
# Effect of hydrogen sulphide on the postharvest metabolism of the green leafy vegetable, pak choy (*Brassica rapa subsp. Chinensis)*

**Hebah Muhsien Sabiah Al Ubeed**

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School of Environmental and Life Sciences

Faculty of Science

University of Newcastle

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# i. Declarations

**Statement of Originality**

I hereby certify that to the best of my knowledge and belief this thesis is the result of original research and contains no material previously published or written by another person except where due reference and acknowledgement is made in the text. It contains no material which has been previously submitted for the award of any other degree or diploma in any university or other tertiary institution.

**Thesis by Publication**

I hereby certify that this thesis is in the form of five papers. I have included as Appendix 1 a written statement from each co-author, endorsed in writing by the Faculty Assistant Dean (Research Training), attesting to my contribution to any jointly authored papers.

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Hebah Muhsien Sabiah Al Ubeed Date

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## iii. Dedication

Every challenging work needs self-efforts as well as the guidance of elders especially those who were very close to our heart.

My humble effort I dedicate to my sweet and loving

Mother

Whose affection, love, encouragement and prayers for day and night have allowed me to achieve such success and honour.

Along with all my hard working and respected
Supervisors

Thank you

## iv. List of publications included in the thesis

Refereed research papers

1. **H.M.S. Al Ubeed**, R.B.H. Wills, M.C. Bowyer, Q.V. Vuong, J.B. Golding. Interaction of exogenous hydrogen sulphide and ethylene on senescence of green leafy vegetables. Postharvest Biology and Technology(2017),133,81-87,https://doi.org/10.1016/j.postharvbio.2017.07.010.
2. **H.M.S. Al Ubeed**, R.B.H. Wills, M.C. Bowyer, J.B. Golding. Comparison of hydrogen sulphide with 1-methylcyclopropene (1-MCP) to inhibit senescence of the leafy vegetable, pak choy. Postharvest Biology and Technology (2018), 137, 129– 133, https://doi.org/10.1016/j.postharvbio.2017.11.020.
3. **H.M.S. Al Ubeed**, R.B.H. Wills, M.C. Bowyer, J.B. Golding. Interaction of the hydrogen sulphide inhibitor, propargylglycine (PAG), with hydrogen sulphide on postharvest changes of the green leafy vegetable, pak choy. Accepted for publication in Postharvest Biology and Technology.
4. **H.M.S. Al Ubeed**, R.B.H. Wills, M.C. Bowyer, J.B. Golding. Inhibition of postharvest senescence of green leafy vegetables, by exogenous D-cysteine and L- cysteine as precursors of hydrogen sulphide. Journal of Horticultural Science and Biotechnology (submitted).

Refereed proceedings of conference oral presentation (accepted)

1. **H.M.S. Al Ubeed**, R.B.H. Wills, M.C. Bowyer, Q.V. Vuong, J.B. Golding. Effect of hydrogen sulphide, nitric oxide and ethylene on the postharvest deterioration of pak choy International Society for Horticultural Science (ISHS) VI Postharvest Unlimited Conference. Madrid, Spain October 2017. (Oral presentation) Accepted for publication in Acta Horticulture.

A statement of the contribution of the candidate and other authors is detailed in Appendix 1.

## Table of Contents

## 1. Abstract

Postharvest senescence of leafy green vegetables is characterised by leaf yellowing and changes to a range of other physio-chemical factors, which together, limit market life. The plant hormone ethylene is traditionally associated with accelerating senescence in postharvest produce and its effects have been closely studied. In recent years, a range of other gases including hydrogen sulphide ($H_2S$), carbon monoxide and nitric oxide (NO) have been identified as plant signaling agents and have been shown to be associated with senescence processes in horticultural produce. This thesis examined the ability of $H_2S$ to inhibit senescence in a range of leafy green vegetables, with pak choy the main target species.

The initial study of the thesis determined that fumigation of pak choy with 250 µL/L $H_2S$ for 4 hr was as the optimal concentration for extending postharvest life at 10ºC. Fumigation was found to affect a range of physiological properties including endogenous ethylene production, rate of chlorophyll degradation, respiration, weight loss, ion leakage and antioxidant activity. It was considered that $H_2S$ inhibited both the favourably affected exogenous ethylene action and endogenous action of ethylene.

Studies involving fumigation with $H_2S$ and 100 µL $L^{-1}$ NO in the presence of 0.1µL/L ethylene were conducted and were found to successfully inhibit chlorophyll loss and decrease respiration in pak choy, with individual gas studies showing $H_2S$ fumigation alone to be the more effective treatment.

The inhibition of senescence of pak choy by $H_2S$ was then studied in comparison with 1- MCP a known inhibitor of ethylene action. In the absence of exogenous ethylene both $H_2S$ fumigation and 1-MCP treatment were found to be equally effective, while in the presence of exogenous ethylene, 1-MCP proved a more effective anti-senescence treatment. It was concluded that for pak choy, 1-MCP was a more effective inhibitor of ethylene action than $H_2S$.

The interaction of exogenous $H_2S$ fumigation with PAG, a known inhibitor of endogenous $H_2S$ production in both plants and mammals, was examined. In these experiments, fumigation with $H_2S$ gas significantly extended the postharvest life of pak choy, while treatment with PAG (as a spray) accelerated senescence. Sequential treatment involving application of $H_2S$ followed by PAG produced varied responses in the individual senescence factors assessed. The findings suggest that the action of PAG was not solely limited to action on endogenous $H_2S$ production pathways, presumably because of its known

mode of action in inactivating pyridoxal phosphate, a cofactor form many biochemical processes.

Potential commercial applications involving the use of $H_2S$ gas a fumigant present significant logistical and regulatory barriers. D- and L-Cysteine are the exclusive metabolic precursors of endogenous $H_2S$ in plants. L-cysteine has identified Generally Regarded as Safe (GRAS) status. Experiments assessing spray treatment of pak choy leaves with 10 mmol D-, L- or DL-cysteine were conducted and found to be equally effective in inhibiting senescence and are considered to have commercial potential.

# 2. Overview

## 2.1 Thesis Objective

To examine the ability of hydrogen sulphide, $H_2S$ precursors, investigate the action of PAG on $H_2S$ and comparison the effect of $H_2S$ with 1-MCP or NO in order to delay the emergence of senescence characteristics in non-climacteric leafy green vegetables using pak choy as the principal model.

## 2.2 Background

Ethylene has long been known to affect postharvest behaviour in plants by regulating the ripening of climacteric fruit and accelerating the senescence of non-climacteric fruit and vegetables. However, the relatively recent discovery that NO and then $H_2S$ were synthesised in mammals and the even more recent finding that these gases are also metabolised in plants has stimulated interest in their interaction with ethylene and hence in their resulting effect on postharvest metabolism. The effect of $H_2S$ on postharvest metabolism was first reported by Zhang et al. (2011) on cut flowers. It has since been found to extend the postharvest life of a limited range of fruits and vegetables. To date however, little attention has been paid to the relationship between $H_2S$ and ethylene (both endogenous and exogenous). Several studies have emerged in this area during the course the studies conducted in this project (Li et al., 2014, Zheng et al., 2016), all of which point to the existence of a strong interaction between $H_2S$ and ethylene, be it biosynthetically generated or applied from an external source. These studies did not however attempt to measure the concentration of ethylene around the produce post-fumigation with $H_2S$, nor did they expose produce to different concentrations of the ethylene during the storage, which would help to assess the relative impact of edogenous verses exogenous effect. The exception was Ge et al. (2017) who treated bananas with both Ethephon (a commercial ethylene source) and sodium hydrosulphide (NaHS) solutions, finding reduced ethylene biosynthesis, a result consistent with the outcomes of our studies. A full assessment of the literature on $H_2S$ as a postharvest treatment is outlined in the Literature Review (Section 3).

The work presented in this thesis was carried out using pak choy as the principal model, but small studies were also conducted with other green leafy vegetables (including parsley, kale and basil) to demonstrate general applicability. The experiments measured the effect of applied treatments on a range of physiochemical factors associated with produce senescence. Aspects studied were:

- Optimising the concentration of H2S that inhibits senescence.
- Interaction between ethylene NO and $H_2S$.
- Comparison of $H_2S$ and 1-MCP, an inhibitor of ethylene action.
- Interaction of $H_2S$ and PAG, an inhibitor of $H_2S$ synthesis.
- Ability of cysteine, a metabolic precursor of $H_2S$, to replicate the beneficial effects of $H_2S$.

### 2.3 Experimental Design

The initial trials exposed pak choy to a range of concentrations of ethylene and $H_2S$, but in subsequent trials, pak choy (and other green leafy vegetables) were fumigated with the optimum concentration of $H_2S$. The $H_2S$ gas was spontaneously and quantitatively generated from the reaction of solid sodium hydrosulphide (NaHS) with water, with the produce fumigated for four hours. After treatment, the leaves were stored at 10 °C in a flowing air stream that was either ethylene-free (<0.001 µL/L) or contained 0.1 µL/L ethylene to similar commercial storage conditions.

The effect of applied treatments was assessed on a range of senescence factors over time. Factors assessed were:

- Respiration rate, as an indicator of overall metabolic activity, was measured as evolved $CO_2$ by thermal conductivity (TCD) gas chromatography,
- Leaf colour, as an indicator of consumer acceptability, was assessed visually with time to a fixed colour score denoted as the market life. In some studies, leaf colour was also assessed as chlorophyll content by spectrophotometry,
- Ion leakage, as an indicator of cell permeability, was measured as the conductivity of an extract solution,
- Ethylene production was measured by flame ionisation (FID) gas chromatography,
- Stomatal status was assessed with a light microscope.
- Total phenolic content (TPC) and antioxidant activity, as DPPH and FRAP, were measured by spectrophotometry, and ascorbic acid by the indophenol titration method. These factors are an indicator of oxidative status.

### 2.4 Research findings

The initial trial fumigated pak choy with a range of $H_2S$ concentrations for four hours followed by storage at 10 °C in air alone or air containing a range of ethylene concentrations (1, 0.1, 0.01 and <0.001 µL/L). A market life increase of approximately 50% was observed

when the ethylene concentration was decreased from 1 to <0.001 µL/L. Produce fumigated with 50, 100 and 250 µL/L $H_2S$ and ventilated in air containing 0.1 µL/L ethylene produced varying results with the greatest extension in market life observed for the 250 µL/L treatment. Assessment of a range of other parameters at this concentration found reduced endogenous ethylene production, reduced respiration rate, decreased chlorophyll degradation, lower weight loss and ion leakage and enhanced total phenolic compounds and assessed antioxidant factors. A similar inhibition of senescence, as expressed by increased market life and reduced respiration rate, by $H_2S$ was also found for the leafy vegetables, kale and basil.

A comparison was then made of the effect on pak choy of fumigation with 250 µL/L $H_2S$ or with 100 uL/L nitric oxide (NO), which preliminary trials showed was the optimum concentration to inhibit pak choy senescence. Fumigation with $H_2S$ or NO increased the market life and decreased the respiration rate during storage in air with and without 0.1 µL/L ethylene but the beneficial effects of both gases were more pronounced in the presence of ethylene. The application of $H_2S$-NO was similar to $H_2S$ alone and dual application of NO- NO or $H_2S$-$H_2S$ did not increase the beneficial effects.

A comparison was then made of the effect on pak choy senescence by fumigating with 250 µL/L $H_2S$ or 10 µL/L 1-MCP. Fumigation with $H_2S$ and 1-MCP gave a similar increase in shelf life and decreased respiration rate, ethylene production and ion leakage of pak choy stored in ethylene-free air. When leaves were stored in 0.1 uL/L ethylene, 1-MCP generated a greater extension in market life and reduction in respiration rate, ion leakage and ethylene production than $H_2S$. However, sequential fumigation with 1-MCP followed by $H_2S$ showed no difference in any senescence factor to produce fumigated with only 1-MCP.

Pak choy was then fumigated with 250 µL/L $H_2S$ or sprayed with a 2 mM PAG solution and stored in air with and without the addition of 0.1 µL/L ethylene. Fumigation with $H_2S$ increased the shelf life and decreased respiration rates, ethylene production, ion leakage and enhanced antioxidant activity. Spraying the leaves with PAG generated the opposite effects for all senescence factors. Treatment with PAG + $H_2S$ showed different effects on the various senescence factors: market life was similar to PAG alone and less than the untreated control, respiration was similar to $H_2S$ alone, antioxidant activity was less than for PAG but higher than for control, and ethylene production and ion leakage were similar to control

The potential for cysteine to be a substitute treatment for fumigation with $H_2S$ was examined by spraying pak choy leaves with D-, L- or racemic DL-cysteine and comparing the effect with $H_2S$. All forms of cysteine inhibited loss of green colour, reduced respiration

rate and ethylene production similar to the effect achieved with H2S. L-cysteine was also evaluated against parsley and peppermint leaves and found to inhibit leaf colour loss and respiration compared to untreated leaves.

**2.5 Advances in scientific knowledge**

Fumigation of pak choy with $H_2S$ was shown to inhibit senescence as exhibited by reduced loss of green colour, respiration, ion leakage, and total phenols and increased antioxidant activity with 250 µL/L, the optimum $H_2S$ concentration. Since kale and basil also showed a similar response to $H_2S$, the use of $H_2S$ would seem to be of potential benefit for many green leafy vegetables. A possible mode of action of $H_2S$ was considered to be through the observed reduction in endogenous ethylene production.

NO is known to also be a signalling gas that inhibits senescence with inhibition of ethylene well demonstrated as a mode of action. This is the first study comparing the effects of $H_2S$ and NO. It was found that while NO inhibited the senescence of pak choy, $H_2S$ was more effective than NO in increasing market life and decreasing respiration. Thus, for pak choy, fumigation with $H_2S$ would seem to be a preferred treatment over fumigation with NO.

This is the first reported study comparing the effects of 1-MCP and $H_2S$ and the findings indicate that for pak choy, 1-MCP was a more reliable inhibitor of senescence than $H_2S$. While both were equally effective when the pak choy was stored in ethylene-free air, 1- MCP was more effective when produce was exposed to 0.1 uL/L ethylene. In addition, sequential fumigation with 1-MCP then $H_2S$ showed no added benefit over fumigation with only 1-MCP. It was concluded that the mode of action of $H_2S$ included inhibiting the action of ethylene as well as inhibiting endogenous ethylene production.

Comparison of the effect of $H_2S$ and PAG showed the expected result that $H_2S$ inhibited all aspects of senescence and PAG enhanced senescence. However, sequential application of PAG then $H_2S$ showed differential responses between senescence factors in a manner that indicates PAG had effects on metabolism that were not linked to the action of endogenous $H_2S$. Thus, PAG was not acting exclusively as an inhibitor of endogenous $H_2S$ production and the additional actions of PAG were postulated to be through its inhibition of PHP, which is a coenzyme for numerous enzyme systems.

While $H_2S$ is effective in inhibiting senescence, it poses logistical and safety issues that would impede obtaining regulatory approval for commercial use. The finding that spraying pak choy leaves with D-, L- or DL-cysteine inhibited senescence similarly to $H_2S$ and that parsley and peppermint leaves also had inhibited senescence from spraying with L- cysteine has the potential to be an acceptable commercial treatment for leafy vegetables in

general due to the GRAS status of L-cysteine. While L-cysteine is known as an inhibitor of browning in fresh-cut produce, this is the first study with D and DL-cysteine on postharvest produce.

### 2.6 Summary of advances in knowledge

The advances in scientific knowledge generated in this thesis are:

- Fumigation with $H_2S$ inhibits senescence of pak choy, kale and basil,
- $H_2S$ is more effective than NO in inhibiting senescence of pak choy,
- 1-MCP is a more reliable inhibitor of senescence of pak choy than $H_2S$,
- PAG does not exclusively act by inhibiting $H_2S$ production with additional actions possibly through inhibition of PHP activity,
- The mode of action of $H_2S$ is by inhibition of ethylene production and the action of ethylene,
- Cysteine captures the benefit of $H_2S$ but is more acceptable for commercial use.

# 3. Literature review

## 3.1 Postharvest Technology

The act of harvesting produce from the parent plant isolates it from its nutrient source. The postharvest life is then determined by the ability of the produce to maintain cellular integrity and function. Fruit and vegetables can be divided into two broad metabolic groups; climacteric and non-climacteric produce. The classification relates primarily to changes in respiration patterns but also to production of ethylene ($C_2H_4$) during maturation, ripening and senescence. Climacteric produce experience a pronounced increase in respiration and ethylene production during ripening, while non-climacteric produce show no marked change in these characteristics during maturation (Wills & Golding, 2016).

### 3.1.1 Changes in climacteric fruit during ripening

Changes in respiration rate and ethylene production are characterised by a marked rise in both to a peak during the climacteric followed by a decline for both when over-ripe.
In addition, ripening of climacteric fruit involves a sequence of metabolic changes that transform fruit colour, texture and flavour characteristics of the fruit, to be edible. Textural changes largely relate to flesh softening, which arises as a consequence of changes to cell wall structures and constituent components, particularly of pectic substances.

Colour changes often include loss of green colour arising from chlorophyll degradation, leading to the emergence of underlying pigments or synthesis of new pigments such as carotenoids (Finney, 1973; Katz et al., 2004).

Flavour is a combination of aroma and taste, where aroma involves the synthesis of a range of volatile organic compounds, while taste is mainly due to a balance of sugar and acid. The major sugars in ripe fruit are sucrose, fructose and glucose (Paliyath et al., 2009). The major acids, which generally decrease during ripening are malic and citric acid although some fruit have other acids such as tartaric acid (e.g. grapes) as the dominant compound. The major organic acid in apple is malic acid, citric acid in tomato and tartaric acid in grapes (Nip et al., 2008). A major compositional change during ripening is often the hydrolysis of starch to sugar, which affects both texture and taste (Davies et al., 1981) .

### 3.1.2 Senescence of non-climacteric produce

Quality changes that occur in non-climacteric fruit and vegetables are categorised as senescence, which can be defined as the last stage in the natural plant development cycle and is associated with irreversible events that lead to cellular disorganisation and eventual tissue collapse. Factors that accelerate senescence and hence loss of quality are aligned with poor handling resulting in damage to tissue and storage conditions such as elevated temperatures, high atmospheric levels of ethylene and low relative humidity. In green leafy vegetables, senescence is generally characterised by leaf yellowing and loss of cellular turgor (Kader, 1985).

Methods for extending postharvest life of non-climacteric produce reduce the rate of metabolism, minimise exposure to postharvest diseases and limit handling-related damage. An important indicator of metabolic activity is respiration, which involves the oxidative degradation of carbohydrates and organic acids. Of particular importance is the oxidation of glucose, which consumes oxygen and releases carbon dioxide and energy as shown in Figure 3.1.

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + ATP$$

Figure 3.1. Cellular respiration of glucose (Kader & Saltveit, 2003)

Postharvest produce with higher respiration rates tend to have a shorter shelf life (Wills & Golding, 2016). While a number of techniques have been developed to inhibit senescence, the most effective and possibly the earliest discovery in postharvest science is reducing the storage temperature (Wills & Golding, 2016). The associated increase in postharvest life is a consequence of reduced respiration and metabolism in general. A lower storage temperature also reduces the rate of water loss from produce, the rate of chlorophyll degradation, sensitivity to ethylene and pathogenic activity (Snowdon, 2010). While reducing the storage-temperature increases postharvest life, prolonged exposure of some produce to low temperatures can be detrimental. For example, chilling injury occurs in some tropical and sub-tropical produce when they are stored below a critical threshold temperature. Injury manifests as a consequence of metabolic imbalances that result in a loss of cellular compartmentalisation, leading to irreversible cell collapse. The threshold temperature for chilling injury development is in the range 10-15°C for tropical produce, and about 5°C for sub-tropical produce (Wills et al., 1999).

### 3.2 Ethylene

Ethylene is a colourless gas and is a metabolised by all plants (Yang & Hoffman, 1984). While the action of ethylene on postharvest produce was known for many years it was not until the 1930s that Gane (1934) identified ethylene as the agent that initiated ripening. It is now known that ethylene action lies at the heart of all stages of plant development including senescence (Buchanan-Wollaston, 1997).

