Postharvest application of brassica meal-derived allyl-isothiocyanate to kiwifruit: effect on fruit quality, nutraceutical parameters and physiological response

Luisa Ugolini¹ · Laura Righetti¹ · Katya Carbone² · Roberta Paris¹ ·
Lorena Malaguti¹ · Alessandra Di Francesco³ · Laura Micheli⁴ · Mariano Paliotta² ·
Marta Mari³ · Luca Lazzeri¹

Revised: 25 January 2017 / Accepted: 31 January 2017 / Published online: 14 February 2017
© Association of Food Scientists & Technologists (India) 2017

Abstract The use of natural compounds to preserve fruit quality and develop high value functional products deserves attention especially in the growing industry of processing and packaging ready-to-eat fresh-cut fruit. In this work, potential mechanisms underlying the effects of postharvest biofumigation with brassica meal-derived allyl-isothiocyanate on the physiological responses and quality of ‘Hayward’ kiwifruits were studied. Fruits were treated with 0.15 mg L⁻¹ of allyl-isothiocyanate vapours for 5 h and then stored in controlled atmosphere (2% O₂, 4.5% CO₂) at 0 °C and 95% relative humidity, maintaining an ethylene concentration <0.02 µL L⁻¹. The short- and long-term effects of allyl-isothiocyanate on fruit quality traits, nutraceutical attributes, glutathione content, antiradical capacity and the activity of antioxidant enzymes were investigated. The treatment did not influence the overall fruit quality after 120 days of storage, but interestingly it enhanced the ascorbic acid, polyphenols and flavan-3-ol content, improving the antioxidant potential of kiwifruit. The short-term effect of allyl-isothiocyanate was evidenced by an increase of superoxide dismutase activity and

of oxidative glutathione redox state, which were restored 24 h after the treatment. The expression levels of genes involved in detoxification functions, ethylene, ascorbate and phenylpropanoid biosynthesis, were also significantly affected upon allyl-isothiocyanate application. These results suggest that allyl-isothiocyanate treatment probably triggered an initial oxidative burst, followed by an induction of protective mechanisms, which finally increased the nutraceutical and technological value of treated kiwifruits.

Keywords Kiwifruits · Brassica meal · Allyl-isothiocyanate · Antioxidant system · Ascorbic acid · Nutraceutical parameters

Introduction

Kiwifruit is a fruit crop successfully adopted by international markets, with China, Italy and New Zealand being the top three producers worldwide (<http://faostat3.fao.org/browse/Q/QC/E>). Despite the spread of new varieties developed from different species, the most popular cultivar is the green-fleshed ‘Hayward’ (*Actinidia deliciosa*), whose distinctive appearance and flavour are highly appreciated by consumers. The great economic interest in kiwifruit is linked to its high nutraceutical values, together with the long-term storage capability and consequent exportability. After harvest, fruits are usually stored at 0 °C under controlled atmosphere (CA), where the removal of ethylene increases the storage time up to 4 months, preserving both fruit quality and flavour (Latocha et al. 2014). Kiwifruits are an excellent source of minerals (e.g. potassium), dietary fibres, and bioactive compounds, such as vitamins E and C (L-ascorbic acid, AsA), flavonoids, anthocyanins, and carotenoids (Drummond 2013). *A. deliciosa*, in particular, is rich in AsA

✉ Luisa Ugolini
luisa.ugolini@crea.gov.it

¹ Consiglio per la ricerca in agricoltura e l’analisi dell’economia agraria, Centro di Ricerca per le Colture Industriali, CREA-CIN, Via di Corticella 133, 40128 Bologna, Italy

² Consiglio per la ricerca in agricoltura e l’analisi dell’economia agraria, Centro di Ricerca per la Frutticoltura, CREA-FRU, Via Fioranello 52, 00134 Rome, Italy

³ CRIOF, Università di Bologna, Via Gandolfi 19, 40057 Cadriano, Bologna, Italy

⁴ Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy

and polyphenols that contribute to the total antioxidant capacity and to the capability of limiting oxidative stress due to reactive oxygen species (ROS). These properties make kiwifruit a ‘functional food’ with preventive effects against certain types of cancers, vascular diseases and gastro-intestinal disorders (Padmanabhan and Paliyath 2016). In plants, ROS are involved in fruit senescence, as excessive production causes lipid peroxidation and membrane damage, but they are also response factors for the activation of defence mechanisms after abiotic and biotic stress. Whether they act as toxic or signalling factors in response to environmental changes depends on the balance between their production and scavenging, and a complex network of mechanisms is involved in ROS homeostasis. L-ascorbic acid and glutathione are the main key players involved in this process, the first as direct antioxidant or as cofactor of enzymes participating in the biosynthesis of secondary metabolites and plant hormones, the second with a primary role in xenobiotic detoxification, redox balance, plant development and defence (Foyer and Noctor 2011). Together with those non-enzymatic antioxidant compounds, enzymes as catalases (CAT), superoxide dismutases (SOD) and peroxidases (POD) are also involved in ROS scavenging and play an important role in fruit physiological changes during ripening and stress response (Xia et al. 2016). Besides, even though kiwifruits contain low levels of proteins, two of the most abundant soluble proteins (actinidin, that is a cysteine proteinase and a thaumatin-like protein, PR5) can affect fruit quality and have beneficial effects on humans. In particular, actinidin contributes to the digestive process, while PR5 is known as a sweetener and flavour modifier, inducible by various agents from ethylene to pathogens. Nonetheless, several pre- and postharvest factors, such as the physiological state of the fruit at harvest, genotype, cultivation techniques, storage period and temperature management, can greatly affect both qualitative and nutritional aspects of kiwifruits (Tavarini et al. 2008; Oz 2010). Among postharvest techniques, the use of natural preservatives, as plant secondary metabolites, could represent a valid option, within an integrated management, for the shelf-life extension of products. Allyl-isothiocyanate (AITC) is the hydrolysis product of the 2-propenyl glucosinolate (sinigrin), a secondary metabolite of the myrosinase–glucosinolate (MYR–GLs) system typically present in plants of the *Brassicaceae* family. The AITC is largely known for its biocidal activity against several plant pests and pathogens (Matthiessen and Kirkegaard 2006; Lazzeri et al. 2011) and it is a volatile molecule ‘generally recognized as safe’ (GRAS) by the Food and Drug Administration (FDA) in the United States since 2006. Allyl-isothiocyanate derived from plant sources is authorized as a natural food preservative in Japan, and the European Food Safety Authority (EFSA, Parma, Italy) has evaluated its safety as a food additive

