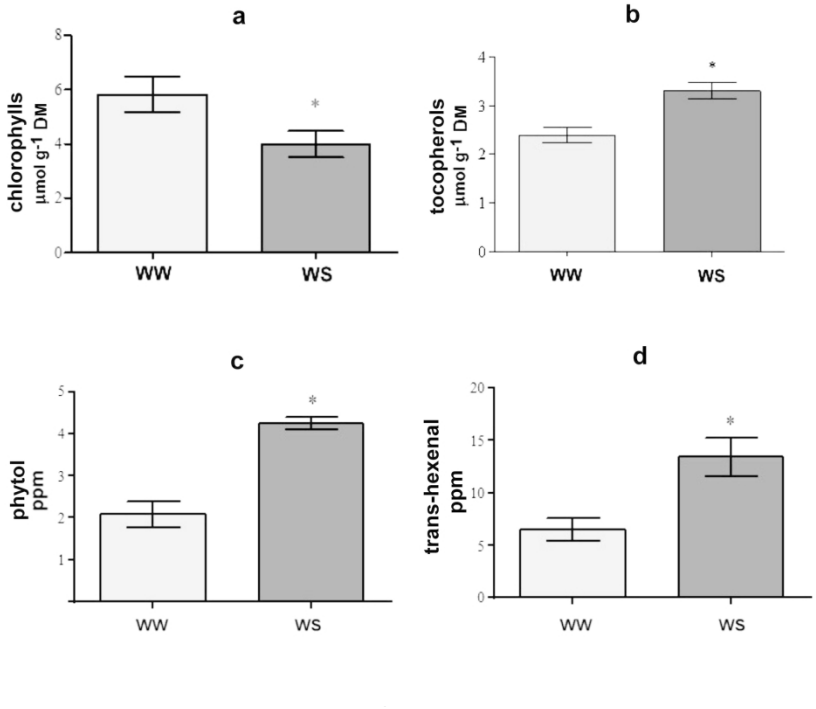

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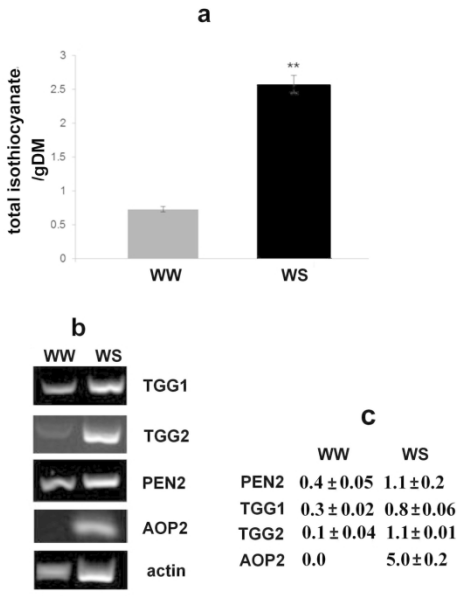

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Table 1. Primers used for detecting several genes involved in the glucosinolate-system pathway by semiquantitative reverse transcription

	F 5'	Rew 5'
TGG1	TCTTAACGTGTGGGATGGCT	CCTCCTTTGTTCACTCCCCT
TGG2	AGATGTGCTGGACGAACTCA	CGGCGTAACAGGTAGGATCA
PEN2	GCATCATCATCCAACAGCGT	ACGCCTTGATCAGTTCTCCA
AOP	CCAGGAAGTGAGAAGTGGGT	TAGCACCATCACCAGCATCA
Actin	AATGGTACCGGAATGGTCAA	AGTTGCTCACAACACCATGC

Table 2. Physiological parameters measured in well-watered (WW) and water stressed (WS) curly kale. Data are the means of three leaves from five plants (±SE or SD) for each experimental condition. Statistical analysis was performed by Student's test. * Significance level at $P \leq 0.05$. ** Significance level at $P \leq 0.001$.

	Well-watered (WW)	Water stressed (WS)
Photosynthesis (A) (μmol CO_2 m^{-2} s^{-1})	17.11 ± 0.23	9.98 ± 1.59 *
Stomatal conductance (g_s) mmol H_2O m^{-2} s^{-1})	0.25 ± 0.01	0.09 ± 0.02 **
F_v/F_m	0.81 ± 0.01	0.79 ± 0.01
NPQ	0.63 ± 0.06	1.48 ± 0.17 **