Climacteric fruit show a surge in ethylene production and respiration during ripening, with ethylene considered responsible for regulating the onset of ripening (Wills & Golding, 2016). Non-climacteric produce differ in that they do not exhibit a surge in either ethylene production or respiration during ripening or maturation (Azzolini et al., 2005). Non- climacteric fruits and vegetables are harvested at the stage of maturation designated to be most desirable for eating and this can be at a physiologically immature stage for some vegetables (such as peas) or at a mature stage, such as for root vegetables (Wang, 2010). The effect of ethylene on non-climacteric produce is to accelerate the senescence process. In leafy vegetables, the effect of ethylene often manifests as accelerated yellowing and leaf abscission. Ethylene also accelerates the loss of firmness of non-climacteric fruits such as cucumber and strawberry, increases spear toughness of asparagus, imparts undesirable flavours in sweet potato and carrots (Martínez-Romero et al., 2007).

#### 3.2.1 Ethylene biosynthesis

The ethylene biosynthetic pathway is now known as the Yang cycle (Figure 3.2) (Miyazaki & Yang, 1987). A key element of ethylene biosynthesis is the conversion of the amino acid methionine to S-adenosyl-L-methionine (SAM), which is subsequently transformed to 1-aminocyclo-propane-1-carboxylic acid (ACC) by the action of the enzyme ACC synthase (ACS). Conversion of ACC to ethylene takes place in the presence of a second enzyme 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) (Sandhya, 2010). The activity of ACS and ACO are considered to be key step steps in ethylene production (Pech et al., 2010).

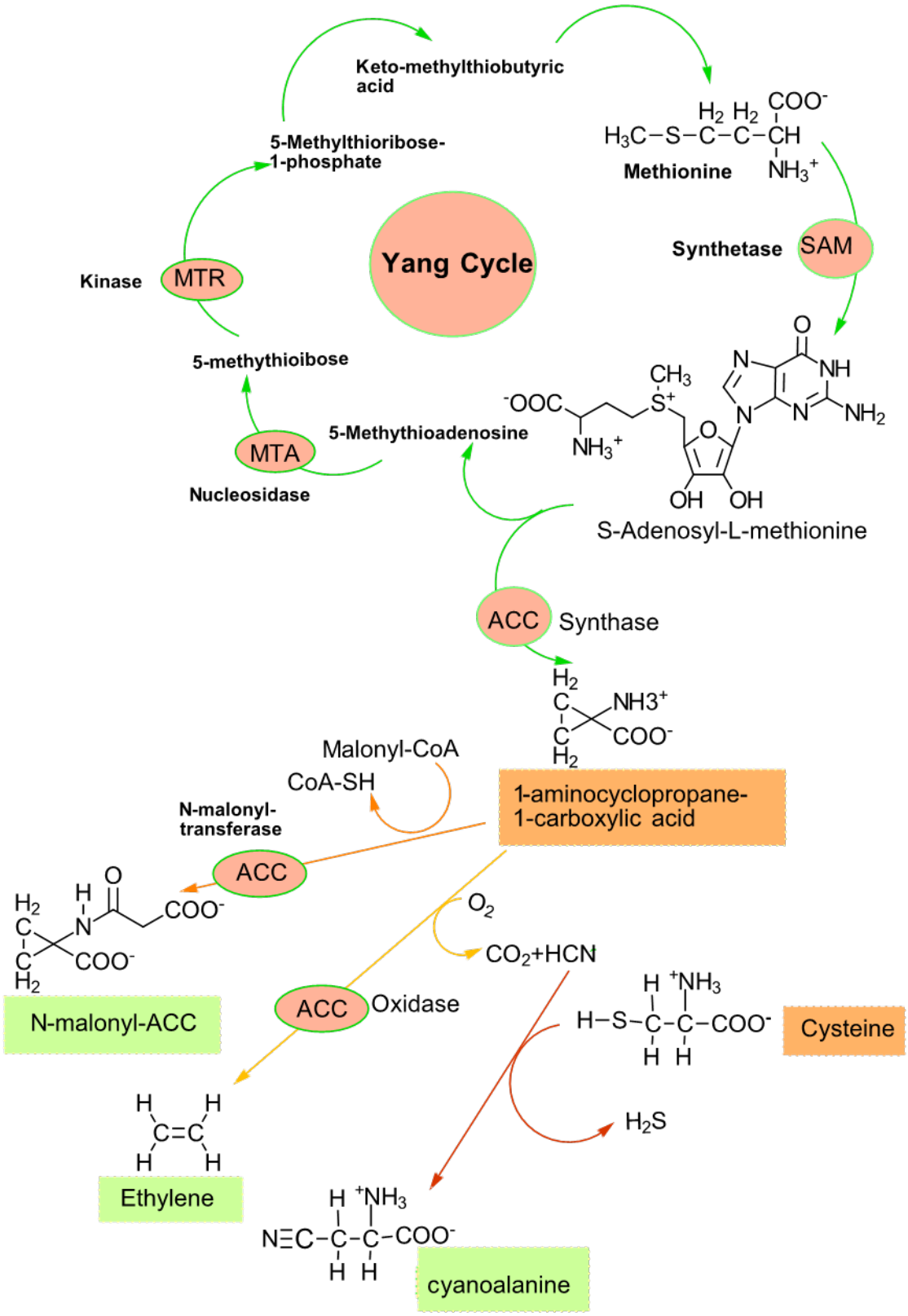


Figure 3.2. The Yang cycle for the synthesis of ethylene (Yang & Hoffman, 1984)

### 3.2.2 Ethylene sensing in plants

The ethylene receptor complex occurs in the endoplasmic reticulum (ER) membrane. The hydrophobic nature of ethylene itself allows for rapid diffusion of the gas within lipid membranes (Chang et al., 1993).

Binding of ethylene to the receptor results in cleavage of the Ethylene Insensitive2 (EIN2) protein in the complex. EIN2 has a key signalling role in regulating gene expression of characteristics associated with senescence (Lin et al. 2008). In Arabidopsis, ethylene binds to five specific receptors; ERS1, ERS2, ETR1, ETR2 and EIN4 which are all interrelated in function (Bleecker et al., 1988; Chang et al., 1993; Sakai et al., 1998).

The ethylene receptors consist of disulphide linked dimers, each of which is capable of binding an individual ethylene unit. Binding itself is facilitated by a copper I cofactor present within the 3-N-terminal transmembrane active site of all receptors. Binding is thought to produce an allosteric change in the shape of the receptor which results in inactivation of the ethylene receptor-constitutive triple response CTR complex (Rodrıguez et al., 1999). Ethylene binding releases a second message which acts in the nucleus, resulting in transcription of new mRNA material which is followed by modification of polyribosome to produce polypeptides that are eventually converted to a new enzyme protein. Proteins coded by this m-RNA are responsible for the activation of enzymes that lead to ripening and senescence (Sisler et al., 1983; Watkins, 2002).

The number of genes associated with the production of ethylene receptors in various fruit and vegetables include six genes in tomato (*Lycopersicon esculentum*) (Klee, 2002), and three in cucumber (*Cucumis sativus*) (Yamasaki et al., 2000), pear (El-Sharkawy et al., 2003) and passionfruit (*Passiflora edulis*) (Mita et al., 1998).

### 3.2.3 Sources and impact of exogenous ethylene

Ethylene is not only produced by normal metabolism of fruit and vegetables but can also arise as a defence response to the action of external stimuli such as attack from microorganisms growing on the plant (Dangl & Jones, 2001). In addition, atmospheric ethylene is derived from a variety of sources including aircraft and motor emissions, cigarette smoke and various industrial activities (Morgott, 2015). The rapid diffusion of ethylene into plant tissues means that exogenous ethylene is as effective as endogenous ethylene in modifying metabolic and physiological behaviour. Ethylene contamination can also arise as

a consequence of the mixed transportation or storage of produce (i.e. high ethylene emitting produce stored with ethylene sensitive produce (Yahia et al., 2011).

### 3.2.4 Inhibiting ethylene action

Inhibition of ethylene action in the senescence process may be approached in a number of ways including (i) competitive inhibition of the ethylene receptor, (ii) displacement of the copper (I) cofactor by a metal of similar electronic character, (iii) scrubbing of atmospheric ethylene through chemical or physical means, or (iv) alteration of signalling pathways or chemical steps associated with ethylene biosynthesis.

#### 3.2.4.1 Competitive inhibition

A range of compounds have been found to competitively inhibit the activity of ethylene, but most interest in recent years has been with 1-methylcyclopropene (1-MCP). The beneficial effects of 1-MCP on ethylene action was pioneered by Sisler and colleagues, with U.S. patent protection granted to Sisler and Blankenship (1996). There are many reviews of 1-MCP including by Watkins (2015), which collate many papers finding that 1-MCP is effective at low part per million concentrations for a few hours to inhibit the action of ethylene-in many commodities. The structural similarity between ethylene and 1-MCP allows it to bind irreversibly to ethylene receptors and thus act as a highly effective inhibitor of ethylene. 1-MCP has been found to be effective in delaying a range of senescence characteristics such as respiration rate, synthesis of plant volatiles, tissue softening, loss of acidity and chlorophyll degradation and chilling injury (Watkins, 2015). There are limitations to the use of 1-MCP as its effects are not uniform across all produce with variation between cultivars and stage of maturity of produce, for example with avocado (Hershkovitz et al., 2005), banana (Golding et al., 1998) and strawberry (Jiang et al., 2001).

#### 3.2.4.2 Displacement of the copper (I) cofactor by a metal

Beyer (1976) extended the vase life of cut flowers with application of silver (I) ions (as a nitrate or thiosulfate salt solution) to the vase solution, with the mode of action proposed to be through displacement of the Cu (I) cofactor by $Ag^{+}$ ions. Both elements belong to the same transition metal group, have similar oxidation behaviour and possess the ability to form complexes with ethylene. The difference in ionic radius between copper I and silver I is considered give rise to a different allosteric effect so that silver fails to initiate the cascade of events leading to senescence. However, use of the treatment on edible produce is not feasible due to toxicity issues associated with silver. Environmental concerns surrounding the use of

silver dipping solutions in the cut flower industry have also led to the practice falling from favour in many countries (Olivier et al., 1998).

#### 3.2.4.3 Scrubbing atmospheric ethylene by chemical or physical methods

The simplest technology to reduce the concentration of atmospheric ethylene surrounding stored horticultural produce is by ventilation of the storage container with ambient air which has a very low ethylene concentration**,** usually <0.01 µl/L (Warton et al., 2000). Wills et al. (2012) showed that ventilation with ambient air was effective in removing endogenous ethylene from bananas packed in cartons during simulated transport, thereby delaying ripening during carriage to market. The technique was used in Australia in the 1950s for the long-distance transport of bananas in louvered rail vans where the moving van stimulated ambient air to flow across the bananas (Hicks, 1957) but fell from favour with the advent of refrigerated trucks.

A range of chemical and physical techniques have also been utilised to remove atmospheric ethylene from around produce. Early investigations focused on adsorption techniques, utilising high surface area materials such as activated charcoal, brominated charcoal and diatomaceous earth (Pratt & Goeschl, 1969). Success, however, proved limited because of insufficient of ethylene adsorption capacity. More recently, the use of metal catalysts (such as 1% palladium adsorbed into activated carbon) has been applied to improve the efficiency of carbon-based reactivity (Martínez-Romero et al., 2007). Other innovative technologies such as halloysite nanotubes (HNTs) have also been utilised. Incorporated into packaging materials, the surface and structural characteristics of these materials allows for the adsorption of ethylene and the lowering of atmospheric oxygen and water vapour transmission rates to increase the shelf life of produce (Tas et al., 2017).

Potassium permanganate ($KMnO_4$) is a strong oxidising agent that reacts with ethylene to yield carbon dioxide and water (Hodgkinson, 1970). The first reported use of $KMnO_4$ adsorbed onto an inert substrate to control atmospheric ethylene levels in storage was by Scott et al. (1970) who showed that bananas stored in sealed polyethylene bags had significantly lower ethylene concentrations and exhibited delayed ripening. Potassium permanganate is now widely used to reduce ethylene around produce with many commercial products available.

Ozone ($O_3$) is an allotropic form of atmospheric oxygen ($O_2$) and has high reactivity including the oxidation of ethylene (Roshchina & Roshchina, 2013). Ozone can be generated by passing a high voltage through an oxygen atmosphere to yield highly reactive oxygen

radicals ($O^{\bullet}$) which rapidly combined with molecular oxygen ($O_2$) to form ozone ($O_3$). However, care is required as ozone is highly toxic with a permitted limit of human exposure of 0.1 µL/L (Tiwari & Muthukumarappan, 2012). A range of ozone generators are now available to control ethylene in fruit and vegetable storage chambers (Skog & Chu, 2001).

### 3.2.5 Biochemical disruption to ethylene synthesis

The conversion of SAM to ACC in the Yang cycle can be disrupted by a range of compounds. Two compounds that were of considerable interest were aminooxyacetic acid (AOA) and aminoethyoxyvinylglycine (AVG). AVG blocks the conversion of SAM to ACC by inhibiting the coenzyme pyridoxal phosphate (PHP) which is required for the activity of ACS (Adams & Yang, 1979). AOA is a specific inhibitor of ACS that reacts with enzyme cofactor pyridoxal 5′-phosphate to form a stable oxime which deactivates ACC synthase through competitive inhibition (Hu et al., 2013). While this limits endogenous ethylene production, such treatments are ineffective against the effects of exogenous ethylene (Morgan & Drew, 1997). In addition, both of these compounds have been shown to have potential toxicity on humans and have been withdrawn from commercial use on fresh produce.

While ethylene was long considered the only gaseous signalling molecule in plants, a range of new gaseous signalling agents have recently been discovered in mammals and were even more recently identified as being synthesised in plants and of having an effect on wide range of metabolic systems. Of particular interest to this study is the role of nitric oxide (NO) and hydrogen sulphide ($H_2S$) in inhibiting senescence and these gases are examined in the following sections.

## 3.3 Nitric Oxide

Nitric oxide is a highly reactive, free radical gas that has traditionally been considered as an industrial pollutant (Flagan & Seinfeld, 2012), but this dramatically changed in the 1990s. The principal function of NO in human physiology is now considered to be as an endothelium-derived relaxing factor (EDRF), which controls the dilation of human arteries, a key factor in regulating blood pressure and blood distribution. NO is also involved in the regulation of diverse physiological and pathophysiological mechanisms in cardiac, nervous and immunological system (Mayer & Hemmens, 1997).

The first reported study on NO and plants was by Churchill and Klepper (1979) who identified endogenous NO emissions from growing soybean plants. However, the impetus to postharvest applications for NO was by Leshem and Haramaty (1996) who demonstrated that

endogenous NO production increased in pea seedings subjected to stress conditions. They also added a NO-donor compound and found greater emission of NO than ethylene but when the ethylene precursor ACC was added both NO and ethylene emissions increased. This led to the suggestion that NO could regulate ethylene production in plants.

The first study on effects of NO on postharvest produce was by (Leshem et al., 1998) who showed that short-term NO fumigation of strawberry, broccoli, cucumber, Chinese broccoli, kiwi fruit and mushroom extended postharvest life. This extension in postharvest life by NO was confirmed by Wills et al. (2000) with strawberry and Soegiarto and Wills (2004) with the leafy vegetables broccoli, green beans and pak choy.

The ability of NO to inhibit senescence and delay fruit ripening has now be shown for a wide range of commodities. Wills (2015) in a review states that while NO inhibits a wide range of factors that lead to senescence or ripening, a key role is as a signalling molecule to regulate endogenous ethylene biosynthesis. For example, Zaharah and Singh (2011) found that mangoes treated with NO experienced downregulated ACS and ACO activity, which in turn reduced levels of ACC. However, NO seems to also act independently on other metabolic systems, for example, Millar and Day (1996) showed that NO can inhibit the action of cytochrome oxidase in plant mitochondria.

### 3.4 Hydrogen sulphide

Hydrogen sulphide is a flammable, colourless, toxic gas with a characteristic odour of rotten eggs (Daldal et al., 2010). Natural sources of $H_2S$ in the atmosphere are diverse and include volcanic and geothermal emissions (Aneja et al., 1982), emissions from coal gasification plants and waste water treatment facilities (Olafsdottir et al., 2014) .

#### 3.4.1 Action of hydrogen sulphide

Even more recently than NO, was identification of $H_2S$ as a signalling agent in mammals (Wang, 2002) and it has now been found to control range of physiological conditions. In general, there are many protective effects of the $H_2S$ including neurons against hypoxic injury, in the respiratory system via decreased lung injury and pulmonary oedema and infiltration of polymorphonuclear cells (Grommes & Soehnlein, 2011). $H_2S$ also has anti-inflammatory action against bacteria in the colon and relaxing the muscles in the colon and intestines (Linden, 2014), vasodilatory action on blood vessels (Bhatia, 2005). $H_2S$ also has a protective role in the kidney through decreased blood pressure (Olson, 2011).

Many papers have now been published to show that $H_2S$ is involved in a range of plant physiological processes including plant growth, germination, stomatal movement and alleviating environment stress. Factors of potential relevance to postharvest behaviour include enhanced chloroplast development (Chen et al., 2011), enhanced abscisic acid receptor expression (Jin et al. (2011), maintenance of plasma membrane integrity (Zhang et al. 2008; 2010) and elevated activity of antioxidant enzymes (Zhang et al. 2010).

The first report on the postharvest action with horticultural produce of $H_2S$ was by Zhang et al. (2011) who used a solution of NaHS as the donor source of $H_2S$, finding that it found delayed senescence in a range of cut flowers. This led to increasing interest in the effect of $H_2S$ on fruit and vegetables, with many journal articles published since the start of this study. Hu et al. (2012) showed that exogenous $H_2S$ gas reduced the incidence of rot, lowered respiration and improved the antioxidant action and firmness of strawberry fruits and prolonged the shelf life of mulberry fruit (Hu et al. 2014), while Zhu et al. (2014) established that $H_2S$ fumigation maintained membrane integrity of kiwifruit during storage. $H_2S$ has also been found inhibit growth of *Aspergillus niger and Penicillium expansum* in pear fruit (Hu et al., 2014), to reduce browning in fresh-cut lotus root by inhibition of oxidative damage (Sun et al., 2015) and to decrease senescence in broccoli (Li et al., 2014). $H_2S$ has been found to regulate the expression of six genes associated with chlorophyll degradation in water spinach (Hu et al., 2015), and upregulation of one gene and downregulation of five genes associated with senescence onset in grape and apple slices (Ni et al., 2016; Zheng et al., 2016).

Most papers on the postharvest effects of $H_2S$ have assessed produce characteristics that are associated with senescence but only a few studies have controlled or even measured the concentration of ethylene around produce, let alone examining the relationship between $H_2S$ and ethylene. Li et al. (2016) found that treatment with $H_2S$ reduced chilling injury in banana and it was associated with a decreased ethylene production.

Zheng et al. (2016) found that treatment of fresh-cut apple slices with $H_2S$ regulated four ethylene biosynthesis genes and six signal transduction genes while Ge et al. (2017) sequentially treated banana daily with both ethylene and $H_2S$ finding this combined treatment decreased oxidative stress compared to bananas treated with ethylene only. Treatment also inhibited endogenous ethylene production by downregulating the expression of four ethylene-related genes, *MaACS1*, *MaACS2*, *MaACO1* and pectate lyase *MaPL*, as well enhancing the expression of two ethylene receptor genes, *MaETR* and *MaERS1*.