(EFSA 2010). Recently, several studies have been directed towards the use of AITC incorporated in antimicrobial food packaging systems (Hendrix et al. 2012). The use of AITC vapours to control postharvest fungal diseases has been successfully applied on different fruit species (Mari et al. 2008; Ugolini et al. 2014; Chen et al. 2015), but the effect of the molecule on plant and the cellular and molecular mechanisms of action have been little investigated until now. AITC is a highly reactive molecule binding thiols, such as cysteine or glutathione, thus modifying protein structure and function. Khokon et al. (2011) observed that AITC induced stomata closure in *Arabidopsis thaliana* via production of ROS and nitric oxide, probably with the function of preventing water loss and enhancing plant defence against microorganisms. At higher doses it also caused oxidative stress and ROS production in *Arabidopsis* seedlings, while expression of glutathione *S*-transferase (GST)-encoding genes and reversible GSH depletion were observed at low doses, as a potential detoxifying mechanism (Øverby et al. 2015). Besides, some works speculate on the AITC function as ethylene analogue or inhibitor based on their structural similarity, but this action has not been fully investigated yet (Nagata 1996). At present, from the standpoint of fruit crops, postharvest AITC biofumigation has been successfully applied to contrast fungal decay in different kinds of berries (e.g. raspberries, strawberries, blackberries, blueberries), apparently due to its direct effect on fungi and pro-oxidant activities. Wang et al. (2010) suggested that the observed increased ROS production in blueberries could be a possible defence mechanism induced in AITC-treated fruits. Nevertheless, the treatment was reported to have a detrimental effect on the antioxidant phytochemical content of these fruits even though a global fruit quality improvement was observed (Chen et al. 2015; Chanjirakul et al. 2006). To the best of our knowledge, there is no literature data about the effect of AITC on the nutraceutical characteristics of kiwifruit. This work was therefore focused on the evaluation of the effects of an AITC treatment on kiwifruit physicochemical parameters, antioxidant enzymes, glutathione content, and expression of genes involved in ripening, stress response and secondary metabolism, in order to assess the potential application of the treatment to improve fruit quality and nutraceutical properties.

Materials and methods

Plant material

Kiwifruits (*Actinidia deliciosa*, cv. ‘Hayward’) were harvested at commercial maturity stage from an orchard located in the production district of Emilia Romagna Region (Italy) and managed according to the Italian

integrated production guidelines. Fruits homogenous in maturity stage and size were carefully selected, excluding those with physical injury, diseases, or incidence of pests.

Isothiocyanate

Allyl-isothiocyanate was produced in situ from the endogenous GL (sinigrin)—MYR system of *Brassica nigra* defatted seed meal (DSM), provided by Agrium Italia SpA (Livorno, Italy) and opportunely formulated (Lazzeri et al. 2010). DSM used for the trials had defined characteristics as 5.1% moisture content, 7.9% residual oil and sinigrin content of 153 $\mu\text{mol g}^{-1}$ (on dry matter, DM) determined with standard procedures (Franco et al. 2016).

AITC treatment

Selected kiwifruits were distributed in 3 trays (26 fruits each), placed in a 0.1 m³ airtight cabinet and treated by AITC vapours produced by the DSM plus water added in an external reactor as described in Mari et al. (2008). Allyl-isothiocyanate concentration was maintained at 0.15 mg L⁻¹ for 5 h. The atmosphere in the cabinet was monitored by an Agilent 7820AC GC system equipped with an Agilent HP-5 column (30 m length, 0.32 mm inner diameter, 0.25 μm film thickness) and FID detector (Agilent Technologies, Santa Clara, USA). Injector and detector temperatures were set at 200 and 300 °C respectively; the oven program started at 60 °C and raised to 120 °C at a rate of 10 °C min⁻¹; the carrier gas (He) flow rate was 1 mL min⁻¹ and the splitless injection mode was selected. The AITC amount was determined on the basis of a previously defined calibration curve using a pure AITC standard (Sigma-Aldrich, Saint Louis, USA). Treated fruits and non-treated control fruits were stored at 12–15 °C overnight and then for 120 days in CA (2% O₂, 4.5% CO₂) at 0 °C and 95% relative humidity, maintaining ethylene concentration <0.02 $\mu\text{L L}^{-1}$ by scrubbing the gas with KMnO₄. Six to ten fruits were randomly sampled per each time point: (1) at harvest, (2) at the end of the treatment (d0), (3) one day after the treatment (d1) and (4) at the end of the storage (d120). All analyses were performed on three pools of fruits.

Kiwifruit quality traits

Quality trait evaluation was performed on fruits at harvest at d120. Kiwifruits were weighed and peeled, and the homogenised samples were analysed for:

Soluble solid content (SSC), determined by a digital refractometer (Refracto 30 PX, Mettler Toledo, Milan, Italy), (expressed as °Brix);

Total titratable acidity (TA), measured by titration of kiwi juice acids with 0.1 N sodium hydroxide (expressed as meq L⁻¹);

pH value, measured using a digital pH-meter (785 DMP, Methrom, Milan, Italy).

Firmness was measured on fresh fruit by a penetrometer (Fruit Pressure Tester FT011, TR snc, Forlì, Italy), using a 10 mm tip (expressed as Newton, 1 N = 9.8 kg) and *moisture content* (unbound water) was evaluated by weight loss after drying fresh samples at 105° ± 1 °C, until a constant weight was reached (expressed as %dry matter, DM).

Extraction and analysis of phytochemical content and antiradical capacity of kiwifruits

A mixed sample of fresh fruits at harvest and at d120 were peeled, cut into small pieces, freeze-dried and stored at -80 °C until analysis. Freeze-dried samples (0.5 g) were extracted with 10 mL of a solution of methanol: water (80:20 v/v) acidified with 0.1% HCl (v/v) to determine fruit phytochemical content and antiradical capacity (AC). The extraction was carried under magnetic stirring (300 rpm), for 30 min, at room temperature in the dark. The mixture was then submitted to an ultrasound-assisted extraction for another 30 min (40 kHz, 10 °C) and centrifuged at 6792g, for 15 min at 4 °C. After a second extraction, supernatants were collected together and immediately analysed.