### 3.4.2 Metabolism of $H_2S$ in plants

Most of the $H_2S$ production in the plants and animals is derived from cysteine (2-amino-3-sulphanylpropanoic acid), which is unusual in that it exists as both the L- and D-enantiomers (Prabhakar, 2012). In the early 1990's, $H_2S$ production in mammals was shown to arise by the action of four PLP dependent enzymes: catabolism of cysteine by desulfuration reaction via cystathionine-γ-synthase (CSE); transamination by cysteine aminotransferase (CAT); substitution of the thiol group in L-cysteine by cystathionine-ß- synthase (CBS) (Kimura et al., 2015; Stipanuk, 2004); or through a novel pathway which involves conversion to D-Cysteine by D-amino acid oxidase (DAO) (Huang et al., 1998). In addition, $H_2S$ production can also occur via the action of 3-mercaptopyruvate sulfurtransferase (3MST), which transports sulphur from 3-mercaptopyruvate to hydropersulfide (MST-SSH) followed by a non-enzymatic reaction that results in the release of $H_2S$ from the active site of MST-SSH (Kimura et al., 2015; Stipanuk, 2004).

The pathways for metabolism of $H_2S$ in plants is summarised in Figure 3.3. and encompasses the transformation of inorganic forms of sulphide from the soil through to the organic form of cysteine via a reducing enzyme. The initial step of cysteine production involves conversion of L-serine by serine acetyltransferase (SAT) and O-acetyl serine (thiol) lyase (OASTL) (Takahashi et al., 2011). There is general agreement (Jin & Pei, 2015; Papenbroak et al., 2007; Rennenberg et al., 1987) that L- and D-cysteine are catalysed by different enzymes. According to Papenbroak et al. (2007), L-cysteine is catalysed by cysteine desulfhydrase (L-CD) while Rennenberg et al. (1987) claimed D-Cysteine is metabolised by D-cysteine desulfhydrase (D-CD). However, recently, Jin & Pei (2015) reported D-cysteine desulfhydrase 2 (D-CD2) degrades both cysteine enantiomers simultaneously. Figure 3.3 also shows that cysteine metabolism is linked to the synthesis of ethylene and the antioxidant glutathione (Romero et al., 2014).

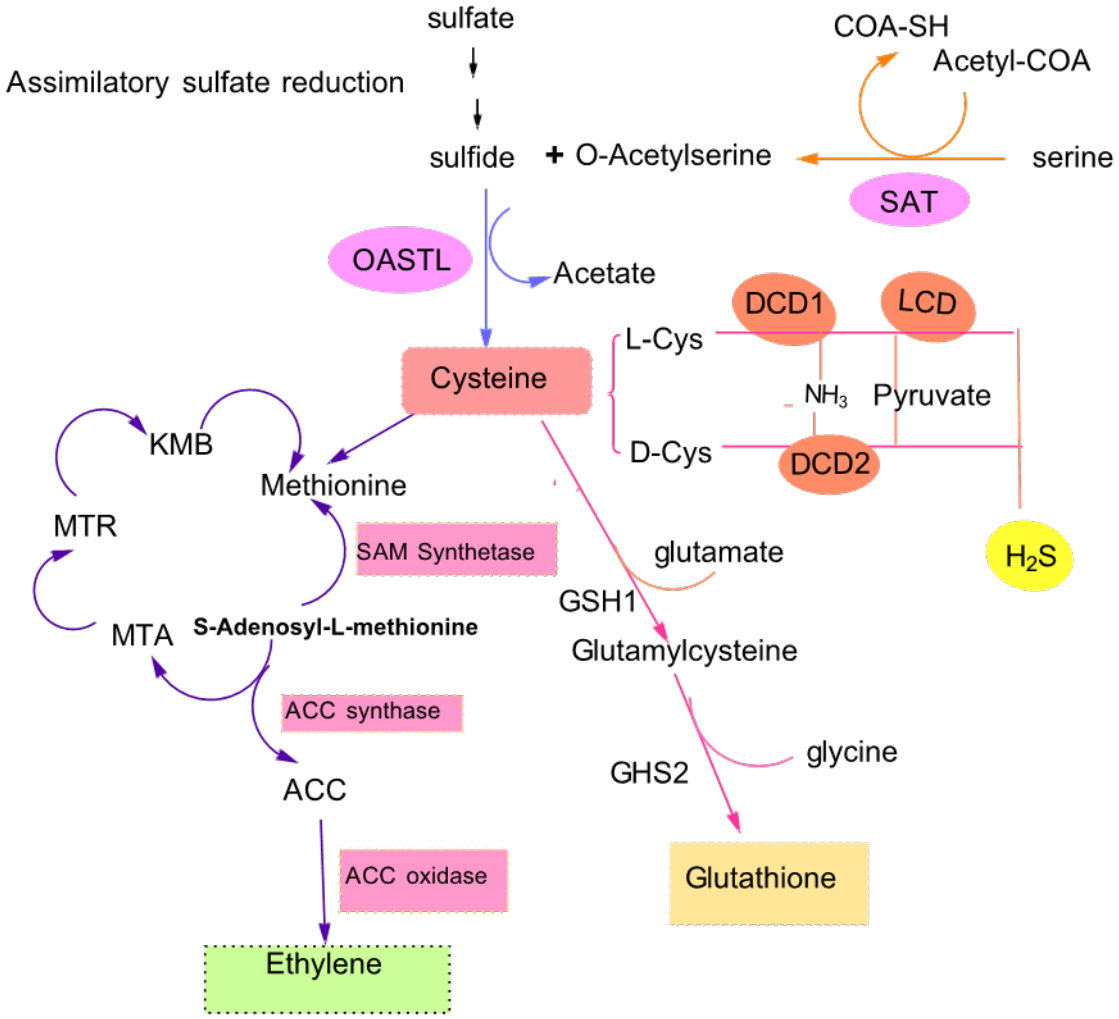


Figure 3.3 Biosynthesis and metabolism of cysteine in plants (Romero et al., 2014)

### 3.4.3 Inhibition of $H_2S$ activity with L-propargylglycine (PAG)

Many compounds have been found to inhibit the synthesis of the $H_2S$ in mammals including L-propargylglycine which interacts with catalytic action of the PLP-dependent enzyme CSE (Qu et al. 2006; Di Salvo et al., 2011). The mode of action of L- propargylglycine has been suggested as irreversibly reacting with the PLP cofactor of CSE (Sun et al., 2009).

However, research studies invariably apply DL-propargylglycine which we will designate as PAG. As with other aspects of $H_2S$ research, studies on PAG were conducted with mammalian cell culture studies and found to inhibit the production and physiological impact of $H_2S$ (Fiorucci et al., 2005; Sun et al., 2009). PAG was first examined in preharvest produce. García-Mata and Lamattina (2010) showed that PAG treatment of *Vicia faba* and *Arabidopsis thaliana* inhibited L-cysteine desulfhydrase activity resulting in reduced $H_2S$ production which partially blocked ABA-dependent stomatal closure. A similar finding was obtained by Lai et al. (2014) but it also blocked the effects of NaCl on Super oxide dismutase (SOD) or Catalase (CAT) activity in alfalfa seedling roots.

Studies on the effect PAG on postharvest quality was reported by Hu et al. (2015) for water spinach (*Ipomoea aquatica*) with PAG increasing yellowing, while fumigation with $H_2S$ after spraying with PAG only protecting chlorophyll degradation by inhibiting the action of chlorophyll degrading enzymes such as chlorophyllase. Li et al. (2014) found the exogenous $H_2S$ treatment of broccoli florets treated with PAG directly lowered LCD and DCD activities, thereby reducing endogenous $H_2S$ levels leading to accelerated senescence. Liu et al. (2017) found similar effects in daylily flowers, with treated with PAG decreasing vase life through aggravated lipid peroxidation and lowered energy status.

### 3.4.4 Methods for application of $H_2S$

In the air, $H_2S$ has a half-life of 12-37 hours depending on atmospheric conditions. Unlike NO, it is thus feasible to expose postharvest produce to atmospheric $H_2S$ over a reasonable period of time.

The first application of $H_2S$ gas to horticulture produce was reported by Thompson and Kats (1978) who diluted a $H_2S$ gas stream with nitrogen which was then injected into a carbon-filtered air stream. However, few researchers have used a diluted gas stream due to problems in controlling the concentration of gas and toxicity concerns (Zhao et al., 2014).

The most used method for the controlled generation of small quantities of $H_2S$ gas is with inorganic sulphide salts such as sodium sulphide ($Na_2S$) or sodium hydrogen sulphide

(NaHS). Both salts, when treated with water, quantitatively and immediately released one mole equivalent of $H_2S$ gas, making them an ideal method of *in situ* gas generation, The use of sulphide salts in mammalian studies was introduced by Kruszyna et al. (1985) and with plants by Zhang et al. (2011).

4-methoxyphenyl morpholino-phosphinodithioate acid (GYY4137) is one of a new generation of highly water soluble (>1 mg/mL at pH 7.4) (Rose et al., 2015) organic $H_2S$-releasing compounds that were developed to treat a range of medical conditions in humans (Caliendo et al., 2010). GYY4137 has been utilised in a range of biomedical applications where high water solubility and slow release $H_2S$ characteristics are desirable (Hartle & Pluth, 2016; Wallace, 2007). The main advantage of organic-based $H_2S$ releasing agents over inorganic sulphide salts is the more favourable release kinetics, which allows a constant, low- level release of $H_2S$ over an extended time period. Release kinetics from GYY4137 are pH sensitive, meaning gas release can be further controlled (Lee et al., 2011). However, the application of organic $H_2S$ donors such as GYY4137 in plant physiology is limited. Lisjak et al. (2010, 2011) compared the action of GYY4137 and NaHS in plants, finding that both produced stomatal opening in the light and blocked stomatal closure in the dark in *Arabidopsis thaliana* and *Capsicum annum* and caused a similar inhibition of abscisic acid induced aggregation of NO and interrupted NO signalling in the leaf.

### 3.5 Use of cysteine as a postharvest treatment

L-Cysteine is a semi-essential amino acid and has Generally Regarded as Safe (GRAS) status which has allowed USDA approval as an additive to baking flour (USDA, 2017). While D-cysteine does not currently have GRAS status, it is a naturally occurring non- proteogenic amino acid, with a similar toxicological profile of 500 mg/kg/day (Shibui et al., 2017).

Research into the application of L-cysteine to postharvest produce has mainly been with fresh-cut produce. Many studies have reported L-cysteine alone or in combined with other compounds as an effective anti-browning agent on a range of fresh cut produce. According to Sharma and Rao (2013), the rationale for such use is due to cysteine being known as an anti-browning agent as it can inhibit peroxidase and polyphenol oxidase (PPO) activity and found it extended the shelf life due to reduced weight loss and flesh browning and total phenolic content with increased TSS and TA. Bico et al. (2009) used an L-cysteine dip to increase the shelf life of banana slices, while Gorny et al. (2002) reported dipped Bartlett pear slices had increased storage life through inhibited browning but the flavour was

not acceptable. Inhibition of browning has also been reported for fresh-cut peach (Colelli et al., 2013), fresh-cut avocado (Dorantes-Alvarez et al. (1998) and apple (Rojas-Grau et al., 2006). Guerrero-Beltran et al. (2005) also decreased browning in Mango puree with a reduced activity of PPO.

The only reported study of L-cysteine on whole fruit and vegetables was by Ali et al. (2016) with litchi fruit. They found inhibition of aril breakdown (observed as tissue browning) with a better retained level of antioxidant activity and a range of other senescence related parameters such as total phenol content, ascorbic acid, ion leakage and catalase and superoxide dismutase activity. There was also reduced activity of peroxidase (POD) and polyphenol oxidase (PPO) enzymes.

No published information was found on the use of D-cysteine or DL-cysteine on Foods.

### 3.6 Pak choy

Most of the research conducted in this thesis was with the non-climacteric green leafy vegetable, Pak choy (*Brassica rapa var. Chinesis*), also known as bok or pok choy, choi or tsoi.

Figure 3.4 Non- climacteric green leafy vegetable pak choy

Pak choy is grown widely in south-east and East Asia but nowadays is commonly available in most western countries. Leaf yellowing is primary major cause of senescence of pak choy after harvest (Able et al., 2003) although quality loss can occur through wilting, rotting and browning as a consequence of postharvest handling and storage (Kader, 2002).

Pak choy is often considered to have a low sensitivity to exogenous ethylene (Able et al., 2005; Luo, 2016). However, Li et al. (2017) found that decreasing the concentration of

ethylene around produce from 0.1 down to 0.001 µL/L at a temperature from 0 – 20 oC will progressively generate an increase in market life of pak choy. That pak choy is sensitive to ethylene was reinforced by Able et al. (2003) who fumigated a range of Chinese vegetables with 1-MCP in the presence of a controlled level of ethylene and found reduced yellowing in pak choy, Chinese mustard, choy sum and garland chrysanthemum.

# 4. Papers

## 4.1 Effect of $H_2S$ and ethylene on senescence

H.M.S. Al Ubeed, R.B.H. Wills, M.C. Bowyer, Q.V. Vuong, J.B. Golding. Interaction of exogenous hydrogen sulphide and ethylene on senescence of green leafy vegetables. Postharvest Biology and Technology (2017) 133, 81-87. https://doi.org/10.1016/j.postharvbio.2017.07.010

### General background

At commencement of the study, the scientific literature provided limited information about the effect of $H_2S$ on the postharvest senescence of leafy vegetables in the presence of exogenous ethylene. Therefore, a study was conducted to determine the effect on a range of factors associated with senescence of pak choy after fumigation with $H_2S$ at a range of concentrations during storage at 10 °C in air that contained a range of ethylene concentrations. Physiochemical factors assessed were market life (time for loss of green colour), ethylene production, ion leakage, weight loss, total phenolic compounds, antioxidant activity and ascorbic acid. At the conclusion of the study on pak choy, the effect of fumigation with $H_2S$ on senescence of kale and basil was investigated to ascertain if the effect was applicable to other leafy vegetables.

### Summary of Results

Ventilating pak choy with decreasing concentrations of ethylene increased the market life by about 50% when the concentration was reduced from 1 to < 0.001 µL/L. Fumigation of pak choywith 50 and 100 µL/L $H_2S$ showed some inhibition of the effect of ethylene but 250 µL/L $H_2S$ was found to be the optimal concentration to increase market life and reduce the respiration rate. Fumigation with 250 µL/L $H_2S$ also reduced ethylene production, chlorophyll loss, weight loss, ion leakage and total phenolic compounds and enhanced the antioxidant activity as measured by FRAP and DPPH.

Fumigation of kale and basil leaves with 250 and 100 µL/L $H_2S$ found 250 µL/L caused damage to leaves especially with basil. Consequently, 100 µL/L $H_2S$ was considered the optimal concentration for these leafy vegetables and such treatment was found to inhibit senescence, as expressed by increased market life and reduced respiration rate.

**Conclusions**

The action of $H_2S$ in affecting all facets of senescence raises the question as to whether $H_2S$ acts on each factor or with a single factor that triggers the other changes. Ethylene could be the controlling factor for all changes as $H_2S$ inhibited the production of ethylene one day after application. This contrasts with the reduction in respiration, ion leakage and total phenols and increase in antioxidant activity which were observed after some days of storage. Given the effect of $H_2S$ increasing the market life of pak choy, kale and basil, fumigation with $H_2S$ would seem to have potential commercial benefit for many leafy vegetables.

A copy of the paper, published in Postharvest Biology and Technology is appended

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# Interaction of exogenous hydrogen sulphide and ethylene on senescence of green leafy vegetables

H.M.S. Al Ubeed[a,b], R.B.H. Wills[a,*], M.C. Bowyer[a], Q.V. Vuong[a], J.B. Golding[a,c]

[a] School of Environmental and Life Sciences, University of Newcastle, Ourimbah NSW 2558, Australia
[b] Department of Life Sciences, Faculty of Science, Al-Muthana University, Al-Muthana, Iraq
[c] NSW Department of Primary Industries, Ourimbah NSW 2258, Australia

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ABSTRACT

Hydrogen sulphide ($H_2S$) gas has been found to delay the appearance of many characteristics associated with senescence of horticultural produce but little attention has been given to its physiological role or its interaction with ethylene. This study used the green leafy vegetable, pak choy (*Brassica rapa* subsp. *Chinensis*) as the principal test commodity and examined the interaction of fumigation with hydrogen sulphide and storage at 10 °C in the presence of controlled levels of ethylene on endogenous ethylene production and a range of factors associated with postharvest deterioration. It was found that hydrogen sulphide inhibited ethylene production, chlorophyll loss, respiration, weight loss, various antioxidant factors and ion leakage. Hydrogen sulphide also inhibited chlorophyll loss and respiration of other green leafy vegetables/herbs, sweet Italian basil (*Ocimum basilicum*) and green curly kale (*Brassica oleracea* var. *sabellica)*. The results suggest that the mode of action of hydrogen sulphide in delaying senescence could be by inhibiting both the production of ethylene and the action of ethylene. The substantial reduction in the rate of loss of chlorophyll following short-term treatment with hydrogen sulphide may have potential commercial benefit for extension in market life of green leafy vegetables.

## 1. Introduction

Ethylene is well known as a gaseous plant growth regulator that regulates numerous aspects of plant growth and development including promoting senescence and fruit ripening (Abeles et al., 1992; Wills, 2015). A concentration of 0.1 $\mu L\,L^{-1}$ was generally cited as the threshold level for the activity of ethylene on postharvest behaviour (Burg and Burg, 1962; Knee et al., 1985) but this related more to analytical capability than empirical evidence. It has now been shown that a wide range of climacteric and non-climacteric produce are incrementally responsive to ethylene at concentrations greater than 0.001 $\mu L\,L^{-1}$ (Wills et al., 1999, 2001).

In addition to ethylene other gaseous plant growth regulators such as nitric oxide (NO) have now been identified. Nitric oxide has been shown to extend the postharvest life of horticultural commodities (Leshem and Haramaty, 1996; Leshem et al., 1998) with the proposed mode of action being through interaction with ethylene. Nitric oxide has been reported to regulate endogenous ethylene production through suppression of pathways linked to ethylene synthesis and by direct stoichiometric inhibition (Manjunatha et al., 2012).

Another more recently identified gaseous plant growth regulator is hydrogen sulphide ($H_2S$) (Jin and Pei, 2015). Hydrogen sulphide has been linked to diverse plant physiological functions such as germination, stomatal movement, root development and flower senescence (Hancock and Whiteman, 2016). Recognition of the role of hydrogen sulphide as a regulator of postharvest senescence is quite recent. Zhang et al. (2011) reported delayed senescence in cut flowers and shoot explants treated with solutions of the hydrogen sulphide donor sodium hydrogen sulphide (NaHS). Fumigation with hydrogen sulphide was subsequently extended to postharvest fruit and vegetables with an extension in storage life achieved through inhibition of a wide range of senescence characteristics. Horticultural produce that have been shown to benefit from treatment with exogenous hydrogen sulphide include strawberry (Hu et al., 2012), broccoli, (Li et al., 2014), peach (Wang et al., 2014), kiwifruit (Zhu et al., 2014), pear (Hu et al., 2014b), mulberry (Hu et al., 2014a) sweet potato (Tang et al., 2014), lotus root (Sun et al., 2015), water spinach (Hu et al., 2015), grape (Ni et al., 2016) and apple (Zheng et al., 2016). Despite this plethora of recent studies, little work has examined the physiological role of hydrogen sulphide in postharvest behaviour and given the established link between ethylene and postharvest senescence, little attention has been given to understanding the interaction of hydrogen sulphide and

* Corresponding author.
*E-mail address:* Ron.Wills@newcastle.edu.au (R.B.H. Wills).

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ethylene. Li et al. (2014) and Zheng et al. (2016) did show that exogenous hydrogen sulphide treatment of broccoli florets and apple slices, respectively, down regulated the expression of genes associated with ethylene biosynthesis. However, these and other published studies did not monitor the concentration of ethylene around produce following exposure to hydrogen sulphide, nor maintain different levels of ethylene around produce during storage. In this study, we have used the green leafy vegetable, pak choy as the principal test commodity and examined the interaction of fumigation with hydrogen sulphide and storage at 10 °C in the presence of controlled levels of ethylene on postharvest deterioration.

Pak choy (*Brassica rapa* subsp. *Chinensis*) (also known as bok or pok choy, choi or tsoi) is widely grown in south and east Asia but is now also available in most Western countries. As with many green leafy vegetables, quality is dependent on leaves retaining their green colour and there being no other visible sign of deterioration such as wilting, rotting or browning (Able et al., 2005). It is commonly claimed (Anon, 1989; Able et al., 2005; Luo, 2016) that pak choy is not particularly sensitive to ethylene but Li et al. (2017) showed that any reduction in the atmospheric ethylene concentration in the range of 1.0 to 0.001 μL $L^{-1}$ at temperatures from 0 to 20 °C resulted in a substantial increase in market life. The experiments in this study report on the interaction of hydrogen sulphide in the presence of continuous ethylene in the storage of pak choy. The effect of hydrogen sulphide on ethylene production and quality factors associated with senescence of non-climacteric produce such as chlorophyll retention, respiration, weight loss, various antioxidant factors and ion leakage were assessed. In addition, the effect of hydrogen sulphide on chlorophyll retention and respiration of two other green leafy herb vegetables, sweet Italian basil (*Ocimum basilicum*) and green curly kale (*Brassica oleracea* var. *sabellica),* was also examined.

## 2. Materials and methods

### 2.1. Produce and experimental designs

#### 2.1.1. Produce

Pak choy plants (cv. 'Shanghai') were harvested from a local commercial farm at Mangrove Mountain, NSW and transported to the laboratory within two hours. All plants selected for each experiment were of uniform size (10 cm length) and colour and without damage to leaves or stem. Pak choy heads were cut and a specific number of outside leaves (the number varying between different experiments) were selected and gently cleaned with tap water. The leaves from each head were randomly distributed into the required number of treatment units each containing the same weight of produce. All experiments were replicated by obtaining batches of plants on three separate occasions with at least two weeks between batches.

Fresh cut basil and kale plants were obtained from a local market. All the leaves selected for an experiment were uniform in size and colour and without damage and similarly distributed into treatment units as for pak choy.

#### 2.1.2. Effect of ethylene concentration

Each treatment unit consisted of eight leaves (about 150 g) from different pak choy heads placed into a 4 L plastic container that was fitted with inlet and outlet ports in the lid. Containers were placed into a temperature controlled cabinet at 10 °C and polyethylene tubing (5 mm diam.) was connected to the inlet port through which flowed humidified air containing four concentrations of ethylene. The experiment comprised 12 containers and groups of three containers were respectively ventilated with 1, 0.1, 0.01 and < 0.001 μL $L^{-1}$ ethylene at 45 mL $min^{-1}$. The < 0.001 μL $L^{-1}$ concentration can be considered as ethylene-free as the analytical limit of detection was 0.001 μL L. The desired concentrations of ethylene were achieved by mixing compressed air that was made ethylene-free by passing through a tube filled with potassium permanganate adsorbed onto alumina pellets with a regulated flow of ethylene from a cylinder (1 mL $L^{-1}$ ethylene in air, BOC Gases, Sydney). The gas mixtures were humidified by bubbling through water in a 2 L glass jar (height 225 cm) to ensure a high humidity of 97–99% RH was maintained in the gas stream to minimise water loss. The ethylene concentration in gas streams was monitored at regular intervals at the inlet port with a gas sample (1 mL) that was analysed by flame ionization gas chromatography as described by Huque et al. (2013). Leaves in each unit were visually assessed daily for green colour and the time for each unit to develop an unacceptable colour (denoted as the market life) was determined using the scoring scale given in Section 2.2.1.

#### 2.1.3. Effect of hydrogen sulphide concentration

Similar as described above, a treatment unit consisted of eight leaves from different pak choy heads with the experiment comprising a total of 24 containers. The 4 L containers were placed at 10 °C and after two hours, groups of three units were fumigated with hydrogen sulphide vapour at 0, 50, 100 and 250 μL $L^{-1}$. Hydrogen sulphide gas at the desired concentration inside a sealed container was generated *in situ* by placing the required weight of solid $NaHS{\cdot}H_2O$ into a dry beaker that was sealed in a container along with the produce to be treated. Water (2 mL) was then injected into the beaker through a septum in the container lid to generate the hydrogen sulphide. Using this method, the liberation of hydrogen sulphide gas has been shown to be quantitative and immediate (Zhao et al., 2014). The containers remained sealed for 4 h after which time the containers were ventilated with ethylene-free air (< 0.001 μL $L^{-1}$) or 0.1 μL $L^{-1}$ ethylene at 45 mL $min^{-1}$. Leaves in all units were visually assessed daily for green colour and the respiration rate of units was analysed by gas chromatography at periodic intervals during storage.

Basil and kale were similarly evaluated for the effect of hydrogen sulphide but only with three concentrations (0, 50 and 100 μL $L^{-1}$). A preliminary experiment showed that a concentration of 250 μL $L^{-1}$ damaged the leaves, particularly of basil. Damage symptoms were brown-black patches typical of chemical injury. A treatment unit consisted of six plant leaves with a total unit weight of about 125 g. Leaves were assessed for green colour and respiration rate as previously described.

#### 2.1.4. Effect on ethylene production

The effect of hydrogen sulphide on ethylene production of pak choy was examined by fumigating three 4 L containers of pak choy (150 g) with 250 μL $L^{-1}$ hydrogen sulphide with three control containers. Containers were then stored at 10 °C in and ventilated with ethylene-free air or air containing 0.1 μL $L^{-1}$ ethylene. At periodic intervals, the containers were sealed and a gas sample (1 mL) was immediately collected in a syringe and analysed for ethylene concentration by gas chromatography. After two hours, another gas sample was collected and analysed for ethylene concentration. The difference in the two readings was used to calculate the rate of ethylene production for each unit of pak choy. After three and six days, produce were visually assessed for green colour and weight loss.

#### 2.1.5. Evaluation of changes in physio-chemical attributes

Analysis during storage of a number of physio-chemical factors required a larger sample size of pak choy. In these experiments, a total of 36 treatment units comprising 30 leaves (about 400 g) from different pak choy heads were placed in 4 L containers which were stored at 10 °C. Half the containers were sealed and fumigated with hydrogen sulphide (250 μL $L^{-1}$) for four hours as detailed above. The remaining 18 containers were sealed for 4 h and became the control treatment. After four hours, all containers were ventilated with humidified air containing 0.1 μL $L^{-1}$ ethylene at 45 mL $min^{-1}$. Eight leaves in each treatment unit were randomly assigned to be visually assessed daily for leaf colour throughout the storage period while 12 leaves were analysed

after three days at 10 °C for chlorophyll, carotenoids and ion leakage (five leaves), ascorbic acid (two leaves), total phenols and antioxidant activity (five leaves) using the methods detailed below. The remaining 12 leaves were similarly analysed after six days.

### 2.2. Physio-chemical assessments

#### 2.2.1. Visual leaf colour (market life)

Visual assessment of the progressive change in leaf colour (green to yellow) of individual leaves was conducted daily using a scoring scale of 0–5 where 0 = green, 1 = 10%, 2 = 20%, 3 = 30%, 4 = 50% and 5 = > 70% loss of original green colour (Li et al., 2017). The mean colour score of all leaves in a treatment unit was calculated daily. An average colour score of 3.0 was considered to be the limit of consumer acceptability and the time for leaves to reach a mean score of 3.0 was designated as the market life. Assessment of a unit was terminated when the mean score of 3.0 was attained.

#### 2.2.2. Respiration rate

Respiration, as carbon dioxide production, was measured for each 4 L container. A container was sealed to allow the accumulation of a measureable concentration of carbon dioxide and a gas sample (1 mL) was collected in a syringe after six hours. The concentration of carbon dioxide in the gas sample was determined by injecting into a thermal conductivity gas chromatograph as described by Huque et al. (2013). The respiration rate was calculated as ng carbon dioxide evolved $kg^{-1}\ s^{-1}$.

#### 2.2.3. Ethylene

The concentration of ethylene in the atmosphere was determined by a collecting a gas sample (1 mL) and analysed by flame ionization gas chromatography as described by Huque et al. (2013). Samples were collected at frequent intervals from the gas streams to ensure the desired ethylene concentration was maintained. Samples were also obtained from ventilated treatment units just before sealing the container and after one hour, and the difference between readings was used to calculate the rate of ethylene production as ng $kg^{-1}\ s^{-1}$.

#### 2.2.4. Weight loss

Containers with pak choy leaves were weighed just before being placed into storage at 10 °C and reweighed after a period of storage. The weight difference was calculated as g $kg^{-1}$.

#### 2.2.5. Chlorophyll and carotenoids

Samples for the analysis of chlorophyll and carotenoids were collected as a cross-section of tissue that was cut from the top half (distal) of five leaves after removing the stems. A leaf sample (1 g) was selected and immediately ground using a mortar and pestle with acetone (10 mL) and left at −18 °C for 24 h. The mixture was then blended in the dark using a Vortex mixer for 2 min running on maximum speed and then filtered through filter paper (Whatman Grade 5) into a volumetric flask (25 mL). The filter paper was then washed with small quantities of acetone to reach to required solution volume. The level of chlorophyll *a*, chlorophyll *b* and total carotenoids was determined using a UV–vis spectrophotometer (Varian Australia, Victoria, Australia) to measure absorbance at 662, 644 and 470 nm, respectively and quantified as mg $L^{-1}$ using the conversion factors given by Lichtenthaler (1987).

#### 2.2.6. Ion leakage

The same five leaves that were used for chlorophyll determination were also used to determine the ion leakage using the method described by Lu (2007). This involved collecting two disks (50 mm diameter) with a cork borer from each leaf. The disks were immediately immersed in double distilled water (40 mL) in glass vials which were incubated for two hours at 25 °C. The conductivity of the solution was then measured with a conductivity meter (Model 4071, Jenway, Staffordshire, UK). The solution was then boiled for 15 min and after cooling to room temperature, the total conductivity was re-measured. Ion leakage was calculated as the percentage of the initial to final value.

#### 2.2.7. Ascorbic acid

Ascorbic acid was determined by the indophenol titration method adapted from Nielsen (2003). Two leaves (with the stems attached) from a treatment unit (about 10 g) were weighed and ground using mortar and pestle with metaphosphoric acid-acetic acid solution (20 mL). Metaphosphoric acid-acetic acid solution (5 mL) was added to 2 mL of the ground extract and the mixture filtered through two layers of cheesecloth. The samples were titrated with the 2,6-dichloroindophenol dye solution until a light rose pink persisted for 5 s. A blank titration without pak choy extract was performed as the control. The amount of dye used in the titration was used to calculate the ascorbic acid content.

#### 2.2.8. Total phenolic content

Total phenolic content was determined according to the Folin-Ciocalteu (FC) method as described by Vuong et al. (2013). Two samples of leaf (each about 4 g) were cut from the distal part of five leaves and ground using a mortar and pestle then mixed with 50% methanol (50 mL). The mixture was placed in an ultrasonic bath (Soniclean, Australia) set at 35 °C and 100 W for 30 min. The mixture was filtered through filter paper (Whatman Grade 5) and the solution placed at −20 °C until further analysis. Each extract was analysed for total phenolic compounds. Briefly, diluted extract (1 mL) was added into 10% Folin-Ciocalteau phenol reagent (5 mL) with 7.5% sodium carbonate solution (4 mL) and kept in the dark for 60 min. The absorbance at 765 nm was noted and quantified as gallic acid equivalents (GA) mg $kg^{-1}$ fresh pak choy from a standard curve of gallic acid against absorbance.

#### 2.2.9. Antioxidant activities

The same five leaves, sample selection and extraction procedure used for total phenols were also used for analysis of antioxidant activities. Antioxidant activity was determined using two assays, 2,2-diphenyl-picrylhydrazine (DPPH) and the ferric reducing antioxidant power (FRAP) assay, using the methods described by Vuong et al. (2013) and Thaipong et al. (2006), respectively.

*2.2.9.1. DPPH.* A stock solution of DPPH (24 mg/100 mL methanol) was stored at −20 °C until required. The working solution was freshly prepared by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance at 515 nm of 1.1 ± 0.02. Pak choy extracts (150 μL) were mixed with DPPH solution (2.85 mL) and kept in the dark for one hour. The absorbance at 515 nm was then noted and quantified as mg Trolox equivalent (TE) $kg^{-1}$ fresh pak choy using a standard curve of Trolox against the absorbance.

*2.2.9.2. FRAP.* A working FRAP solution was prepared by mixing 300 mM acetate buffer, 10 mM TPTZ in 40 mM HCl and 20 mM ferric chloride in the ratio of 10:1:1 and warmed at 37 °C in a water bath. Pak choy extracts (150 μL) were allowed to react with the FRAP working solution (2.85 mL) for 30 min in the dark. The absorbance at 593 nm was then noted and converted to mg TE $kg^{-1}$ fresh pak choy from a standard curve of Trolox.

#### 2.2.10. Stomatal status

The appearance of leaf stomates was determined on fully expanded pak choy leaves 1 h after arrival at the laboratory and one hour and 24 h after being placed in the dark at 10 °C. The epidermal peels were detached from the abaxial surface of the leaf with a scalpel and placed on a microscope slide. A drop of methylene blue was added to stain the tissue and the stomates observed under a light microscope fitted with a

**Table 1**
The market life (time to colour score 3) of pak choy ventilated with different ethylene concentrations during storage at 10 °C.

| Ethylene (μL $L^{-1}$) | Market life (days) |
|---|---|
| 1 | 7.3 |
| 0.1 | 10.2 |
| 0.01 | 10.7 |
| < 0.001 | 10.8 |
| *LSD* | *0.45* |

Each value is the mean of nine units (3 batches × 3 replicates). The LSD is at $P = 0.05$.

digital image camera (Leica DM 500 and ICC50 HD, Heerbrugg, Switzerland) at 10–20× magnification.

### 2.3. Statistical analysis

Data were analysed by two-way analysis of variance (ANOVA) and where a significant difference between treatments was found the least significant difference (LSD) of the mean values at P = 0.05 was calculated. Statistical procedures were performed using SPSS for Microsoft version 22.0 software package (SPSS Chicago, IL).

## 3. Results and discussion

### 3.1. Effect of ethylene concentration on leaf colour (market life)

The results presented in Table 1 show that the time for pak choy leaves to lose 30% of green colour intensity (score 3), which was designated as the market life, significantly increased as the concentration of ethylene in the ventilating air stream decreased ($P < 0.001$). There was about a 50% increase in market life as the ethylene concentration in the ventilating air stream was reduced from 1 μL $L^{-1}$ to < 0.001 μL $L^{-1}$. The data confirm the findings of Li et al. (2017) that pak choy is sensitive to ethylene and should be taken into consideration in evaluating the effect of other compounds on postharvest behaviour.

From these results, an ethylene concentration of 0.1 μL $L^{-1}$ in the ventilating air stream was used in subsequent studies as the concentration that would accelerate loss of postharvest quality. In addition, based on a review of the literature, Li et al. (2017) concluded that 0.1 μL $L^{-1}$ was a background ethylene level commonly present in fruit and vegetable environments. The interaction of hydrogen sulphide with 0.1 μL $L^{-1}$ ethylene would therefore have commercial relevance.

### 3.2. Interaction of hydrogen sulphide and ethylene on market life and respiration

Fumigation with hydrogen sulphide significantly increased the market life of pak choy as assessed by visual green colour ($P < 0.001$), with the greatest benefit observed at the highest concentration of hydrogen sulphide, 250 μL $L^{-1}$ (Fig. 1). Overall, ventilation with air containing 0.1 μL $L^{-1}$ ethylene into the storage container significantly decreased market life compared to ventilation with ethylene-free air ($P < 0.001$) but there was a significant interaction between ethylene and hydrogen sulphide concentration ($P < 0.05$). Fig. 1 shows that fumigation with 50 and 100 μL $L^{-1}$ hydrogen sulphide partially mitigated the effect of ethylene while 250 μL $L^{-1}$ hydrogen sulphide had fully overcome the deleterious effect of ethylene on market life.

The respiration rate of pak choy was significantly reduced by fumigation with hydrogen sulphide ($P < 0.001$) and increased by the presence of ethylene ($P < 0.001$). However, there was a significant interaction between ethylene concentration, hydrogen sulphide concentration and storage time ($P < 0.01$). Fig. 2 shows that for pak choy ventilated with 0.1 μL $L^{-1}$ ethylene without exposure to hydrogen sulphide, respiration increased with storage time while pak choy

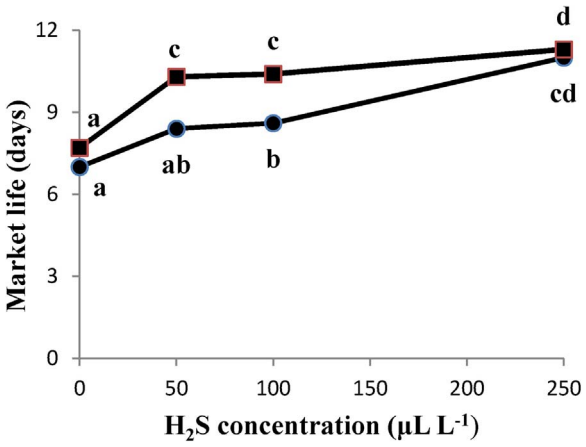


**Fig. 1.** Effect of fumigation with hydrogen sulphide on the market life of pak choy leaves that were subsequently stored at 10 °C and ventilated with air that was ethylene-free (■) or contained 0.1 μL $L^{-1}$ ethylene (●).
Each point is the mean of nine assessments (3 units × 3 batches of produce). The LSD at $P = 0.05$ of 0.87 was applied to the mean values and those not sharing the same superscript are significantly different.

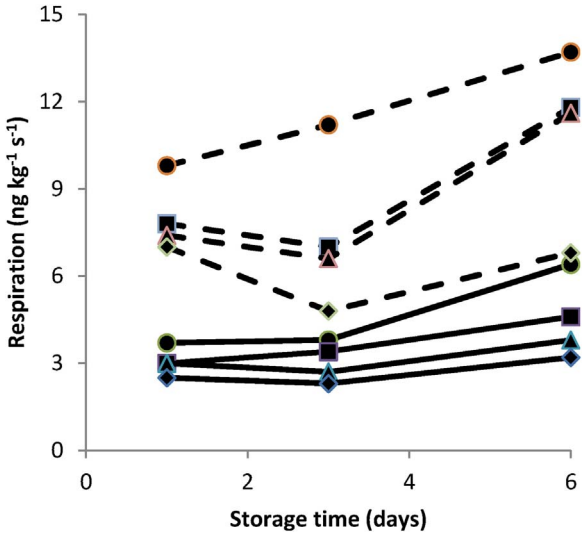


**Fig. 2.** Respiration rate of pak choy after fumigation with hydrogen sulphide at 0 (●), 50 (■), 100(▲) and 250 (♦)μL$L^{-1}$ and ventilated with air that was ethylene-free (solid lines) or contained 0.1 μL$L^{-1}$ ethylene (dash lines) during storage at 10 °C. Values are the mean of nine assessments (3 containers × 3 batches of produce). LSD at $P = 0.05$ was 1.6.

fumigated with hydrogen sulphide showed decreased respiration from one day to three days then increased at six days. Pak choy that was ventilated with ethylene-free air and no exposure to hydrogen sulphide showed a similar increase in respiration during storage but produce fumigated with hydrogen sulphide showed no significant difference in respiration between one day and three days storage but then increased from three days to six days.