Total polyphenol content (TPC) and *flavan-3-ol content (FLC)* of extracts were determined according to Carbone and Mencarelli (2015). Data were expressed as mg of gallic acid equivalents (GAE) and mg catechin equivalents (CAE) per g on DM for TPC and FLC, respectively.

L-ascorbic acid content (AsAC) of kiwifruits was determined by Differential Pulse Voltammetry (DPV) and Cyclic Voltammetry (CV). Briefly, the DPV measurements were performed using a computer-controlled system, AUTO-LAB model PGSTAT 12 with GPES software (ECO-CHEMIE, the Netherlands). Voltammetric measurements employed a screen-printed electrode produced in house, which consisted of a three electrode configuration with graphite working and counter electrodes and a silver pseudo-reference electrode printed onto a plastic support film. All measurements were carried out in 0.1 M KCl aqueous solutions at room temperature. The DPV and CV were performed according to Cavalieri et al. (2013) using 60 μL of AsA standard solutions and extracted fruit samples in order to observe the electrochemical behaviour of each analyte and the matrix effect. The quantitative determination of AsA in kiwifruits was extrapolated using the calibration curves obtained with increasing amounts (0–125 × 10⁻⁶ M) of AsA (standard solutions) in 0.1 M KCl using DPV.

Fig. 1 Relative transcript abundance by RT-qPCR of genes belonging to different metabolisms in kiwifruits treated with AITC at the end of the treatment (d0) and 24 h afterwards (d1). **a** Ethylene synthesis and perception. SAM, *S*-adenosylmethionine synthase; ACO1, ACC oxidase 1; ETR2, ethylene receptor; **b** cell wall and starch degradation. PG, polygalacturonase; BAM, beta-amylase; **c** phenyl propanoid pathway and phenol oxidation. PAL, phenylalanine ammonia-lyase; CHS3, chalcone synthase; PPO, polyphenol oxidase; **d** ascorbate/glutathione biosynthesis and metabolism. GPP, *L*-galactose-1-phosphate phosphatase; GalDH, *L*-galactose dehydrogenase; MDHAR, monodehydroascorbate reductase; GST, tau class glutathione transferase 5; **e** others. Act d1, actinidin; PR5, thaumatin-like protein. Each bar represents the ratio between expression in AITC treated fruits and their untreated control at the specified time point. The results were normalized to multiple reference genes and expressed as $2^{-\Delta\Delta C_t}$. Stars indicate significant difference according to REST software analysis

Antiradical capacity (AC) was assessed measuring the ability of the extracts to scavenge synthetic radicals [i.e. 2,2-diphenyl-1-picryl-hydrazyl-hydrate, DPPH $\cdot$ and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), ABTS $\cdot^+$]. Both DPPH $\cdot$ and ABTS $\cdot^+$ quenching capacities were estimated spectrophotometrically according to Carbone and Mencarelli (2015), and expressed in terms of EC₅₀.

Antioxidant enzyme activity assays

Fruit pericarps, collected at d0 and d1, were cut, frozen, grinded in liquid nitrogen by a Waring Commercial laboratory blender (Waring products division Torrington, CT, USA) and stored at -80°C . Enzyme extraction and analysis were performed according to Yang et al. (2013), unless otherwise stated. Samples were homogenised with 0.5 M potassium phosphate (pH 7.0), 1 mM ethylene diamine tetraacetic acid (EDTA), 50 g L⁻¹ polyvinylpyrrolidone and 10 mL L⁻¹ Triton X-100. After centrifugation (31.500g, 20 min, 4 °C), the supernatant was used for enzyme spectrophotometric assays carried out in a Varian Cary 300 Bio Uv/Vis spectrophotometer (Agilent Technologies).

Catalase (CAT) activity was determined by monitoring the decrease of absorbance at 240 nm of the reaction mixture containing 50 mM phosphate buffer (pH 7), 10 mM of H₂O₂ and the kiwifruit extract.

Guaiacol peroxidase (POD) activity was determined by monitoring the increase in absorbance at 470 nm of the mixture composed by 50 mM potassium phosphate (pH 7), 20 mM guaiacol (Sigma-Aldrich), 10 mM H₂O₂, and kiwifruit extract.

One unit (U) of CAT or POD activity was defined as the amount of enzyme causing a change in absorbance of 0.01 min⁻¹.

Superoxide dismutase (SOD) activity was determined by using an Infinite M200 PRO microplate reader (Tecan Austria GmbH). The reaction mixture containing 50 mM

potassium phosphate (pH 7.8), 13 mM methionine, 75 μM nitrobluetetrazolium (NBT), 2 μM riboflavin (Sigma-Aldrich), 0.1 mM EDTA and enzyme extract was exposed 10 min to light from a fluorescent lamp (Sylvania F15T8-CW Cool White fluorescent lamp). The absorbance was

read at 560 nm and subtracted from blank samples with and without kiwifruit extracts, illuminated or not. One U of SOD was defined as the amount of enzyme that inhibits the photoreduction of NBT by 50% under the assay conditions. Specific enzyme activities were expressed as U g⁻¹ protein. Extract protein concentration was determined using the Bio-Rad dye reagent (Bio-Rad, Munchen, Germany).

Glutathione content determination of kiwifruit

Frozen ground pericarp samples collected from fruit at d0 and d1 were extracted with cold 5% 5-sulfosalicylic acid (SSA), 1:10 w/v, at 4 °C by using a TissueLyser (Retsch GmbH, Haan, Germany), and subsequently centrifuged at 31,500 g for 15 min at 4 °C. Total glutathione (reduced + oxidized glutathione, GSH + GSSG) spectrophotometric analysis was performed by using the GSH assay kit (Sigma-Aldrich), with GSH reductase, 5,5'-dithiobis(2-nitrobenzoic acid (DTNB) and NADPH, according to the provided protocol on a Infinite M200 PRO microplate reader. GSSG was determined by the same method in the presence of GSH masking agent 1-methyl-2-vinylpyridinium triflate (M2PV) (Shaik and Mehvar 2006). Results are expressed as nmol g⁻¹ FW. GSH was estimated as the difference between total and oxidized form and GSH redox state calculated as the percentage of GSH over total: GSH/(GSH + GSSG) × 100.