### 3.3. Effect of hydrogen sulphide on ethylene production

The data in Table 2 show that the rate of ethylene production by pak choy was significantly reduced ($P < 0.001$) by fumigation with 250 μL $L^{-1}$ hydrogen sulphide compared to control leaves, with the effect present at all storage times and with produce ventilated with air and 0.1 μL $L^{-1}$ ethylene. There was no significant interaction between treatment and storage time which indicates hydrogen sulphide had induced a reduced ethylene production by one day after application. Table 2 also shows that the decrease in ethylene production effected by

**Table 2**
Ethylene production of pak choy after fumigation with 250 μL $L^{-1}$ hydrogen sulphide and ventilated with air that was ethylene-free or contained 0.1 μL $L^{-1}$ ethylene during storage at 10 °C.

| $H_2S$ concn (μL $L^{-1}$) | $C_2H_4$ production (ng $kg^{-1}$ $s^{-1}$) | | | | Green colour score | |
|---|---|---|---|---|---|---|
| | 1 day | 3 days | 6 days | Mean[a] | 3 days | 6 days |
| Ventilated with ethylene-free air | | | | | | |
| 0 | 0.204 | 0.171 | 0.131 | $0.171^{d}$ | 1.2 | 2.5 |
| 250 | 0.154 | 0.097 | 0.006 | $0.107^{c}$ | 1.0 | 1.5 |
| Ventilated with 0.1 μL $L^{-1}$ ethylene | | | | | | |
| 0 | 0.029 | 0.021 | 0.018 | $0.023^{b}$ | 1.4 | 2.8 |
| 250 | 0.021 | 0.010 | 0.010 | $0.014^{a}$ | 1.0 | 2.0 |
| *LSD* | | | | *0.008* | | |

[a] Mean values for treatments are from 27 assessments (3 containers × 3 storage times × 3 batches of produce). LSD value was applied to the means and those not sharing the same superscript in the same column or row are significantly different at $P = 0.05$. Colour scores are the mean of nine assessments (3 containers × 3 batches of produce).

hydrogen sulphide preceded the loss of green colour.

It can also be seen that the rate of ethylene production by pak choy significantly decreased in all treatments with increasing storage time ($P < 0.001$), which is consistent with the findings of Able et al. (2005). In addition, ethylene production was significantly lower from pak choy ventilated with 0.1 μL $L^{-1}$ ethylene compared to ventilation with ethylene-free air ($P < 0.001$). This is consistent with findings of Vendrell and McGlasson (1971) and Philosoph-Hadas et al. (1989) that the presence of ethylene in the atmosphere inhibits endogenous production of ethylene although both studies exposed produce to a much higher concentration of 10 μL $L^{-1}$ ethylene.

Since the rate of senescence of pak choy is greater in the presence of 0.1 μL L ethylene than in ethylene-free air, it would seem that the absolute concentration of exogenous ethylene is driving senescence rather than the rate of endogenous production. However, the data indicate that hydrogen sulphide seems to be multifactorial, acting on both the synthesis (Table 2) and action of ethylene (Tables 1 and 2) in driving senescence.

### 3.4. Effect of hydrogen sulphide on other physio-chemical factors

Changes in a range of physio-chemical factors which are known to be associated with postharvest senescence were assessed to determine the effect of fumigation with 250 μL $L^{-1}$ hydrogen sulphide of pak choy leaves during storage at 10 °C in an air stream containing 0.1 μL $L^{-1}$ ethylene.

#### 3.4.1. Weight loss

The data in Table 3 show that, as expected, weight loss, which is mainly loss of water, increased during storage ($P < 0.05$). There was also a significant interaction between treatment and storage time ($P < 0.05$) where weight loss was similar in both treatments at three days but after six days there was a significant difference between

**Table 3**
Weight loss of pak choy after fumigation with 250 μL $L^{-1}$ hydrogen sulphide and ventilated with air containing 0.1 μL $L^{-1}$ ethylene during storage at 10 °C.

| Treatment | Weight loss (g $kg^{-1}$) | | |
|---|---|---|---|
| | 3 days | 6 days | Mean[a] |
| Control | 0.22 | 0.42 | $0.32^{c}$ |
| $H_2S$ | 0.20 | 0.28 | $0.\ 24^{b}$ |
| Mean[a] | $0.21^{a}$ | $0.32^{b}$ | *LSD = 0.05* |

[a] Mean values for treatments and storage time are from 18 assessments (3 containers × 2 storage times × 3 batches of produce). LSD values were applied to the means and those not sharing the same superscript in the same column or row are significantly different at $P = 0.05$.

**Table 4**
Chlorophyll and carotenoid levels and ion leakage of pak choy fumigated with 250 μL $L^{-1}$ of hydrogen sulphide and ventilated with air containing 0.1 μL $L^{-1}$ ethylene during storage at 10 °C.

| Treatment | Amount of quality factor | | |
|---|---|---|---|
| | 3 days | 6 days | Mean[a] |
| Chlorophyll *a* (mg $L^{-1}$) | | | |
| Control | 18.2 | 14.6 | $16.4^{a}$ |
| $H_2S$ | 21.6 | 17.4 | $19.5^{b}$ |
| Mean[a] | $19.9^{b}$ | $16.0^{a}$ | *LSD = 2.7* |
| Chlorophyll *b* (mg $L^{-1}$) | | | |
| Control | 25.5 | 18.3 | $21.9^{a}$ |
| $H_2S$ | 35.0 | 22.8 | $28.9^{b}$ |
| Mean[a] | $30.3^{b}$ | $20.6^{a}$ | *LSD = 2.53* |
| Total chlorophyll (mg $L^{-1}$) | | | |
| Control | 43.7 | 32.9 | $38.2^{a}$ |
| $H_2S$ | 56.6 | 40.1 | $48.4^{b}$ |
| Mean[a] | $50.2^{b}$ | $36.5^{a}$ | *LSD = 4.37* |
| Total carotenoids (mg $L^{-1}$) | | | |
| Control | 2.2 | 6.2 | $4.2^{a}$ |
| $H_2S$ | 4.6 | 8.0 | $6.3^{b}$ |
| Mean[a] | $3.4^{a}$ | $7.1^{b}$ | *LSD = 1.00* |
| Ion Leakage (%) | | | |
| Control | 28 | 79 | $53^{b}$ |
| $H_2S$ | 24 | 44 | $34^{a}$ |
| Mean[a] | $26^{a}$ | $61^{b}$ | *LSD = 13.2* |

[a] Mean values for treatments and storage time are from 36 assessments (6 containers × 2 storage times or treatments, respectively × 3 batches of produce). LSD values were applied to the means and those not sharing the same superscript in the same column or row are significantly different at $P = 0.05$.

treatments where weight loss was lower in hydrogen sulphide treated pak choy. A potential source of water loss from leafy vegetables is through stomates. However, visual examination of the pak choy leaves used in this study showed that the stomates were closed when produce arrived in the laboratory (within two hours of harvest) and remained closed during storage at 10 °C. The reduction in water loss would thus appear to occur by an inhibition of transpiration/metabolism rather than through open stomates.

#### 3.4.2. Chlorophyll and carotenoids

The levels of chlorophyll *a*, chlorophyll *b* and total chlorophyll in pak choy leaves after three and six days at 10 °C in the presence of 0.1 μL $L^{-1}$ ethylene are presented in Table 4. As expected, the levels of all three chlorophyll moieties significantly decreased during storage ($P < 0.001$) but fumigation with hydrogen sulphide significantly inhibited the loss of these chlorophylls ($P < 0.001$) with the effect present at both three and six days storage. Visual assessment of the loss of green colour was also conducted and confirm the earlier findings (Fig. 1) that leaves fumigated with hydrogen sulphide lost visual green colour at a slower rate than control leaves. The time to reach score 3.0 (market life) was about eight days for control leaves and about 11 days for hydrogen sulphide-treated leaves. The data therefore validates the use of visual colour assessment as an estimate of relative chlorophyll content.

The results presented in Table 4 also show that total carotenoids content increased during storage ($P < 0.001$) and the increase was greater for leaves fumigated with hydrogen sulphide ($P < 0.001$). This result is consistent with studies on broccoli which found carotenoid levels to be preserved in hydrogen sulphide-treated produce (Li et al., 2014). The result, however, contradicts findings by Ni et al. (2016) who reported reduced carotenoid accumulation in the rachis and pulp of hydrogen sulphide-treated Kyoho grapes relative to the control. However, carotenoid levels in leaves maintain steady state concentration through the competing processes of biosynthesis and photo-oxidative degradation, while in many fruits and vegetables, carotenoids accumulate during ripening (Hu et al., 2015).

### 3.4.3. Ion leakage

The data in Table 4 show that the percent ion leakage significantly increased with storage time ($P < 0.001$) which is consistent with increased electrolyte leakage from cells with increasing leaf senescence (Rolny et al., 2011). Fumigation with hydrogen sulphide significantly decreased ion leakage ($P < 0.05$), a finding similar to that reported by Hu et al. (2015) who found that NaHS treatment significantly delayed membrane deterioration in water spinach. However, in our study, there was also a significant interaction between storage time and treatment ($P < 0.05$) where ion leakage was similar in both fumigated and control leaves at three days but untreated leaves showed a marked increase at six days with hydrogen sulphide-treated leaves showing a significantly smaller increase. The effect of hydrogen sulphide inhibiting ion leakage thus showed a similar pattern in treatment effects as weight loss. This suggests a better retention of cellular integrity with hydrogen sulphide treatment which may directly relate to a reduced rate of respiration and transpiration.

### 3.4.4. Antioxidant-related activity

The measures of antioxidant activity, DPPH and FRAP, both showed a significant decline from three days to six days ($P < 0.001$) but there was also a significant interaction between storage time and treatment ($P < 0.001$) (Table 5). This was due to levels in both treatments being similar at three days but at six days there was a smaller decrease in antioxidant activity in produce that had been fumigated with hydrogen sulphide.

The total phenol content showed a significant effect of storage time and treatment ($P < 0.001$) but no significant interaction between treatment and time (Table 5). Total phenols increased from three days to six days but pak choy leaves fumigated with hydrogen sulphide had a lower level of total phenols at both storage times. These findings contrast with the work of Sun et al. (2015) who found higher DPPH and ABTS radical scavenging activities and elevated phenol metabolism in hydrogen sulphide-treated lotus root slices relative to the control treatment.

For ascorbic acid (Table 5), there was a significant decrease from three days to six days storage ($P < 0.05$) but no significant effect due to hydrogen sulphide treatment although there was a trend ($P < 0.1$) to a higher level at six days. Li et al. (2014) did find a higher ascorbic acid level in broccoli fumigated with hydrogen sulphide.

**Table 5**
Changes in compounds related to antioxidant activity of pak choy fumigated with 250 μL $L^{-1}$ of hydrogen sulphide and ventilated with air containing 0.1 μL $L^{-1}$ ethylene during storage at 10 °C.

| Treatment | Amount of metabolite | | |
|---|---|---|---|
| | 3 days | 6 days | Mean[a] |
| FRAP (mg $kg^{-1}$) | | | |
| Control | 0.73 | 0.54 | $0.63^{a}$ |
| $H_2S$ | 0.74 | 0.62 | $0.68^{b}$ |
| Mean[a] | $0.74^{b}$ | $0.58^{a}$ | *LSD = 0.02* |
| DPPH (mg $kg^{-1}$) | | | |
| Control | 0.61 | 0.38 | *$0.50^{a}$* |
| $H_2S$ | 0.64 | 0.52 | *$0.58^{b}$* |
| Mean[a] | $0.63^{b}$ | $0.45^{a}$ | *LSD = 0.01* |
| Total phenols (mg $kg^{-1}$) | | | |
| Control | 0.57 | 0.74 | *$0.66^{b}$* |
| $H_2S$ | 0.45 | 0.61 | *$0.53^{a}$* |
| Mean[a] | $0.51^{a}$ | $0.67^{b}$ | *LSD = 0.12* |
| Ascorbic acid (mg 100 $g^{-1}$) | | | |
| Control | 43.7 | 37.0 | *40.3* |
| $H_2S$ | 44.2 | 40.0 | *42.1* |
| Mean[a] | $44.0^{b}$ | $38.5^{a}$ | *LSD = 4.55* |

[a] Mean values for treatments and storage time are from 72 assessments (2 samples × 6 containers × 2 treatments or storage times, respectively × 3 batches of produce). LSD values were applied to the means and those not sharing the same superscript in the same column or row are significantly different at $P = 0.05$.

**Table 6**
Market life of basil and kale leaves after fumigation with hydrogen sulphide and ventilation with air containing 0.1 μL $L^{-1}$ ethylene during storage at 10 °C.

| Produce | $H_2S$ (μL $L^{-1}$) | Market life (days)[a] |
|---|---|---|
| Basil | 0 | $7.8^{a}$ |
| | 50 | $7.7^{a}$ |
| | 100 | $9.6^{b}$ |
| | *LSD* | *0.48* |
| Kale | 0 | $6.3^{a}$ |
| | 50 | $7.7^{b}$ |
| | 100 | $8.7^{c}$ |
| | *LSD* | *0.60* |

[a] Each value for market life is the mean of nine assessments (3containers × 3 batches of produce).

### 3.5. Effect of hydrogen sulphide on market life and respiration of basil and kale

The data in Table 6 show that the market life of basil was significantly extended when leaves were fumigated with 100 μL $L^{-1}$ hydrogen sulphide ($P < 0.001$) and ventilated with air containing 0.1 μL $L^{-1}$ ethylene. The market life of kale was significantly increased by fumigation with 50 μL $L^{-1}$ and further increased by 100 μL $L^{-1}$ ($P < 0.001$).

The effect of hydrogen sulphide on the respiration rate of basil and kale is presented in Fig. 3 with both produce showing a similar pattern of change as pak choy. A significant interaction between the treatment and storage time ($P < 0.05$) showed that basil and kale not exposed to hydrogen sulphide had an increased respiration rate during storage, but that the respiration rate of produce fumigated with 100 μL $L^{-1}$ hydrogen sulphide declined from two days to four days storage and remained lower than control produce at six days.

## 4. Conclusions

The multi-faceted actions of hydrogen sulphide observed in this study raises the question as to whether hydrogen sulphide acts directly and independently on each of these quality and physiological factors, or interacts with a single entity that triggers the emergence of characteristics associated with postharvest deterioration. Ethylene is an obvious potential controlling factor and this study clearly showed that hydrogen sulphide inhibited the production of ethylene and inhibited the action of ethylene, at least on loss of chlorophyll and respiration. The impact of hydrogen sulphide on reducing ethylene production was rapid with significant change observed one day after application. The interaction of exogenous hydrogen sulphide with ethylene could therefore be the mechanism whereby hydrogen sulphide inhibits senescence albeit that such interaction may be on more than one metabolic system. Further, the reduction in weight loss, respiration, ion leakage and total phenols and an increase in antioxidant activity effected by hydrogen sulphide were mostly observed after some days of storage suggesting they could have resulted from exposure to a reduced concentration of ethylene.

Given the direct effect of hydrogen sulphide treatment increasing the market life of pak choy, kale and basil, the findings suggest potential commercial benefit from the short-term fumigation of edible leafy produce with hydrogen sulphide. Reducing the rate of loss of chlorophyll together with reductions in weight loss, respiration, ion leakage and an increase in antioxidant activity are additive to extending market life and improving consumer acceptability. While investigations are at an early stage, findings to date suggest the effect of hydrogen sulphide on chlorophyll to be ubiquitous, with examples studies reported for postharvest produced such as broccoli (Li et al., 2014) and water spinach (Hu et al., 2015) as well as for living plants (Zhang et al., 2009). A potential impediment to the use of hydrogen sulphide is the potential for an adverse sensory impact due to its objectionable odour.

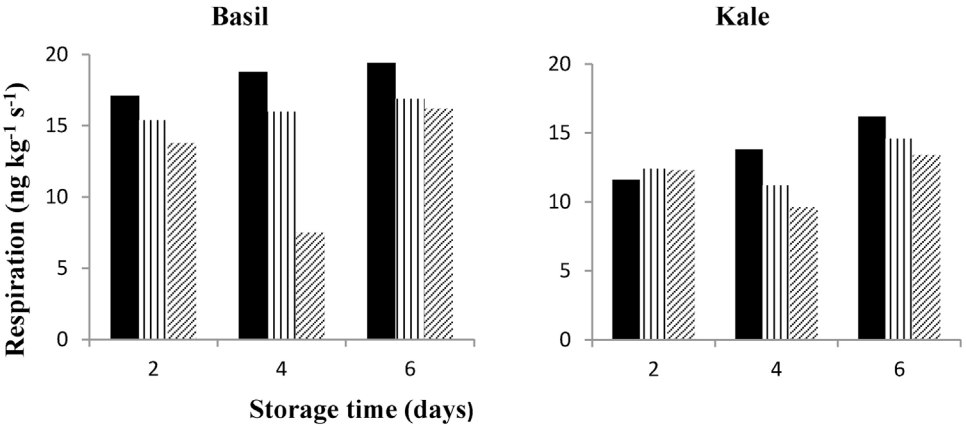


**Fig. 3.** Respiration rate of basil and kale after fumigation with hydrogen sulphide at 0 (solid bar), 50 (vertical lines) and 100 (45° lines) μL $L^{-1}$ and ventilation with air containing 0.1 μL $L^{-1}$ ethylene during storage at 10 °C.
Values are the mean of nine assessments (3 containers × 3 batches of produce). LSD at $P = 0.05$ was 1.0 for basil and 1.4 for kale.

However, while a noticeable odour was present on leaves when removed from the fumigation chamber but no odour was detected the following day.

Future studies using inhibitors of ethylene action such as 1-methylcyclopropene (1-MCP) could be useful to uncouple the effect of hydrogen sulphide on the synthesis and action of ethylene. The more fundamental question of the involvement of endogenous hydrogen sulphide in ethylene metabolism or action also needs investigation.

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## References

Abeles, F.B., Morgan, P.W., Saltveit, M.E., 1992. Ethylene in Plant Biology. Academic Press, New York.
Able, A.J., Wong, L.S., Prasad, A., O'Hare, T.J., 2005. The physiology of senescence in detached pak choy leaves (*Brassica rapa* var. *chinensis*) during storage at different temperatures. Postharvest Biol. Technol. 35, 271–278.
Anon, 1989. Guide to Food Transport: Fruit and Vegetables. Mercantila Publishers, Copenhagen, pp. 178–179.
Burg, S.P., Burg, E.A., 1962. Role of ethylene in fruit ripening. Plant Physiol. 37, 179–189.
Hancock, J.T., Whiteman, M., 2016. Hydrogen sulfide signaling: interactions with nitric oxide and reactive oxygen species. Ann. N. Y. Acad. Sci. 1365, 5–14.
Hu, L.Y., Hu, S.L., Wu, J., Li, Y.H., Zheng, J.L., Wei, Z.J., Liu, J., Wang, H.L., Liu, Y.S., Zhang, H., 2012. Hydrogen sulfide prolongs postharvest shelf life of strawberry and plays an antioxidative role in fruits. J. Agric. Food Chem. 60, 8684–8693.
Hu, H., Shen, W., Li, P., 2014a. Effects of hydrogen sulphide on quality and antioxidant capacity of mulberry fruit. Int. J. Food Sci. Technol. 49, 399–409.
Hu, K.D., Wang, Q., Hu, L.Y., Gao, S.P., Wu, J., Li, Y.H., 2014b. Hydrogen sulfide prolongs postharvest storage of fresh-cut pears (*Pyrus pyrifolia*) by alleviation of oxidative damage and inhibition of fungal growth. PLoS One 9, e85524. http://dx.doi.org/10.1371/journal.pone.0085524.
Hu, H., Liu, D., Li, P., Shen, W., 2015. Hydrogen sulfide delays leaf yellowing of stored water spinach (*Ipomoea aquatica*) during dark-induced senescence by delaying chlorophyll breakdown, maintaining energy status and increasing antioxidative capacity. Postharvest Biol. Technol. 108, 8–20.
Huque, R., Wills, R.B.H., Pristijono, P., Golding, J.B., 2013. Effect of nitric oxide (NO) and associated control treatments on the metabolism of fresh-cut apple slices in relation to development of surface browning. Postharvest Biol. Technol. 78, 16–23.
Jin, Z., Pei, Y., 2015. Physiological implications of hydrogen sulfide in plants: pleasant exploration behind its unpleasant odour. Oxid. Med. Cell. Longev. 2015, 6, Article ID 397502. . http://dx.doi.org/10.1155/2015/397502.
Knee, M., Proctor, F.J., Dover, C.J., 1985. The technology of ethylene control: use and removal in post-harvest handling of horticultural commodities. Ann. Appl. Biol. 107, 581–595.
Leshem, Y.Y., Haramaty, E., 1996. The characterization and contrasting effects of the nitric oxide free radical in vegetative stress and senescence of *Pisum sativum* Linn. foliage. J. Plant Physiol. 148, 258–263.
Leshem, Y.Y., Wills, R.B.H., Ku, V.V.V., 1998. Evidence for the function of the free radical gas– nitric oxide (NO)–as an endogenous maturation and senescence regulating factor in higher plants. Plant Physiol. Biochem. 36, 825–833.
Li, S.P., Hu, K.D., Hu, L.Y., Li, Y.H., Jiang, A.M., Xiao, F., Han, Y., Liu, Y.S., Zhang, H., 2014. Hydrogen sulfide alleviates postharvest senescence of broccoli by modulating antioxidant defense and senescence-related gene expression. J. Agric. Food Chem. 62, 1119–1129.
Li, Y., Wills, R.B.H., Golding, J.B., 2017. Interaction of ethylene concentration and storage temperature on postharvest life of the green vegetables pak choi, broccoli, mint, and green bean. J. Hortic. Sci. Biotechnol. 92, 288–293. http://dx.doi.org/10.1080/14620316.2016.1263545.
Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzym. 148, 350–382.
Lu, S., 2007. Effect of packaging on shelf-life of minimally processed bok choy (*Brassica chinensis* L.). LWT-Food Sci. Technol. 40, 460–464.
Luo, Y., 2016. Bok choy. In: Gross, K.C., Wang, C.Y., Saltveit, M. (Eds.), The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks, Agriculture Handbook 66. U.S. Department of Agriculture, Agricultural Research Service, Washington DC (p. 243).
Manjunatha, G., Gupta, K.J., Lokesh, V., Mur, L.A., Neelwarne, B., 2012. Nitric oxide counters ethylene effects on ripening fruits. Plant Signaling Behavior 7, 476–483.
Ni, Z.J., Hu, K.D., Song, C.B., Ma, R.H., Li, Z.R., Zheng, J.L., Zhang, H., 2016. Hydrogen sulfide alleviates postharvest senescence of grape by modulating the antioxidant defenses. Oxid. Med. Cell. Longev. 2016, 14, Article ID 4715651. . http://dx.doi.org/10.1155/2016/4715651.
Nielsen, S.S., 2003. Vitamin C determination by indophenol method. In: Nielsen, S.S. (Ed.), Food Analysis Laboratory Manual. Springer, Boston, MA, pp. 45–50.
Philosoph-Hadas, S., Pesis, E., Meir, S., Reuveni, A., Aharoni, N., 1989. Ethylene-enhanced senescence of leafy vegetables and fresh herbs. Acta Hortic. 258, 37–45.
Rolny, N., Costa, L., Carrión, C., Guiamet, J.J., 2011. Is the electrolyte leakage assay an unequivocal test of membrane deterioration during leaf senescence? Plant Physiol. Biochem. 49, 1220–1227.
Sun, Y., Zhang, W., Zeng, T., Nie, Q., Zhang, F., Zhu, L., 2015. Hydrogen sulfide inhibits enzymatic browning of fresh-cut lotus root slices by regulating phenolic metabolism. Food Chem. 177, 376–381.
Tang, J., Hu, K.D., Hu, L.Y., Li, Y.H., Liu, Y.S., Zhang, H., 2014. Hydrogen sulfide acts as a fungicide to alleviate senescence and decay in fresh-cut sweet potato. HortSci. 49, 938–943.
Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., Byrne, D.H., 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J. Food Compos. Anal. 19, 669–675.
Vendrell, M., McGlasson, W.B., 1971. Inhibition of ethylene production in banana fruit tissue by ethylene treatment. Aust. J. Biol. Sci. 24, 885–895.
Vuong, Q.V., Hirun, S., Roach, P.D., Bowyer, M.C., Phillips, P.A., Scarlett, C.J., 2013. Effect of extraction conditions on total phenolic compounds and antioxidant activities of *Carica papaya* leaf aqueous extracts. J. Herbal Med. 3, 104–111.
Wang, W., Shen, Y.G., Zhu, L.Q., Zhang, W., Wu, S.F., Du, H.Y., 2014. Effects of exogenous H2S on preservation of peaches and reactive oxygen metabolism. J. Fruit Sci. 31, 302–307.
Wills, R.B.H., Ku, V.V.V., Shohet, D., Kim, G.H., 1999. Importance of low ethylene levels to delay senescence of non-climacteric fruit and vegetables. Aust. J. Expt. Agric. 39, 221–224.
Wills, R.B.H., Warton, M.A., Mussa, D.M.D.N., Chew, L.P., 2001. Ripening of climacteric fruits initiated at low ethylene levels. Aust. J. Expt. Agric. 41, 89–92.
Wills, R.B.H., 2015. Low ethylene technology in non-optimal storage temperatures. In: Wills, R.B.H., Golding, J.B. (Eds.), Advances in Postharvest Fruit and Vegetable Technology. CRC Press, Boca Raton FL, pp. 167–190.
Zhang, H., Ye, Y.K., Wang, S.H., Luo, J.P., Tang, J., Ma, D.F., 2009. Hydrogen sulfide counteracts chlorophyll loss in sweet potato seedling leaves and alleviates oxidative damage against osmotic stress. Plant Growth Regulat. 58, 243–250.
Zhang, H., Hu, S.L., Zhang, Z.J., Hu, L.Y., Jiang, C.X., Wei, Z.J., Liu, J., Wang, H.L., Jiang, S.T., 2011. Hydrogen sulfide acts as a regulator of flower senescence in plants. Postharvest Biol. Technol. 60, 251–257.
Zhao, Y., Biggs, T.D., Xian, M., 2014. Hydrogen sulfide (H 2S) releasing agents: chemistry and biological applications. Chem. Commun. (Camb.) 50, 11788–11805.
Zheng, J.L., Hu, L.Y., Hu, K.D., Wu, J., Yang, F., Zhang, H., 2016. Hydrogen sulfide alleviates senescence of fresh-cut apple by regulating antioxidant defense system and senescence-related gene expression. HortSci. 51, 152–158.
Zhu, L.Q., Wang, W., Shi, J.Y., Zhang, W., Shen, Y.G., Du, H.Y., 2014. Hydrogen sulfide extends the postharvest life and enhances antioxidant activity of kiwifruit during storage. J. Sci. Food Agric. 94, 2699–2704.

### 4.2 Effect of hydrogen sulphide and nitric oxide on pak choy

H.M.S. Al Ubeed, R.B.H. Wills, M.C. Bowyer, Q.V.Vuong, J.B. Golding. Effect of hydrogen sulphide, nitric oxide and ethylene on the postharvest deterioration of pak choy. International Society for Horticultural Science (ISHS) VI Postharvest Unlimited Conference. Madrid, Spain October 2017. (Oral presentation) Accepted for publication in Acta Horticulture.

**General background**

NO and $H_2S$ are now accepted as gaseous signaling molecules in plants. The involvement of NO in postharvest metabolism was known for about 15 years prior to investigations involving $H_2S$ and has been strongly linked with ethylene metabolism and action. There is no published information that compares the effects of NO and $H_2S$ on postharvest senescence, particularly in the presence of exogenous ethylene. A study was therefore conducted to determine the effect of short-term fumigation with $H_2S$ or NO alone or in sequence on senescence of pak choy during subsequent storage at 10 °C in the presence of 0.1 µL/L ethylene.

**Summary of Results**

Pak choy fumigation with 250 µL/L $H_2S$ or 100 µL/L NO showed increased market life (i.e. reduced loss of green colour) and decreased respiration rate during storage with ethylene-free air or 0.1 µL/L ethylene but the beneficial effects of both gases were more pronounced in the presence of ethylene. $H_2S$ showed a greater increase in market life and decrease in respiration rate than NO. The application of $H_2S$-NO was similar to $H_2S$ alone and dual application of NO-NO or $H_2S$-$H_2S$ did not increase the beneficial effects over a single application.

**Conclusions**

While fumigation with $H_2S$ and NO increased the market life and reduced ethylene production of pak choy, the magnitude of improvement was greater for $H_2S$ and treatment with $H_2S$ + NO did not generate any greater benefit than $H_2S$ alone. This prompted all further studies in this thesis to concentrate on the potential benefits to be derived from the use of $H_2S$ and its mode of action.

A copy of the conference paper Accepted for publication in Acta Horticulture is appended.

# Effects of hydrogen sulphide, nitric oxide and ethylene on postharvest deterioration of pak choy

H.M.S. Al Ubeed [1,2,*], R.B.H. Wills [1], M.C. Bowyer [1], Q.V. Vuong [1] and J.B. Golding [1,3]
[1] School of Environmental and Life Sciences, University of Newcastle, Ourimbah NSW 2558, Australia.
[2] Department of Life Sciences, Faculty of Science, Al-Muthana University, Al-Muthana, Iraq.
[3] NSW Department of Primary Industries, Ourimbah NSW 2258, Australia.

* Corresponding author. E-mail address: hebah.alubeed@uon.edu.au (H.M.S. Al Ubeed)

**Keywords**: Fumigation, $H_2S$, NO, *Brassica rapa*, respiration, senescence; leafy vegetable

## ABSTRACT

**Ethylene, hydrogen sulphide ($H_2S$) and nitric oxide (NO) are signaling molecules that affect plant metabolism. Postharvest fumigations with these gases have been shown to interfere with a range of factors associated with postharvest senescence. This study reports on postharvest studies with pak choy *(Brassica rapa subsp.Chinensis)* that examined the effect of fumigation with hydrogen sulphide and nitric oxide and subsequent storage at 10 °C in air with and without the addition of 0.1 µL $L^{-1}$ ethylene. The results showed that treatment with nitric oxide increased the shelf life and decreased respiration rates for the pak choy heads during the storage. However, fumigation with $H_2S$ alone or in combination with NO resulted in greater inhibition of respiration rate and extension in market life, and this effect was more pronounced in the presence of 0.1 µL $L^{-1}$ ethylene.**

## INTRODUCTION

Nitric oxide (NO) and hydrogen sulphide ($H_2S$) have been identified as important signalling compounds in plants and have also been studied in relation to their effects on postharvest quality (Wills et al., 2000, Hu et al., 2012). NO is thought to act as a downstream secondary messenger in many signaling processes (Wilson et al., 2008) and has been considered to be an ethylene antagonist extending the postharvest life of many horticultural products (Wills et al., 2015). Leshem (1996) first showed that NO reduced the effects of ethylene in pea seedlings. However, subsequent studies have shown that NO extends the postharvest life of many horticultural products such as strawberry (Wills et al., 2000), carnation flower (Bowyer et al., 2003), broccoli, green beans and pak choy (Soegiarto and Wills, 2004).
Hydrogen sulphide ($H_2S$) has also been recognized as a gaseous metabolic regulator in plant systems (Jin and Pei, 2015). $H_2S$ has been linked to diverse plant physiological functions such as germination, stomatal movement, root development and flower senescence (Hancock and Whiteman, 2016). Recognition of the role of $H_2S$ as a regulator of senescence is a more recent phenomenon. Zhang et al. (2011)reported delayed senescence in cut flowers and explant leaves treated with solutions of the $H_2S$ donor sodium hydrogen sulphide(NaHS). $H_2S$ fumigation was subsequently shown to extend the postharvest life of many fruit and vegetables with a broad range of senescence characteristics, such as strawberry (Hu et al., 2012), broccoli (Li et al., 2014), pear (Hu et al., 2014c), kiwifruit (Zhu et al., 2014), apple (Zheng et al., 2016) and pak choy (Al Ubeed et al., 2017).
The effects of $H_2S$ and other signaling molecules should not be considered in isolation, as they can interact. There have been studies reported where pretreatment with both $H_2S$ donor and NO donor compounds induced heat tolerance of maize seedling (Li et al., 2013) and reduced the antioxidants with cadmium stress in Bermuda grass (Shi et al., 2014). $H_2S$ has also been shown to have an impact on the metabolism of both reactive oxygen species and reactive nitrogen species (Hancock and Whiteman, 2016; Wilson et al., 2008). In addition, Chang et al. (2014) demonstrated an additive effect when combining both $H_2S$ and NO to extend the shelf life of strawberries.
Pak choy *(Brassica rapa subsp. Chinensis)* (also known as bok or pok choy, choi or tsoi) is widely grown in south and east Asia but is now also available in most Western countries. As with many green leafy vegetables, quality is dependent on leaves retaining their green colour and there being no other visible sign of deterioration such as wilting, rotting or browning (Able et al., 2005). This current study examined

the interaction of NO and $H_2S$ fumigation at 10°C in the presence of a controlled level of ethylene (0.1 uL $L^{-1}$) on the postharvest deterioration of pak choy.

# MATERIALS AND METHODS

## Produce and experimental design

Pak choy plants ('Shanghai') were harvested from a local commercial farm at Mangrove Mountain, NSW (Australia) and transported to the laboratory within two hours (hr). All plants selected for each experiment were of uniform size (10 cm length) and colour, and without damage to leaves or stems. Pak choy heads were selected and gently cleaned with tap water and allowed to stand in ambient air until dry. Each treatment unit consisted of 6 heads of pak choy with a combined weight of between 400-500 g which were placed into a 4 L plastic container that was fitted with inlet and outlet ports in the lid. Each experiment comprised 126 containers and groups of nine containers were respectively ventilated with 0.1 μL $L^{-1}$ ethylene or <0.001 μL $L^{-1}$ ethylene (considered 'air') at 45 mL $min^{-1}$. The containers were placed in a temperature controlled cabinet at 10°C. All experiments were replicated by obtaining batches of plants on three separate occasions with at least two weeks between batches. A treatment unit consisted of six heads pak choy in a single 4L container. Each treatment had three containers per treatment.

## Effect of hydrogen sulphide and nitric oxide concentrations

The following treatments were applied:

- Fumigation with $H_2S$ vapour at 250 μL $L^{-1}$ was conducted inside a sealed container by placing the required weight of solid NaHS.$H_2O$ into a dry beaker that was sealed in a container along with the produce to be treated. Water (2 mL) was then injected into the beaker through a septum in the container lid to generate the hydrogen sulphide. Using this method, the liberation of hydrogen sulphide gas has been shown to be quantitative and immediate for four hr (Zhao et al., 2014).
- Fumigation with NO involved injecting 100 μL $L^{-1}$ of NO gas into the 4 L sealed container through an injection port. The NO gas was obtained from a standard cylinder of NO gas containing 4150±170 μL $L^{-1}$ in nitrogen, (BOC Gases, Sydney) and fumigated for four hr.
- Fumigation with double treatments (NO-NO). This involved exposure of 100 μL $L^{-1}$ NO for four hr followed by one hr in the air then another fumigation with 100 μL $L^{-1}$ NO for another four hr. Other treatments followed a similar time sequence but involved different gas treatment combinations.
- Fumigation with ($H_2S$-$H_2S$) which comprised two exposures to 250 μL $L^{-1}$ $H_2S$.
- Fumigation with ($H_2S$-NO) which was fumigation with 250 μL $L^{-1}$ $H_2S$ then 100 μL $L^{-1}$ NO.
- Fumigation with (NO-$H_2S$) which comprised of the reversed treatment for the previous treatment, i.e. 100 μL $L^{-1}$ NO followed by 250 μL $L^{-1}$ $H_2S$
- The control treatment which involved storage of the containers in the air at 10 °C for nine hr.

All the containers were then ventilated with humidified ethylene-free air (<0.001 μL $L^{-1}$) or 0.1 μL $L^{-1}$ ethylene at 45 mL $min^{-1}$ at 10°C. The quality of leaves was visually assessed daily for green colour, and the respiration rate of units was analysed by gas chromatography at periodic intervals.

## Visual leaf colour (market life)

Visual assessment of the change in leaf colour from green to yellow of individual leaves was conducted daily using a scoring scale of 0-5 where 0 = green, 1 = 10%, 2 = 20%, 3 = 30%, 4 = 50% and 5 =>70% loss of original green colour (Li et al, 2017). The mean colour score of all leaves in a treatment unit was calculated daily. An average colour score of 3.0 was considered to be the limit of consumer acceptability and the time for leaves to reach a mean score of 3.0 was designated as the market life. Assessment of a unit was terminated when the mean score of 3.0 was attained.

## Respiration rate

Respiration rate as carbon dioxide ($CO_2$) production was measured for each 4 L container. A container was sealed to allow the accumulation of a measurable concentration of $CO_2$. A gas sample (1 mL) was

collected in a syringe after four hr from the atmosphere of containers ventilated with ethylene and without ethylene-free air. The concentration of $CO_2$ in the gas sample was determined by injecting into a thermal conductivity gas chromatograph as described by Huque et al. (2013).

## Statistical analysis

Data were analysed by two-way analysis of variance (ANOVA) and where a significant difference between treatments was found the least significant difference (LSD) of the mean values at P=0.05 was calculated. Statistical procedures were performed using SPSS for Microsoft version 22.0 software package (SPSS Chicago, IL).

# RESULTS AND DISCUSSION

## Effect of hydrogen sulphide and nitric oxide on respiration rate

The effects of NO and $H_2S$ treatments with the addition of ethylene into the storage atmosphere on the respiration rates of pak choy are presented in Table1. As expected, there was a significant interaction between the treatment and storage time ($P< 0.001$), and between storage time and ventilation with exogenous ethylene ($P< 0.001$).

Pretreatment with exogenous $H_2S$ alone or in combined with NO caused a significant inhibition in the respiration rate when stored in the ethylene-free air which was higher than when stored with 0.1 μL $L^{-1}$ ethylene (Table 1). These observations are consistent with findings of Chang et al. (2014) who reported that treatment with donor solutions of NO alone, $H_2S$ alone or the combination of NO and $H_2S$ solutions showed these treatments inhibited the respiration rate of treated strawberry fruit. Chang et al. (2014) further showed that the combination treatment of NO and $H_2S$ resulted in significantly lower respiration rates than all other treatments. However, Chang et al. (2014) did not report on the levels of ethylene around the strawberries during storage. These results are in agreement with the previous results for mulberry fruit (Hu et al., 2014a), strawberry fruit (Hu et al., 2012)and water spinach(Hu et al., 2015). They found after applied exogenous $H_2S$ donor alone caused decline in the respiration rate during the storage time and increased the shelf life for this product. Moreover, Zaharah and Singh (2011) reported that applying an exogenous NO donor alone suppressed the respiration rate for mango and papaya fruit (Li et al., 2014b).

With the addition of 0.1 μL $L^{-1}$ ethylene into the storage atmosphere, the respiration rates increased for all treatments, particularly with the control (water treatment), NO-NO and NO-$H_2S$ at all storage times.

Table1. Effect fumigation with different combinations of 250 μL $L^{-1}$ $H_2S$ and 100 μL $L^{-1}$ NO on the respiration rate of pak choy leaves either with ethylene–free air (<0.001 μL $L^{-1}$ ethylene) or with 0.1 μL $L^{-1}$ ethylene and storage at 10°C.