Gene expression analysis: RT-qPCR

Total RNA was isolated from 200 to 300 mg of frozen kiwifruit outer pericarp collected at d0 and d1, following the protocol by Chang et al. (1993), scaled down in order to work in 2 mL tubes. RNA was then cleaned up using MinElute column and on column DNase I treatment (Qiagen), according to manufacturer's instructions. RNA quantity and quality were evaluated with the Infinite 200-PRO NanoQuant system (Tecan, Männedorf, Switzerland) and on 1% agarose gel, respectively. cDNA synthesis was performed starting from 500 ng of RNA in 10 µL reactions, using High-Capacity RNA-to-cDNA Kit (Life Technologies). Gene-specific primers (Table 1) were chosen from literature or designed using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) from EST sequences available at NCBI or gene predictions available at the Kiwifruit Genome database (<http://bioinfo.bti.cornell.edu/cgi-bin/kiwi/home.cgi>, Huang et al. 2013). qPCR was performed using the Rotor-Gene 6000 Instrument (Corbett Life Science) and Sybr Select Master Mix (Life Technologies) with 100 nM specific primer pairs and 2 µL of 1:10 diluted cDNA, in 15 µL total volume. After an initial denaturation at 95 °C for 2 min, 45 amplification cycles were performed (95 °C for 15 s, 60 °C

for 60 s), followed by melting curve analysis. Each analysis was repeated twice. *UBQ*, *UBC9*, and *TubA* were used as reference genes. Calculations were made according to the $2^{-\Delta\Delta C_t}$ method using REST software (Pfaffl et al. 2002).

AITC residue analysis in kiwifruits

AITC quantification was performed on kiwifruits at d120. Fruits were peeled and 0.5–1 g of the epicarp (2–3 mm thick) or 5 g of the outer pericarp were separately cut into small pieces and analysed by solid phase microextraction (SPME) coupled with GC-FID technique as in Ugolini et al. (2014). GC analysis was carried out on a Varian GL-3800 equipped with an Agilent HP-5 column (30 m, 0.25 mm, 0.25 µm), an automatic auto-sampler CombiPAL (CTC Analytics) and FID detector. The analytical conditions were: injector and detector temperature were maintained at 260 and 280 °C respectively; flow of the carrier gas (nitrogen) was set at 1 mL min⁻¹; column oven temperature followed a gradient from 60 to 90 °C, with a rate of 5 °C min⁻¹ and from 90 to 200 °C with a rate of 40 °C min⁻¹. The splitless mode was used for injection. Blank samples of untreated fruit were analysed following the same protocol. A calibration curve with blank sample of the non-treated epicarp and the outer pericarp spiked with AITC hexane dilutions was constructed and the limit of quantification and detection (LOQ and LOD) were determined. Measurements were replicated 5 times for treated fruit and calibration samples.

Statistical analysis

Described analysis were carried out in triplicate and statistical analysis of data expressed as mean ± standard error was performed with the SPSS 17.0 software (SPSS, Inc., Chicago, Illinois), unless differently specified. Data were then subjected to a one-way analysis of variance (ANOVA) employing Tukey's HSD test to assess significant differences between samples analysed ($P < 0.05$). Finally, Pearson's correlation coefficient (r) was used to determine variable correlations ($P < 0.01$).

Results and discussion

Effect of AITC on kiwifruit quality traits, phytochemical content and antiradical capacity

The mean values of the physico-chemical attributes of kiwifruit at the end of storage are shown in Table 2. All fruits were at a adequately ripe stage for consumption ($SSC \geq 12.5$ °Brix) (Beirão-da-Costa et al. 2008), with no

Table 1 List of the primers used for transcript abundance analysis by RT-qPCR, with respective gene identification code (ID) from the Kiwifruit Genome database (<http://bioinfo.bti.comell.edu/cgi-bin/kiwi/search.cgi>, or GeneBank database (<https://www.ncbi.nlm.nih.gov/genbank>)

Short name	Gene description	Type/metabolism	Primer forward 5'→3'	Primer reverse 5'→3'	Gene ID
<i>TUA</i>	Alpha-tubulin	Reference	ACAACACAGCAGTGGCAGAG	AGAGAACTCCCTTCCTCCA	FG466654
<i>UBC9^a</i>	Ubiquitin conjugating enzyme	Reference	CCATTTCAGAGGTGTGCTT	TACTTGTCCGGTCCGTCTT	FG524028
<i>UBQ</i>	Ubiquitin	Reference	CCAAGATCCAGGACAAAGGAG	TGGAGGGTCGATTCTTCTG	FG409945
<i>SAM</i>	S-adenosylmethionine synthase	GOI/ethylene synthesis	CCCAGATTTCACCTGGGAGA	TCTTCTCAGCCAGAAATGGT	ACU17240
<i>ACO1</i>	ACC oxidase 1	GOI/ethylene synthesis	CCGCTTGTGTTGTGTGAAC	TTCTTTCTGGGGAATGCAAC	HQ293204
<i>ETR2</i>	Ethylene receptor	GOI/ethylene perception	AAACGTGTGGGAAAGATGC	CAATCCCGTCATCACACAAC	EU170629
<i>PG</i>	Polygalacturonase	GOI/fruit softening/cell wall degradation	GTTAGGGGTGTGCTCTTCCA	GATTGGGGCAATTTTGTGTG	AF152756
<i>BAM^b</i>	Beta-amylase	GOI/fruit ripening/starch degradation	ATCCTGACCTTGTCTACACA	ATGGGTGTCTTCTCTCTAAG	FG525163
<i>PAL</i>	Phenylalanine ammonia-lyase	GOI/phenyl propanoid pathway	GCTTGGATTACGGGTTCAAA	GAGTTCACATCTTGGTTGTGTG	Achn153791; Achn066341
<i>CHS3</i>	Chalcone synthase	GOI/phenyl propanoid pathway	AGAGAAAGTTCAGCGCATGT	ATTTGGGAATTTCCACCACA	Achn168721
<i>PPO</i>	Polypheol oxidase	GOI/stress response	GGTGATCCGACCTGACAACT	ACAGCTCCGTGATACCCAAC	Achn226891
<i>GPP</i>	L-galactose-1-phosphate phosphatase	GOI/ascorbic acid synthesis	TACCAAAACCAAGCATGTGGA	ACTTGTGGTCAGGGAAATGC	AY87585
<i>GalDH^c</i>	L-galactose dehydrogenase	GOI/ascorbic acid synthesis	TTGGGAAGGATGCGAAAAC	GCAGCCACTTCACAAAAGCA	EU525846
<i>MDHAR^c</i>	Monodehydroascorbate reductase	GOI/ascorbic acid recycling	TCAAATTATCAAGGGAACTGTGGC	ACCAACAACAAACGATGTCCGT	Achn091771; Achn075231; GU339041
<i>GST</i>	Tau class glutathione transferase 5	GOI/glutathione metabolism	TTGCTGGAGCAAAGACAAATG	GGCTTCTCTGAATGATTCCAA	Achn223211
<i>Act d1</i>	Actinidin	GOI/abundant protein in kiwifruit	AGGCTACATGAGGATCCTGC	GGCCATCCTTGTCTCATTTAG	M38422
<i>PR5</i>	Thaumatin-like protein	GOI/abundant protein in kiwifruit	TGATGGGTTCATGTGGCGA	CCAGGGGCACGTAACTCATT	AJ871175