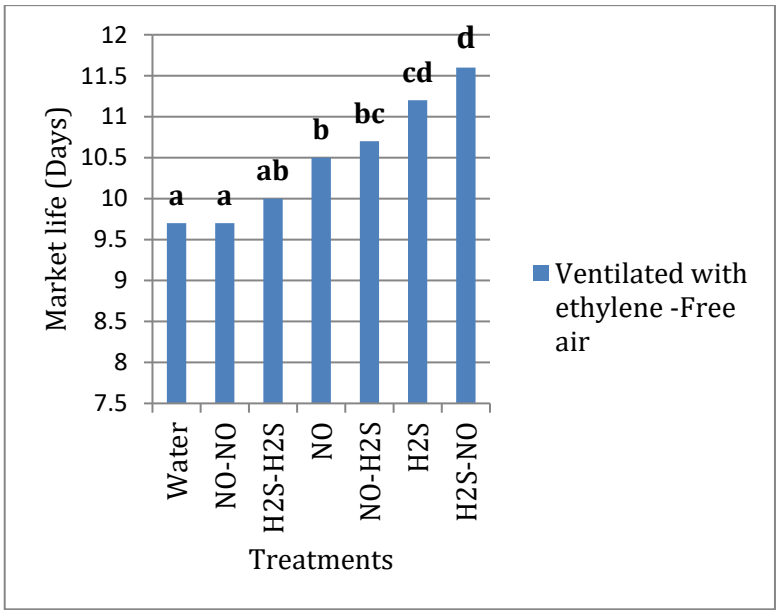
| | Respiration (mL $CO_2$ $kg^{-1}$ $hr^{-1}$) | | | | |
|---|---|---|---|---|---|
| | **Ventilated with** <0.001 μL $L^{-1}$ ethylene | | | | |
| Treatment | Day 1 | Day 2 | Day 4 | Day 6 | Mean |
| Water | 8.6 | 9.4 | 10.0 | 13.3 | $10.3^{b}$ |
| $H_2S$-$H_2S$ | 11.2 | 10.6 | 9.7 | 10.6 | $10.5^{b}$ |
| NO-NO | 11.1 | 12.4 | 11.2 | 14.9 | $12.4^{c}$ |
| NO | 9.4 | 9.1 | 7.9 | 12.9 | $9.8^{b}$ |
| NO-$H_2S$ | 14.1 | 15.2 | 11.4 | 13.8 | $13.6^{d}$ |
| $H_2S$ | 8.2 | 6.7 | 5.9 | 7.1 | $7.0^{a}$ |
| $H_2S$-NO | 10.6 | 7.7 | 6.1 | 8.3 | $8.2^{a}$ |
| Mean | $10.4^{b}$ | $10.1^{b}$ | $8.9^{a}$ | $11.5^{c}$ | |

| | Ventilated with 0.1 µL $L^{-1}$ ethylene | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 4 | Day 6 | |
| Water | 27.6 | 26.4 | 27.3 | 28.0 | $27.3^{d}$ |
| $H_2S$-$H_2S$ | 18.6 | 17.5 | 13.6 | 12.8 | $15.6^{bc}$ |
| NO-NO | 18.8 | 20.2 | 17.0 | 15.7 | $17.9^{c}$ |
| NO | 25.3 | 10.8 | 7.2 | 10.3 | $13.4^{b}$ |
| NO-$H_2S$ | 16.9 | 18.1 | 12.9 | 10.9 | $14.7^{b}$ |
| $H_2S$ | 12.4 | 10.8 | 7.6 | 10.7 | $10.4^{a}$ |
| $H_2S$-NO | 15.9 | 9.2 | 8.9 | 11.7 | $11.4^{ab}$ |
| LSD (*P*=0.05) | 3.82 | | | | 1.91 |
| Mean | $19.4^{c}$ | $16.1^{b}$ | $13.5^{a}$ | $14.3^{ab}$ | |
| LSD | 1.44 | | | | |

Mean Values for treatments stored either in air or ethylene are the mean of 36 readings (3 containers x 4 days of the storage x 3 batches of produce). Mean values for storage time are the average of 63 assessments (3 containers x 7 treatments x 3 batches of produce). LSD values were applied to the means and those not sharing the same superscripts in the same column are significantly different at P<0.05.

## Effect of hydrogen sulphide and nitric oxide on leaf colour (market life)

The effect of NO and $H_2S$ in the absence of added ethylene into the storage atmosphere (<0.001 µL $L^{-1}$ ethylene) on the postharvest life of pak choy is presented in Figure 1. The results showed that treatment and adding ethylene into the atmosphere had significant effects on market life ($p < 0.001$). There was no significant interaction between the treatments and ventilation with ethylene. Fumigation with $H_2S$ and NO with ventilation with ethylene-free air resulted in the highest shelf life for the pak choy compared with ventilated with 0.1 µL $L^{-1}$ of the ethylene. This observation was supported by the findings of Li et al. (2017) who showed that the addition of ethylene to the storage environment, reduced the market life of a range of non-climacteric vegetables, including pak choy. Both the addition of $H_2S$ and the combination of $H_2S$–NO showed increased the market life of pak choy leaves compared to untreated control leaves ventilated with or without ethylene. These observations are in agreement with previous results for effect $H_2S$ alone on postharvest produce such as broccoli (Li et al., 2014) and water spinach (Hu et al., 2015).



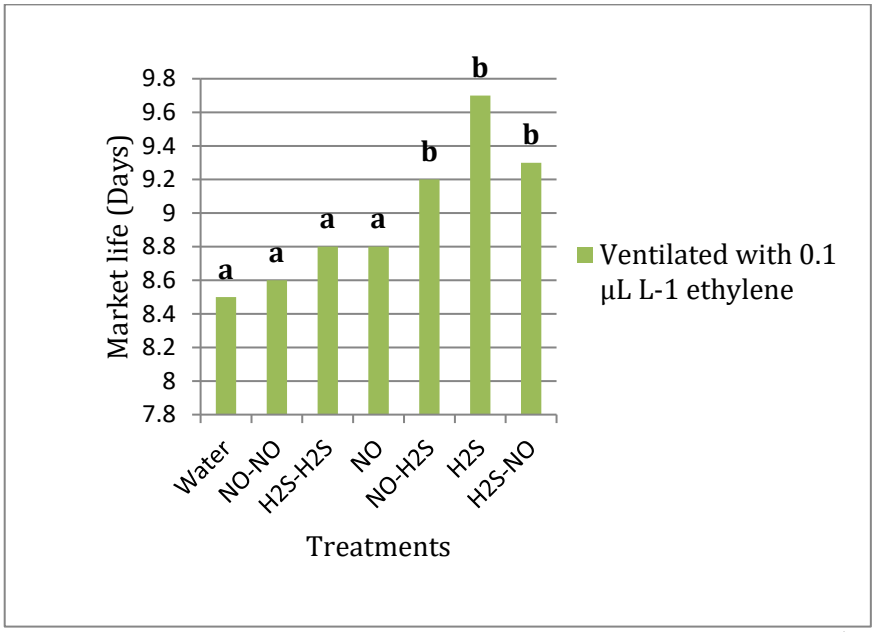



Figure.1 Effect of fumigation with different combinations of 250 µL $L^{-1}$ $H_2S$ and 100 µL $L^{-1}$ NO on the market life of pak choy leaves that were subsequently stored at 10 °C. Each point is the mean of the nine assessments (3 units x 3 batches of produce). The LSD at *P=0.05* of *0.58* and those not sharing the same superscript in the same column are significantly different at *P* <0.05.

## CONCLUSION

Pre-storage treatment with both $H_2S$ and NO in the presence of ethylene resulted in an increase in the market life and reduced the respiration rate during the storage of pak choy. These results indicated that these gases could be commercially used to improve the storage life of pak choy leaves when transport or storage at 10°C.

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## Literature Cited

Able, A.J., Wong, L.S., Prasad, A., and O'Hare, T.J., (2005). The physiology of senescence in detached pak choy leaves (*Brassica rapa* var. *chinensis*) during storage at different temperatures. Postharvest Biol. Technol. 35, 271-278.

Al Ubeed, H.M.S., Wills, R.B.H., Bowyer, M.C., Vuong, Q.V., and Golding, J.B. (2017). Interaction of exogenous hydrogen sulphide and ethylene on senescence of green leafy vegetables. Postharvest Biol. Technol. *133*, 81-87 doi http://dx.doi.org/10.1016/j.postharvbio.2017.07.010.

Bowyer, M.C., Wills, RB.H.., Badiyan, D., and Ku, V.V.V. (2003). Extending the postharvest life of carnations with nitric oxide-comparison of fumigation and *in vivo* delivery. Postharvest Biol. Technol. *30*, 281-286 doi http://dx.doi.org/10.1016/S0925-5214(03)00114-5.

Chang, Z., Jingying, S., Liqin, Z., Changle, L., and Qingguo, W. (2014). Cooperative effects of hydrogen sulfide and nitric oxide on delaying softening and decay of strawberry. Int. J. Agric. Biol. Eng. *7*, 114 doi http://dx.doi.org/10.3965/j.ijabe.20140706.014.

Hancock, J.T., and Whiteman, M. (2016). Hydrogen sulfide signaling: interactions with nitric oxide and reactive oxygen species. Ann. New York Acad. Sci. *1365*, 5-14 doi http://dx.doi.org/ 10.1111/nyas.12733.

Hu, H., Liu, D., Li, P., and Shen, W. (2015). Hydrogen sulfide delays leaf yellowing of stored water spinach (*Ipomoea aquatica*) during dark-induced senescence by delaying chlorophyll breakdown, maintaining energy status and increasing antioxidative capacity. Postharvest Biol. Technol. *108*, 8-20 https://doi.org/10.1016/j.postharvbio.2015.05.003

Hu, H., Shen, W., and Li, P. (2014a). Effects of hydrogen sulphide on quality and antioxidant capacity of mulberry fruit. Int.l J. Food Sci. Technol. *49*, 399-409 doi http://dx.doi.org/ http://10.1111/ijfs.12313
Hu, K.-D., Wang, Q., Hu, L.-Y., Gao, S.-P., Wu, J., Li, Y.-H., Zheng, J.-L., Han, Y., Liu, Y.-S., and Zhang, H. (2014b). Hydrogen sulfide prolongs postharvest storage of fresh-cut pears (*Pyrus pyrifolia*) by alleviation of oxidative damage and inhibition of fungal growth. PloS one *9*, e85524 https://doi.org/10.1371/journal.pone.0085524.
Hu, L.-Y., Hu, S.-L., Wu, J., Li, Y.-H., Zheng, J.-L., Wei, Z.-J., Liu, J., Wang, H.-L., Liu, Y.-S., and Zhang, H. (2012). Hydrogen sulfide prolongs postharvest shelf life of strawberry and plays an antioxidative role in fruits. J.Agric.Food Chem. *60*, 8684-8693 doi http://dx.doi.org/10.1021/jf300728h.
Huque, R., Wills, R.B.H., Pristijono, P., and Golding, J.B. (2013). Effect of nitric oxide (NO) and associated control treatments on the metabolism of fresh-cut apple slices in relation to development of surface browning. Postharvest Biol. Technol. *78*, 16-23. DOI: 10.1016/j.postharvbio.2012.12.006
Jin, Z., and Pei, Y. (2015). Physiological implications of hydrogen sulfide in plants: pleasant exploration behind its unpleasant odour. Oxidative Medicine and Cellular Longevity *2015* doi http://dx.doi.org/10.1155/2015/397502.
Leshem, Y.Y. (1996). Nitric oxide in biological systems. Plant Growth Reg. *18*, 155-159 doi http://dx.doi.org/10.1007/BF00024375.
Li, S.-P., Hu, K.-D., Hu, L.-Y., Li, Y.-H., Jiang, A.-M., Xiao, F., Han, Y., Liu, Y.-S., and Zhang, H. (2014a). Hydrogen sulfide alleviates postharvest senescence of broccoli by modulating antioxidant defense and senescence-related gene expression. J. Agric. Food Chem. *62*, 1119-1129. http://dx.doi.org/10.1021/jf4047122.
Li, X.P., Wu, B., Guo, Q., Wang, J.D., Zhang, P., and Chen, W.X. (2014b). Effects of nitric oxide on postharvest quality and soluble sugar content in papaya fruit during ripening. J.Food Proc.Preserv. *38*, 591-599 doi:10.1111/jfpp.12007.
Li, Y., Wills, R., and Golding, J. (2017). Interaction of ethylene concentration and storage temperature on postharvest life of the green vegetables pak choi, broccoli, mint, and green bean. J. Hortic. Sci. Biotechnol. *92*, 288-293 doi http://dx.doi.org/10.1080/14620316.2016.1263545.
Li, Z.G., Yang, S.Z., Long, W.B., Yang, G.X., and Shen, Z.Z. (2013). Hydrogen sulphide may be a novel downstream signal molecule in nitric oxide-induced heat tolerance of maize (*Zea mays* L.) seedlings. Plant, Cell Environ. *36*, 1564-1572 doi http://doi.org/10.1111/pce.12092.
Shi, H., Ye, T., and Chan, Z. (2014). Nitric oxide-activated hydrogen sulfide is essential for cadmium stress response in bermudagrass (*Cynodon dactylon* (L). Pers.). Plant Physiol. Biochem. *74*, 99-107 doi https://doi.org/10.1016/j.plaphy.2013.11.001.
Soegiarto, L., and Wills, R. (2004). Short term fumigation with nitric oxide gas in air to extend the postharvest life of broccoli, green bean, and bok choy. HortTechnology *14*, 538-540.
Wills, R., Ku, V., and Leshem, Y. (2000). Fumigation with nitric oxide to extend the postharvest life of strawberries. Postharvest Biol. Technol. *18*, 75-79 doi http://dx.doi.org/10.1016/S0925-5214(99)00061-7.
Wills, R., Pristijono, P., and Golding, J. (2015). Nitric oxide and postharvest stress of fruits, vegetables and ornamentals. In Nitric Oxide Action in Abiotic Stress Responses in Plants (Springer), pp. 221-238 doi http:// dx.doi.org/ 10.1007/978-3-319-17804-2_14.
Wilson, I.D., Neill, S.J., and Hancock, J.T. (2008). Nitric oxide synthesis and signalling in plants. Plant Cell Environ. *31*, 622-631 doi http:// dx.doi.org/ 10.1111/j.1365-3040.2007.01761.x
Zaharah, S.S., and Singh, Z. (2011). Postharvest nitric oxide fumigation alleviates chilling injury, delays fruit ripening and maintains quality in cold-stored 'Kensington Pride' mango. Postharvest Biol. Technol. *60*, 202-210. https://doi.org/10.1016/j.postharvbio.2011.01.011
Zhang, H., Hu, S.-L., Zhang, Z.-J., Hu, L.-Y., Jiang, C.-X., Wei, Z.-J., Liu, J., Wang, H.-L., and Jiang, S.-T. (2011). Hydrogen sulfide acts as a regulator of flower senescence in plants. Postharvest Biol. Technol. *60*, 251-257.
Zhao, Y., Biggs, T.D., and Xian, M. (2014). Hydrogen sulfide (H 2 S) releasing agents: chemistry and biological applications. Chemical Commun. *50*, 11788-11805 doi

http://dx.doi.org/10.1039/c4cc00968a
Zheng, J.-L., Hu, L.-Y., Hu, K.-D., Wu, J., Yang, F., and Zhang, H. (2016). Hydrogen sulfide alleviates senescence of fresh-cut apple by regulating antioxidant defense system and senescence-related gene expression. HortScience *51*, 152-158.
Zhu, L., Wang, W., Shi, J., Zhang, W., Shen, Y., Du, H., and Wu, S. (2014). Hydrogen sulfide extends the postharvest life and enhances antioxidant activity of kiwifruit during storage. J. Sci. Food Agric. *94*, 2699-2704 doi http://dx.doi.org/10.1002/jsfa.6613.

### 4.3 Comparison of $H_2S$ and 1-MCP to inhibit senescence of pak choy.

H.M.S. Al Ubeed, R.B.H. Wills, M.C. Bowyer, J.B. Golding. Comparison of hydrogen sulphide with 1-methylcyclopropene (1-MCP) to inhibit senescence of the leafy vegetable, pak choy. Postharvest Biology and Technology (2018) 137, 129– 133. https://doi.org/10.1016/j.postharvbio.2017.11.020.

**General background**

The action of 1-MCP in inhibiting ethylene induced senescence and thereby extending the storage life of many produce including leafy vegetables has been extensively studied. There are, however, no published informations comparing the effect of short-term fumigation of $H_2S$ and 1-MCP alone or in sequence on senescence. This paper reports on such effects on the market life, ion leakage, and ethylene production and respiration rate of pak choy during subsequent storage in air that was ethylene-free or contained 0.1 µL/L ethylene.

**Summary of Results**

$H_2S$ and 1-MCP, were equally effective in increasing market life (i.e. inhibiting loss of green colour) and inhibiting respiration, ion leakage and ethylene production when pak choy was stored in an ethylene-free atmosphere. When produce were ventilated with 0.1 µL/L ethylene, 1-MCP was more effective in inhibiting loss of green colour, respiration and ethylene production than $H_2S$. Sequential fumigation with 1-MCP then $H_2S$ showed no difference in any senescence factor to produce that was fumigated with 1-MCP alone.

**Conclusion**

1- MCP would appear to be a more reliable postharvest treatment for pack choy than $H_2S$ as it was more effective in the presence of exogenous ethylene. The study concluded that the mode of action of $H_2S$ was by inhibiting the action of ethylene as well as by inhibiting ethylene production. In a low ethylene environment, inhibition of ethylene production would be sufficient to inhibition of senescence. However, when exogenous ethylene is present, the primary mode of action of $H_2S$ must be by inhibiting the action of ethylene. It is unlikely that $H_2S$ binds to the same ethylene receptor site as 1-MCP as it would need to deprotonate to bind strongly and there would be a different allosteric effect on the receptor from coordination of $H_2S$ than of 1-MCP. It is noted that a range of ethylene receptors have been documented in the literature. Whatever the bonding site of $H_2S$, it must be less tightly bound than 1-MCP and can be partially displaced by exogenous ethylene to cater for the greater inhibition by 1-MCP in the presence of ethylene. The lack of any cumulative effect of 1-

MCP and $H_2S$ could be due to the stronger binding of 1-MCP to its receptor or that 1-MCP binds to a receptor that is more central to the action of ethylene.

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# Comparison of hydrogen sulphide with 1-methylcyclopropene (1-MCP) to inhibit senescence of the leafy vegetable, pak choy

H.M.S. Al Ubeed[a,b], R.B.H. Wills[a,*], M.C. Bowyer[a], J.B. Golding[a,c]

[a] School of Environmental and Life Sciences, University of Newcastle, Ourimbah NSW 2558, Australia
[b] Department of Life Sciences, Faculty of Science, Al-Muthana University, Al-Muthana, Iraq
[c] NSW Department of Primary Industries, Ourimbah NSW 2258, Australia

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ABSTRACT

Postharvest fumigation with hydrogen sulphide ($H_2S$) can inhibit senescence of a range of fruit and vegetables. It has been suggested that the mode of action of hydrogen sulphide is through inhibiting either the endogenous production and/or the action of ethylene. This study compared the effect of fumigation with 250 μL $L^{-1}$ hydrogen sulphide and 10 μL $L^{-1}$ 1-methylcyclopropene (1-MCP) on a range of factors associated with senescence of the leafy vegetable, pak choy stored at 10 °C and ventilated with ethylene-free air or air containing 0.1 μL $L^{-1}$ ethylene. When pak choy was stored in an ethylene-free atmosphere, hydrogen sulphide and 1-MCP were equally effective in inhibiting the loss of green colour, respiration, ion leakage and endogenous ethylene production. However, for containers ventilated with ethylene, 1-MCP was more effective in inhibiting loss of green colour, respiration and ethylene production than hydrogen sulphide. Sequential fumigation with 1-MCP followed by hydrogen sulphide showed no difference in any quality factor to produce fumigated with 1-MCP. The study concluded that (i) the mode of action of hydrogen sulphide included inhibiting the action of ethylene and (ii) for pak choy, 1-MCP was a more effective fumigation treatment than hydrogen sulphide.

## 1. Introduction

Hydrogen sulphide ($H_2S$) has been identified as a gaseous plant growth regulator that affects diverse plant physiological functions such as germination, stomatal movement, root development and flower senescence (Hancock and Whiteman, 2016). A role for hydrogen sulphide in regulation of postharvest senescence was first reported by Zhang et al. (2011) who found delayed senescence in cut flowers and shoot explants. Beneficial effects of postharvest fumigation with hydrogen sulphide have now been extended to a wide range of fruit and vegetables with an increase in postharvest life achieved through the inhibition of various senescence characteristics (Fotopoulos et al., 2015). However, the mode of action of hydrogen sulphide in inhibiting senescence remains unclear but some interaction with ethylene would seem likely given the central role of ethylene in promoting ripening and senescence (Abeles et al., 1992). The only reported study on hydrogen sulphide and ethylene production was by Al Ubeed et al. (2017) who found that hydrogen sulphide inhibited ethylene production by the green leafy vegetable, pak choy. They suggested that the mode of action of hydrogen sulphide in delaying senescence could be by inhibiting endogenous production of ethylene and/or by inhibiting the action of ethylene.