The gene type (reference or gene of interest, GOI) and the metabolism in which GOIs are involved are specified. Superscript letters indicate the reference of the primers' sequences: a: Günther et al. (2011), b: Richardson et al. (2011), c: Li et al. (2010)

Table 2 Pomological and nutraceutical parameters (mean $\pm$ SE) of kiwifruit treated or not with AITC and determined at the end of storage (d120)

	CTRL	AITC
DM	14.9 $\pm$ 0.4	14.1 $\pm$ 0.2
SSC	13.3 $\pm$ 0.1	12.93 $\pm$ 0.03*
TA	258 $\pm$ 1	261 $\pm$ 2
pH	3.43 $\pm$ 0.02	3.38 $\pm$ 0.02
Firmness	31.5 $\pm$ 0.3	33.8 $\pm$ 0.2
AsAC	69 $\pm$ 2	105 $\pm$ 4*
TPC	3.078 $\pm$ 0.009	3.48 $\pm$ 0.03*
FLC	1.398 $\pm$ 0.007	1.505 $\pm$ 0.002*
EC ₅₀ -DPPH $\cdot$	12.56 $\pm$ 0.01	10.0 $\pm$ 0.1*
EC ₅₀ -ABTS $\cdot^+$	1.029 $\pm$ 0.004	0.86 $\pm$ 0.01*

Results are expressed on a dry matter (DM) basis. DM (%), soluble solid content (SSC, Brix); titratable acidity (TA, meq L⁻¹); firmness (N); ascorbic acid content (AsAC, μ g g⁻¹); total phenolic content (TPC, mg gallic acid equivalents GAE g⁻¹); total flavanol content (FLC, mg catechin equivalents CAE g⁻¹); EC₅₀-DPPH $\cdot$ (mg); EC₅₀-ABTS $\cdot^+$ (mg). AITC treated (AITC) sample means followed by an asterisk are significantly different from untreated control (CTRL) means ($P < 0.05$, Tukey's HSD test)

EC₅₀ = mg of kiwifruit required to obtain 50% DPPH $\cdot$ or ABTS $\cdot^+$ scavenging

decay. Postharvest AITC treatment did not significantly influence the overall quality of kiwifruit except SSC, which decreased in the treated samples as compared to the control after cold storage ($P < 0.05$). These findings agree with those reported in the literature on the ability of AITC in maintaining the postharvest fruit quality (Wang 2003).

Kiwifruits are generally recognised as a valuable source of vitamin C. However, this is a readily degradable compound, so it is of great importance to know how postharvest technologies could affect its content and possibly identify the treatments that reduce AsA losses in ripe fruit. In this study, kiwifruit AsAC was markedly affected by AITC treatment (Table 2). In fact, AsAC strongly decreased with storage (-55% in control fruits) as already observed by Oz (2010), while AITC application significantly reduced AsAC losses, keeping the level similar to those of samples at harvest time ($152 \pm 6 \mu\text{g g}^{-1}$ DM). Kiwifruits are also known for the presence of other valuable compounds as polyphenols (Du et al. 2009). In this study, storage markedly affected the total polyphenol content (TPC) of analysed samples. The highest TPC was found in AITC treated kiwifruits, which was 24% higher compared to that at harvest time ($2.810 \pm 0.007 \text{ mg GAE g}^{-1}$ DM; $P < 0.05$) and also significantly higher than control samples ($+13\%$). FLC was also increased by AITC treatment, which produced a significant accumulation of flavans compared to the control samples ($\sim +8\%$). In this work the antioxidant potential of kiwifruits was also evaluated by means of two

different in vitro assays: the DPPH $\cdot$ and ABTS $\cdot^+$ scavenging methods. The results are expressed as the quantity of antioxidant necessary to reduce the initial radical concentration by 50%, which is the value generally defined as EC₅₀ (Table 2): the higher the antiradical capacity, the lower this value is. In both assays, AITC treated samples showed the highest AC, which was 1.6-fold higher than that at harvest (EC₅₀ = $15.75 \pm 0.08 \text{ mg}$ on DM; EC₅₀ = $1.39 \pm 0.03 \text{ mg}$ on DM, for DPPH $\cdot$ and ABTS $\cdot^+$ scavenging, respectively). Parametric correlation analysis pointed out a very good negative correlation between TPC and AC ($r = -0.96$ and -0.89 , for DPPH $\cdot$ and ABTS $\cdot^+$, respectively) significant at 0.01 level (2-tailed). AC also showed a good negative correlation with AsAC, which was higher in the case of ABTS $\cdot^+$ radical scavenging ($r = -0.59$ and -0.75 , for DPPH $\cdot$ and ABTS $\cdot^+$, respectively; $P < 0.01$, 2-tailed). These findings are in agreement with those reported in literature (Du et al. 2009), highlighting the contribution of vitamin C and polyphenols to the antioxidant potential of kiwifruits. The results show for the first time the AITC effect of prevention of AsAC and TPC loss on kiwifruit during postharvest storage in AC. The exact mechanism of action of the molecule is not known and to have some indication on the possible metabolic pathways and enzymes to be involved, investigations by biochemical and gene expression analysis were performed as described below.

Effect of AITC on antioxidant enzyme activity and glutathione kiwifruit content

The short- and long-term effect of AITC on the activity of antioxidant enzymes in kiwifruit was investigated. The results (Table 3) highlighted a significant increase of SOD activity in treated kiwifruits compared to untreated ones, determined immediately after the treatment (d0). The basal level of activity was quickly restored at 24 h (d1) and no differences were detected at the end of storage (d120). Regarding CAT and POD activities, these were similar to those found by other authors for kiwifruits stored 120 days in cold storage (Yang et al. 2013), but no significant differences were noted between AITC-treated and control kiwifruits as between fruits before and after storage. SOD is the first line of defence against ROS toxicity and is a strong free radical scavenging enzyme that first neutralize the superoxide radical into the less toxic hydrogen peroxide which can be further converted into water by CAT and POD enzymes (Xia et al. 2016). It can be speculated that SOD increased activity might play a protective role against an initial burst-like effect of AITC treatment. This hypothesis was further validated by reduced and oxidized glutathione quantitative determination. Soon after AITC application (d0) reduced glutathione (GSH) content was significantly lower

Table 3 Antioxidant enzyme activities (Unit mg^{-1} of protein) and glutathione content (nmol g^{-1} FW) determined in kiwifruit immediately after AITC treatment (d0), one day later (d1) and after 120 days of storage under controlled atmosphere (d120): superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), reduced glutathione (GSH), oxidized glutathione (GSSG), redox state [GSH/(GSH + GSSG) %]

	CTRL	AITC
SOD		
d0	30.8 $\pm$ 2.4b	40.2 $\pm$ 4.5a
d1	29.6 $\pm$ 3.4b	31.2 $\pm$ 1.7b
d120	34.6 $\pm$ 5.5ab	32.4 $\pm$ 5.7ab
POD		
d0	12.9 $\pm$ 1.7a	17.7 $\pm$ 4.5a
d1	17.9 $\pm$ 3.5a	17.3 $\pm$ 5.7a
d120	22.0 $\pm$ 2.6a	15.8 $\pm$ 10.4a
CAT		
d0	1.4 $\pm$ 0.6a	1.7 $\pm$ 1.2a
d1	1.5 $\pm$ 0.7a	1.4 $\pm$ 0.5a
d120	1.3 $\pm$ 0.3a	1.3 $\pm$ 0.5a
GSH		
d0	158.2 $\pm$ 10.5a	133.3 $\pm$ 11.9b
d1	171.3 $\pm$ 25.7a	153.1 $\pm$ 8.7ab
GSSG		
d0	6.4 $\pm$ 1.0a	8.1 $\pm$ 1.0a
d1	2.9 $\pm$ 0.4b	2.7 $\pm$ 0.1b
GSH/(GSH + GSSG)%		
d0	96.1b	94.3c
d1	98.3 0.2a	98.3 0.2a

Untreated control (CTRL) and AITC treated (AITC) sample means followed by the same letters are not statistically different ($P < 0.05$, Tukey's HSD test)

(−16%) in treated compared to control samples, while oxidized glutathione (GSSG) content was higher in AITC treated samples, even if not significantly. After 24 h (d1), GSH and GSSG levels increased and decreased respectively in both treated and control, and the redox state of the two pools was comparable. Therefore, AITC treated samples showed a more oxidized cellular redox state than control immediately after treatment, suggesting an initial but reversible oxidative stress caused by AITC. The glutathione reversible depletion effect and consequent ROS accumulation has already been observed in AITC treated *Arabidopsis thaliana* seedlings, possibly indicating a role of the glutathione in the detoxification/control of ITCs in plant tissue in accordance with findings in mammalian cells (Øverby et al. 2015). Total glutathione level was also rapidly recovered after a few hours in *Arabidopsis*. Lower GSH content after AITC postharvest treatment has also previously been observed in blueberries and raspberries (Chen et al. 2015; Chanjirakul et al. 2006). GSH/GSSG is the major

cellular redox buffer along with ascorbate/dehydroascorbate and changes in these ratios can reflect cellular toxicity but have also been associated with important redox signalling and regulation of gene expression related to stress defence. The AITC effect on the redox state could be correlated with the induction of protective and antioxidant non-enzymatic systems and could be responsible for the increased AsA, polyphenol and flavan content in AITC treated fruit (Table 2), even though this hypothesis needs further investigation.

Gene expression analyses

The expression level of 14 genes involved in different metabolisms related to fruit ripening and stress responses (as detailed in Table 1) was analysed in response to AITC application, immediately after treatment (d0) and one day later (d1). Results are shown in Fig. 1.

According to our qualitative results on kiwifruit, which indicated a reduced loss of AsA after AITC treatment during postharvest storage (Table 2), it was decided to investigate two genes involved in AsA biosynthesis through the main L-galactose pathway (L-galactose-1-phosphate phosphatase *GPP* and L-galactose dehydrogenase *GaldH*) and one (monodehydroascorbate reductase *MDHAR*) involved in AsA recycling. The increased expression levels of *GPP* and *GaldH* suggest that AITC might compensate for AsA degradation through *de novo* biosynthesis. *MDHAR*, instead, was not affected at all by AITC. The transcriptional behaviour of a tau class glutathione transferase-encoding gene (*GST*) was also assessed in this work as it is linked to AsA through the glutathione–ascorbate cycle. GST enzymes have functions in detoxification as they catalyse the conjugation between GSH and electrophilic xenobiotics and their activity is often up-regulated in response to various types of stress. Øverby et al. (2015) in *A. thaliana* demonstrated that AITC is not only involved in a reversible depletion in GSH-pool, as mentioned above, but also in the elevated expression of GST-encoding genes. In agreement with this report, *GST* gene was up-regulated upon AITC treatment. The different GSH redox state observed early in treated fruit (Table 3) could be part of the signalling transduction induced by AITC that influenced the expression levels of these genes.

In kiwifruits, contrary to tomato and many other climacteric fruits, most physiological changes associated to ripening occur before autocatalytic ethylene production is detectable and ethylene production is associated more to fruit softening and senescence than ripening. In this work, three genes potentially involved in ethylene production and perception (*S*-adenosylmethionine synthase *SAM*, 1-aminocyclopropane-1-carboxylate oxidase 1 *ACO1* and ethylene receptor 2 *ETR2*) were found either slightly

increased or significantly up-regulated in response to AITC, suggesting the activation of ethylene synthesis and perception. SAM synthase genes and enzymes and ethylene receptors have previously been shown to be ethylene-responsive in kiwifruit (Atkinson et al. 2011), therefore AITC could initiate an autocatalytic process of ethylene biosynthesis and response that would normally occur later. It is known that the expression of beta-amylase (BAM) is positively linked to this process and associated to starch degradation and soluble sugar accumulation. Consistently, in our system *BAM* and polygalacturonase *PG*, which is involved in cell wall degradation, were induced one day after treatment, suggesting that cell wall remodelling and soluble sugar accumulation, naturally occurring in kiwifruit during late stages of fruit ripening, would be anticipated by AITC treatment. However, this did not affect final fruit firmness (Table 2), probably because this process was stopped by subtracting ethylene during CA storage.

Phenolic secondary metabolites affect plant-derived food quality characteristics such as appearance, flavour and other health-promoting properties. Their content in foods is affected by many factors that influence phenolic stability, biosynthesis and degradation. In terms of biosynthesis, the key enzyme phenylalanine ammonia-lyase (PAL) is especially relevant, as it can be induced by different stress conditions and ethylene itself can induce both the gene expression and the enzyme activity of *PAL* (Singh et al. 2010). In this work, a 4× induction of *PAL* expression level was observed, probably responsible for the significantly higher TPC value in kiwifruit at the end of storage (Table 2), even though the tested chalcone synthase gene (*CHS3*), a downstream gene in the flavonoid biosynthetic pathway, was not affected by the treatment. Moreover, a polyphenol oxidase (*PPO*), encoding one of the main enzymes responsible for fruit quality loss due to phenolic degradation, was down-regulated.

Finally, the expression level of two-gene coding for the most abundant proteins in kiwifruit was also investigated, actinidin (*Act d1*) and a thaumatin-like protein (*PR5*). Both genes were sensitive to AITC, though with divergent trends of modulation. Interestingly, *Act d1* was three times more expressed in AITC treated samples than in control ones. This could lead to an increased concentration in actinidin, which generally decreases during fruit ripening, contributing to further enhancement of nutritional values and shelf-life of the fruit. The opposite was found for *PR5*, although further studies are required to understand if this could impact on fruit quality.

AITC residues in treated kiwifruit

After a 4-month storage period of kiwifruits, the concentration of AITC residues in the epicarp was below the LOQ

of 0.26 mg kg⁻¹, corresponding to about 0.075 mg kg⁻¹ of the whole fruit, and no detectable residues were found in the outer pericarp (LOD = 0.0096 mg kg⁻¹, corresponding to about 0.0024 mg kg⁻¹ of the whole fruit). According to EFSA, the acceptable daily intake (ADI) for AITC used as food additive is 0.02 mg/kg bw/day (EFSA 2010), so in our case the residues are largely below this limit, even in the case of presumable use of freeze-dried kiwifruit pulp extracts.

Conclusion

A 5-h treatment with 0.15 mg L⁻¹ AITC in combination with CA can preserve ‘Hayward’ kiwifruit quality and enhance the nutraceutical properties in terms of increased antiradical capacity and phytochemical content, without leaving any detectable AITC residues on fruit. GSH cycle involvement in AITC mechanism of action/detoxification was confirmed in kiwifruit and AITC influence on AsAC and ethylene synthesis and PAL was depicted at gene expression level. These findings open new perspectives in the use of this natural compound to enhance phytochemical content and antioxidant properties of fresh and, potentially, minimally processed fruits.

Acknowledgements The trials were performed as a part of the activities of the Project Sistema Integrato di Tecnologie per la valorizzazione dei sottoprodotti della filiera del Biodiesel (VALSO) financed by Ministry of Agriculture, Food and Forestry Policies, MiPAAF (D.M.17533/7303/10 of 29/04/2010) and coordinated by CREA-CIN of Bologna.

References

- Atkinson RG, Gunaseelan K, Wang MY, Luo L, Wang T, Norling CL, Johnston SL, Maddumage R, Schröder R, Schaffer RJ (2011) Dissecting the role of climacteric ethylene in kiwifruit (*Actinidia chinensis*) ripening using a 1-aminocyclopropane-1-carboxylic acid oxidase knockdown line. *J Exp Bot* 62:3821–3835
- Beirão-da-Costa S, Steiner A, Correia L, Leitão E, Empis J, Moldão-Martins M (2008) Influence of moderate heat pre-treatments on physical and chemical characteristics of kiwifruit slices. *Eur Food Res Technol* 226(4):641–651
- Carbone K, Mencarelli F (2015) Influence of short-term postharvest ozone treatments in nitrogen or air atmosphere on the metabolic response of white wine grapes. *Food Bioprocess Technol*. doi:10.1007/s11947-015-1515-y
- Cavalieri F, Micheli L, Zhou M, Tortora M, Palleschi G, Ashokkumar M (2013) Electrochemical investigation of the interaction between lysozyme-shelled microbubbles and vitamin C. *Anal Bioanal Chem* 405(16):5531–5538
- Chang S, Puryear J, Cañey J (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol Rep* 11:113–116
- Chanjirakul K, Wang SY, Wang CY, Siriphanich J (2006) Effect of natural volatile compounds on antioxidant capacity and

- antioxidant enzymes in raspberries. *Postharvest Biol Technol* 40:106–115
- Chen H, Gao H, Fang X, Ye L, Zhou Y, Yang H (2015) Effects of allyl isothiocyanate treatment on postharvest quality and the activities of antioxidant enzymes of mulberry fruit. *Postharvest Biol Technol* 108:61–67
- Drummond L (2013) The composition and nutritional value of kiwifruit. *Adv Food Nutr Res* 68:33–57
- Du G, Li M, Ma F, Liang D (2009) Antioxidant capacity and the relationship with polyphenol and vitamin C in *Actinidia* fruits. *Food Chem* 113:557–562
- EFSA (2010) EFSA panel on food additives and nutrient sources added to food (ANS): scientific opinion on the safety of allyl isothiocyanate for the proposed uses as a food additive. *EFSA J* 8:1943–1983
- Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol* 155:2–18
- Franco P, Spinozzi S, Pagnotta E, Lazzeri L, Camborata C, Roda A (2016) Development of a liquid chromatography–electrospray ionization–tandem mass spectrometry method for the simultaneous analysis of intact glucosinolates and isothiocyanates in *Brassicaceae* seeds and functional foods. *J Chromatogr A* 1428:154–161
- Günther CS, Chervin C, Marsh KB, Newcomb RD, Souleyre EJF (2011) Characterisation of two alcohol acyltransferases from kiwifruit (*Actinidia* spp.) reveals distinct substrate preferences. *Phytochemistry* 72:700–710
- Hendrix KM, Morra JM, Lee H-B, Min SC (2012) Defatted mustard seed meal-based biopolymer film development. *Food Hydrocolloid* 26:118–125
- Huang S, Ding J, Deng D, Tang W, Sun H, Liu D, Zhang L, Niu X, Zhang X, Meng M et al (2013) Draft genome of the kiwifruit *Actinidia chinensis*. *Nat Commun* 4:2640
- Khokon MDAR, Jahan MDSJ, Rahman T, Hossain MA, Muroyama D, Minami I, Munemasa S, Mori IC, Nakamura Y, Murata Y (2011) Allyl isothiocyanate (AITC) induces stomatal closure in *Arabidopsis* plant cell. *Environment* 34:1900–1906
- Latocha P, Krupa T, Jankowskic P, Radzanowskad J (2014) Changes in postharvest physicochemical and sensory characteristics of hardy kiwifruit (*Actinidia arguta* and its hybrid) after cold storage under normal versus controlled atmosphere. *Postharvest Biol Technol* 88:21–33
- Lazzeri L, Leoni O, Manici LM, Palmieri S, Patalano G (2010) Use of seed flour as soil pesticide. US Patent No. 7, 749, 549, July 6
- Lazzeri L, D'Avino L, Ugolini L, De Nicola GR, Cinti S, Malaguti L, Bagatta M, Patalano G, Leoni O (2011) Bio-based products from *Brassica carinata* A. Braun oils and defatted meals by a second generation biorefinery approach. In: 19th EU BC&E conference proceedings, pp 1080–1092
- Li M, Ma F, Liang D, Li J, Wang Y (2010) Ascorbate biosynthesis during early fruit development is the main reason for its accumulation in kiwi. *PLoS ONE* 5:e14281
- Mari M, Leoni O, Bernardi R, Neri F, Palmieri S (2008) Control of brown rot on stonefruit by synthetic and glucosinolate-derived isothiocyanates. *Postharvest Biol Technol* 47:61–67
- Matthiessen JN, Kirkegaard JA (2006) Biofumigation and enhanced biodegradation: opportunity and challenge in soil borne pest and disease management. *Crit Rev Plant Sci* 25:235–265
- Nagata M (1996) Studies on inhibitory mechanism of allyl isothiocyanate on browning and ethylene production of shredded cabbage (*Brassica oleracea*). *Bull Natl Res Inst Veg Ornament Plants Tea Ser A* 11:131–158
- Øverby A, Stokland RA, Åsberg SE, Sporsheim B, Bones AM (2015) Allyl isothiocyanate depletes glutathione and upregulates expression of glutathione S-transferases in *Arabidopsis thaliana*. *Front Plant Sci* 6:1–9
- Oz AT (2010) Effects of harvests date and conditions of storage of 'Hayward' kiwifruits on contents of L-ascorbic acid. *J Food Agric Environ* 8:242–244
- Padmanabhan P, Paliyath G (2016) Kiwifruit. In: *Encyclopedia of Food and Health*, Academic Press, Oxford, pp 490–494. doi:10.1016/B978-0-12-384947-2.00409-8
- Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30:e36
- Richardson AC, Boldingh HL, McAtee PA, Gunaseelan K, Luo Z, Atkinson RG, David KM, Burdon JN, Schaffer RJ (2011) Fruit development of the diploid kiwifruit, *Actinidia chinensis* "Hort16A". *BMC Plant Biol* 11:182
- Shaik IH, Mehvar R (2006) Rapid determination of reduced and oxidized glutathione levels using a new thiol-masking reagent and the enzymatic recycling method: application to the rat liver and bile samples. *Anal Bioanal Chem* 385(1):105–113
- Singh R, Rastogi S, Dwivedi UN (2010) Phenylpropanoid metabolism in ripening fruits. *Compr Rev Food Sci Food Saf* 9:398–416
- Tavarini S, Degl' Innocenti E, Remorini D, Massai R, Guidi L (2008) Antioxidant capacity, ascorbic acid, total phenols and carotenoids changes during harvest and after storage of Hayward kiwifruit. *Food Chem* 107:282–288
- Ugolini L, Martini C, Lazzeri L, D'Avino L, Mari M (2014) Control of postharvest grey mould (*Botrytis cinerea* Per.:Fr.) on strawberries by glucosinolate-derived allyl-isothiocyanate treatments. *Postharvest Biol Technol* 90:34–39
- Wang CY (2003) Maintaining postharvest quality of raspberries with natural volatile compounds. *Int J Food Sci Technol* 38(8):869–875
- Wang SY, Chen CT, Yin JJ (2010) Effect of allyl isothiocyanate on antioxidants and fruit decay of blueberries. *Food Chem* 120:199–204
- Xia Y, Chen T, Qin G, Li B, Tian S (2016) Synergistic action of antioxidative systems contributes to the alleviation of senescence in kiwifruit. *Postharvest Biol Technol* 111:15–24
- Yang Q, Zhang Z, Rao J, Wang Y, Sun Z, Ma Q, Dong X (2013) Low temperature conditioning induces chilling tolerance in 'Hayward' kiwifruit by enhancing antioxidant enzyme activity and regulating endogenous hormone levels. *J Sci Food Agric* 93:3691–3699