The discovery of 1-methylcyclopropene (1-MCP) as a competitive inhibitor of the action of ethylene (Sisler and Blankenship, 1996) was a landmark development in postharvest technology. 1-MCP is a competitive inhibitor of ethylene perception and acts by strongly binding to ethylene receptor sites thus preventing binding and the subsequent signalling that triggers ripening and senescence. Numerous subsequent studies have since shown that 1-MCP can extend the postharvest life of a wide range of commodities (Watkins, 2015). Included in such studies, Able et al. (2005) found that 1-MCP extended the shelf life of pak choy by inhibiting loss of green colour when 1 μL $L^{-1}$ ethylene was present in the atmosphere around produce.

Since both hydrogen sulphide and 1-MCP have been shown to inhibit senescence of pak choy, this study compared the effect of fumigating pak choy (*Brassica rapa* subsp. *Chinensis* − also known as bok or pok choy, choi or tsoi) in both the presence and absence of exogenous ethylene. The study was undertaken by fumigating pak choy leaves with hydrogen sulphide, 1-MCP and sequential fumigation of 1-MCP followed by hydrogen sulphide. Treated produce was then stored at 10 °C and ventilated with ethylene-free air (< 0.001 μL $L^{-1}$ ethylene) or air containing 0.1 μL $L^{-1}$ ethylene. Quality measures determined were loss

* Corresponding author.
*E-mail address:* Ron.Wills@newcastle.edu.au (R.B.H. Wills).

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of green colour (as the primary measure of consumer acceptance), respiration (as a measure of general metabolism), ion leakage (as a measure of cellular integrity), and endogenous ethylene production as the primary driver of senescence,

## 2. Materials and methods

### 2.1. Produce

Pak choy plants (cv. 'Shanghai') were harvested from a local commercial farm at Mangrove Mountain, NSW, Australia, and transported to the laboratory within two hours. Pak choy heads selected for the study were of uniform size (10 cm length) and colour and without damage to leaves or stem. The four outside leaves from each head were selected and gently cleaned under running tap water. The leaves from all heads were randomly distributed into 12 treatment units with each containing a total of 25 leaves that weighed between 250 and 350 g. Experiments were replicated with pak choy obtained from the same farm on three occasions over a one month period.

#### 2.1.1. Treatments and experiment design

The pak choy leaves in each treatment unit were placed into separate 4 L containers that were fitted with inlet and outlet ports and held at 20 °C. After two hours at 20 °C, groups of three units were sealed and treated with one of the following:

- Fumigation with hydrogen sulphide vapour at a concentration of 250 μL $L^{-1}$: Hydrogen sulphide gas was generated *in situ* by adding solid $NaHS.H_2O$ (Sigma Aldrich, Australia) (2.4 mg) into a dry beaker that was placed into a container. The container was then sealed and water (2 mL) was injected into the beaker through a septum in the container lid to instantaneously and quantitatively liberate hydrogen sulphide gas (Zhao et al., 2014). Containers remained sealed for four hours at 20 °C then exposed to ambient air for five hours.
- Fumigation with 1-MCP at a concentration of 10 μL $L^{-1}$: 1-MCP gas at the desired concentration was generated *in situ* by placing SmartFresh™ powder (0.14% of active ingredient) (supplied by AgroFresh, Australia) (66.4 mg) into a dry beaker in a container which was then sealed. Water (2 mL) was injected into the beaker through a septum in the container lid to liberate 1-MCP gas. Containers remained sealed for four hours at 20 °C then exposed to ambient air for five hours.
- Fumigation with 1-MCP at 10 μL $L^{-1}$ followed by fumigation with hydrogen sulphide at 250 μL $L^{-1}$: Using the above methods, containers of produce were fumigated with 1-MCP for four hours, followed by one hour in ambient air then fumigated with hydrogen sulphide for four hours.

The concentrations of hydrogen sulphide and 1-MCP were selected used as the respective optimum concentrations reported by Al Ubeed et al. (2017) and Sonnthida Sambath, University of Newcastle, unpublished data) for pak choy. The control treatment was left untreated in unsealed containers held in ambient air at 20 °C for nine hours.

After nine hours, all the containers were sealed, placed at 10 °C and ventilated with ethylene-free air or air containing 0.1 μL $L^{-1}$ ethylene at a flow rate of 45 mL $min^{-1}$. Ethylene-free air was generated by passing compressed ambient air through a tube filled with potassium permanganate adsorbed onto alumina pellets. The ethylene concentration of the air was analysed as < 0.001 μL $L^{-1}$ which was the analytical limit of detection. The 0.1 μL $L^{-1}$ ethylene gas stream was achieved by mixing the ethylene-free air with a regulated flow of ethylene from a cylinder (1 mL $L^{-1}$ ethylene in air, BOC Gases, Sydney). Both gas streams were humidified by bubbling through water held in a 2 L glass jar (height 2.25 m) to ensure a high humidity of 97–99% RH was maintained in the gas stream to minimise water loss.

### 2.2. Quality assessment

Leaves in each treatment unit were visually assessed daily for green colour and analysed after two, four and six days at 10 °C for respiration rate (as evolved carbon dioxide) and endogenous ethylene production. Ion leakage was destructively assessed after three and six days storage.

#### 2.2.1. Visual leaf colour (market life)

Visual assessment of the change in leaf colour from green to yellow of individual leaves used a scoring scale of 0–5 where 0 = green, 1 = 10%, 2 = 20%, 3 = 30%, 4 = 50% and 5 = > 70% loss of original green colour (Li et al., 2017). The mean colour score of all leaves in a treatment unit was calculated daily. An average colour score of 3.0 was considered to be the limit of consumer acceptability and the time for leaves to reach a mean score of 3.0 was designated as the market life. Assessment of a unit was terminated when the mean score of 3.0 was attained.

#### 2.2.2. Respiration rate

A container of produce was sealed to allow the accumulation of a measurable concentration of carbon dioxide. A gas sample (1 mL) was collected in a syringe from the atmosphere of a sealed container after four hours. The concentration of carbon dioxide in the gas sample was determined by injecting into a thermal conductivity gas chromatograph as described by Huque et al. (2013) and the respiration rate calculated.

#### 2.2.3. Ethylene production

The ethylene concentration in the ventilating gas stream was monitored at regular intervals at the inlet port with a gas sample (1 mL) that was analysed by flame ionization gas chromatography as described by Huque et al. (2013). Endogenous ethylene production of pak choy during storage was determined by sealing a container and immediately collecting a gas sample (1 mL) that was analysed for the exogenous background ethylene concentration. After three hours, another gas sample was collected and analysed for ethylene concentration. The difference in the two readings was used to calculate the rate of endogenous ethylene production.

#### 2.2.4. Ion leakage

At each assessment, five leaves were chosen at random from the 25 leaves in a unit and used to determine the ion leakage using the method described by Lu (2007).which involved collecting two disks (5 mm diameter) from each leaf and immersing the disks in distilled water (40 mL) for two hours at 25 °C when the conductivity of the solution was measured with a conductivity meter (Model 4071, Jenway, Staffordshire, UK). The solution was then boiled for 15 min and after cooling to room temperature (20 °C), the total conductivity was re-measured. Ion leakage was calculated as the percentage change in conductivity from the initial to final value.

### 2.3. Statistical analysis

Data from each experiment were analysed by two-way analysis of variance (ANOVA) and where a significant difference between treatments was found the least significant difference (LSD) at $P = 0.05$ was calculated. Statistical procedures were performed using SPSS for Windows version 22.0 software package (SPSS Chicago, IL).

## 3. Results

### 3.1. Market life

The market life, as designated by the time for pak choy leaves to lose 30% of green colour intensity (score 3), was found to be significantly affected ($P < 0.001$) by hydrogen sulphide and 1-MCP when stored in air or in ethylene at 10 °C. Table 1 shows that the market life

**Table 1**
Market life of pak choy fumigated with hydrogen sulphide and 1-MCP then ventilated with ethylene-free air or 0.1 μL $L^{-1}$ ethylene during storage at 10 °C.

| Treatment | Market life (days) | |
|---|---|---|
| | Stored in air | Stored in ethylene |
| Control | 9.3[a] | 8.0[a] |
| $H_2S$ | 11.8[b] | 10.3[b] |
| 1-MCP | 11.6[b] | 11.4[c] |
| 1-MCP + $H_2S$ | 12.2[b] | 12.1[c] |
| *LSD* | 0.72 | 0.77 |

Different batches of pak choy were stored in air and in ethylene. Each value is the mean of nine treatment units (3 replicates × 3 batches of produce). Treatments in each column not sharing the same superscript letter are significantly different at $P = 0.05$.

of pak choy stored in air was increased by the fumigation treatments but there was no significant difference between hydrogen sulphide, 1-MCP and hydrogen sulphide + 1-MCP. As expected, the market life of pak choy of control leaves was reduced by the addition of ethylene into the storage atmosphere but there was a differential effect between the applied treatments; fumigation with hydrogen sulphide + 1-MCP and 1-MCP were not significantly different but were both greater than the market life of leaves fumigated with hydrogen sulphide.

### 3.2. Respiration rate

The respiration rate was also measured on the same leaves that were used to determine market life. There was a significant interaction between the treatment and storage time for pak choy stored in air ($P < 0.01$) and in ethylene ($P < 0.05$). The results presented in Table 2 firstly show that the respiration rate of untreated pak choy leaves held in air increased during storage. There was no significant difference between control and fumigated leaves after two days storage in air but all fumigation treatments had a significantly lower respiration rates than control leaves after four and six days. The only significant difference between the three fumigation treatments was at six days where leaves fumigated with hydrogen sulphide has a higher respiration rate than leaves fumigated with 1-MCP or 1-MCP + hydrogen sulphide which were not signficantly different.

Table 2 also shows that the addition of ethylene into the storage atmosphere increased the respiration rate of all treatments. Untreated control leaves showed an increase in respiration during storage but the fumigation treatments showed a different response. All fumigation treatments had a significantly lower respiration rate than control leaves

**Table 2**
Respiration rate of pak choy fumigated with hydrogen sulphide and 1-MCP then ventilated with ethylene-free air or 0.1 μL $L^{-1}$ ethylene during storage at 10 °C.

| Treatment | Respiration rate (ng $kg^{-1}$ $s^{-1}$) | | |
|---|---|---|---|
| | 2 d | 4 d | 6 d |
| Stored in air | | | |
| Control | 4.3[c] | 5.4b | 6.2[a] |
| $H_2S$ | 3.4[cde] | 3.0[de] | 4.3[c] |
| 1-MCP | 3.8[cd] | 2.5[e] | 3.0[de] |
| 1-MCP + $H_2S$ | 3.6[cd] | 2.2[e] | 3.0[de] |
| *LSD* | | 1.2 | |
| Stored in ethylene | | | |
| Control | 6.8[c] | 8.0[b] | 9.1[a] |
| $H_2S$ | 5.3[d] | 3.8[ef] | 4.9[de] |
| 1-MCP | 4.2[ef] | 3.7[f] | 3.8[f] |
| 1-MCP + $H_2S$ | 4.0[ef] | 3.3[f] | 3.7[f] |
| *LSD* | | 1.1 | |

Each value is the mean of nine treatment units (3 replicates × 3 batches of produce). Values in each storage atmosphere not sharing the same superscript letter are significantly different at $P = 0.05$.

**Table 3**
Ion leakage of pak choy fumigated with hydrogen sulphide and 1-MCP then ventilated with ethylene-free air or 0.1 μL $L^{-1}$ ethylene during storage at 10 °C.

| Ventilating gas | Treatment | Ion leakage (%) | | |
|---|---|---|---|---|
| | | 3 d | 6 d | Mean[a] |
| Air | Control | 26.2 | 35.0 | 30.6[b] |
| Air | $H_2S$ | 16.4 | 21.0 | 18.7[a] |
| Air | 1-MCP | 16.0 | 20.9 | 18.5[a] |
| Air | 1-MCP + $H_2S$ | 14.6 | 21.9 | 18.2[a] |
| | *LSD* | | | 4.8 |
| Air | Control (air) | 20.8 | 42.2 | 31.5[a] |
| $C_2H_4$ | Control ($C_2H_4$) | 32.8 | 68.8 | 50.8[b] |
| $C_2H_4$ | $H_2S$ | 24.2 | 40.7 | 32.4[a] |
| $C_2H_4$ | 1-MCP | 25.6 | 40.9 | 33.2[a] |
| $C_2H_4$ | 1-MCP + $H_2S$ | 19.5 | 42.9 | 31.2[a] |
| | *LSD* | | | 11.2 |

Different batches of pak choy were stored in air and in ethylene. [a] Mean values for treatments are from 18 assessments readings (3 containers × 2 storage times × 3 batches of produce). LSD values are at $P = 0.05$ and mean values in each experiment not sharing the same superscript are significantly different.

at the three storage times but leaves fumigated with hydrogen sulphide had a higher respiration at two and six days than leaves fumigated with 1-MCP and 1-MCP + hydrogen sulphide which were not signficantly different.

### 3.3. Ion leakage

For leaves exposed to ethylene-free air, the level of ion leakage in pak choy leaves was significantly affected by treatment and storage time ($P < 0.001$) but there was no significant interaction between treatment and storage time. The data in Table 3 show that, as expected, ion leakage increased with increasing time and that it was significantly lower in the three fumigation treatments compared to control leaves with no significant difference between the different fumigation treatments.

The experiment was repeated on leaves that were ventilated with 0.1 μL $L^{-1}$ ethylene during storage but with the inclusion of an additional control treatment that was ventilated with air. There was a significant effect of treatment and storage time ($P < 0.001$) and the data in Table 3 show that the three fumigation treatments exhibited a similar reduction in ion leakage compared to the ethylene-ventilated control. Ion leakage of the air-ventilated control was significantly higher than that of the ethylene-ventilated control but was not significantly different to the three fumigation treatments that were ventilated with ethylene. Thus hydrogen sulphide and 1-MCP were equally effective in negating the deleterious effect of ethylene in increasing ion leakage.

### 3.4. Ethylene production

The rate of endogenous ethylene production of leaves stored in air and in ethylene showed a significant effect of treatment and storage time for pak choy stored in air and in ethylene ($P < 0.001$) but there was no significant interaction between treatment and storage time. The data in Table 4 show that for pak choy stored in air, the three fumigation treatments had a significantly lower rate of endogenous ethylene production than control leaves but with no significant difference between the fumigation treatments.

The addition of ethylene into the storage atmosphere markedly decreased the rate of endogenous ethylene production in all treatments. However, control leaves still had a significantly higher endogenous ethylene production than the fumigation treatments but fumigation with hydrogen sulphide was not as effective as fumigation with 1-MCP and 1-MCP + hydrogen sulphide in reducing ethylene production.

Thus, fumigation with hydrogen sulphide was not as effective in

**Table 4**
Ethylene production of pak choy fumigated with hydrogen sulphide and 1-MCP then ventilated with ethylene-free air or 0.1 μL $L^{-1}$ ethylene during storage at 10 °C.

| Treatment | $C_2H_4$ production (ng $kg^{-1}$ $s^{-1}$) | | | |
|---|---|---|---|---|
| | 2 d | 4 d | 6 d | Mean[a] |
| Stored in air | | | | |
| Control | 0.24 | 0.17 | 0.09 | 0.17[b] |
| $H_2S$ | 0.13 | 0.09 | 0.07 | 0.09[a] |
| 1-MCP | 0.15 | 0.10 | 0.07 | 0.10[a] |
| 1-MCP + $H_2S$ | 0.10 | 0.08 | 0.06 | 0.08[a] |
| *LSD* | | | | *0.024* |
| Stored in ethylene | | | | |
| Control | 0.096 | 0.062 | 0.048 | 0.065[c] |
| $H_2S$ | 0.068 | 0.045 | 0.034 | 0.049[b] |
| 1-MCP | 0.044 | 0.029 | 0.024 | 0.032[a] |
| 1-MCP + $H_2S$ | 0.042 | 0.030 | 0.028 | 0.033[a] |
| *LSD* | | | | 0.015 |

Different batches of pak choy were stored in air and in ethylene. [a] Mean values for treatments are from 27 assessments (3 replicates × 3 storage times × 3 batches of produce). LSD values are at $P = 0.05$ and mean values in each storage atmosphere not sharing the same superscript are significantly different.

reducing ethylene production as fumigation with 1-MCP while for pak choy stored in air all three fumigation treatments were equally effective in decreasing ethylene production.

## 4. Discussion

For pak choy stored in an ethylene-free air stream, fumigation with hydrogen sulphide or 1-MCP was found to be equally effective in inhibiting senescence as exhibited by extending the market life (i.e. inhibiting loss of green colour) and reducing the respiration rate and ion leakage. Sequential fumigation with 1-MCP followed by hydrogen sulphide did not generate any added benefit over a single fumigation of either compound.

When pak choy was stored in an exogenous ethylene stream, untreated produce showed a decrease in market life and an increase in respiration rate and ion leakage as would be expected from ethylene enhancing the rate of senescence. While all three fumigation treatments showed a beneficial response compared to untreated produce, fumigation with hydrogen sulphide was less effective than with 1-MCP in extending market life and reducing respiration while for ion leakage, hydrogen sulphide and 1-MCP were equally effective in inhibiting the increase during storage. For all three quality characteristics there was no added benefit from the combined fumigation of 1-MCP followed by hydrogen over that achieved by 1-MCP alone.

Comparison of the effects of the fumigation treatments when stored in air and ethylene showed that the market life and ion leakage of leaves fumigated with 1-MCP were not significantly different in the two ventilating atmospheres. This indicates that 1-MCP was fully able to negate the effect of exogenous ethylene on loss of chlorophyll and loss of cellular integrity. The increase in respiration rate due to the presence of ethylene, however, was not fully negated by 1-MCP. In contrast, hydrogen sulphide only fully negated the effects of exogenous ethylene for ion leakage.

Endogenous ethylene production of pak choy stored in air was decreased by all three fumigation treatments with no significant difference between treatments. When pak choy was stored in exogenous ethylene, endogenous ethylene production of leaves in control and all fumigation treatments was greatly reduced compared to leaves stored in air. Such an effect has been shown for a range of postharvest produce (Saltveit, 1999) including for pak choy (Al Ubeed et al., 2017). However, in addition, there was a greater reduction in loss of pak choy quality attributes of all fumigated leaves relative to control but the reduction in ethylene production achieved by hydrogen was not as great as with 1-MCP alone or in combination with hydrogen sulphide. This additional reduction in endogenous ethylene production by the fumigation treatments relative to control is consistent with an added negative feedback mechanism in operation for hydrogen sulphide and for 1-MCP. While the primary action of 1-MCP is considered to be through blocking the action of ethylene, the literature is ambivalent on the effect of 1-MCP on ethylene production. For leafy vegetables, Sun et al. (2012) reported that ethylene production of Chinese kale (*Brassica. alboglabra*) was strongly inhibited by 1-MCP whereas Kenigsbuch et al. (2007) reported mint leaves had increased ethylene production.

Thus for pak choy, hydrogen sulphide and 1-MCP appear to inhibit both the production of endogenous ethylene and the action of ethylene. In a low ethylene environment, inhibition of ethylene production could lead to inhibition of senescence. Indeed, Li et al. (2014) and Zheng et al. (2016) have shown that fumigation with hydrogen sulphide down regulated the expression of genes associated with ethylene biosynthesis of broccoli florets and apple slices, respectively. However, the inhibition of senescence by hydrogen sulphide in the presence of exogenous ethylene indicates that the primary mode of action of hydrogen sulphide could be by inhibiting the action of ethylene. One possibility is that hydrogen sulphide binds to the same ethylene receptor genes as 1-MCP. However, 1-MCP is reputed to bind through a copper I cofactor that requires a cysteine residue ($Cys_{65}$) to be present to successfully coordinate in the ETR1 binding domain (Lacey and Binder, 2014). While hydrogen sulphide could theoretically bind to the copper ion in the receptor, as it is of similar size to ethylene, it would likely need to deprotonate to bind strongly. In addition, the allosteric (shape change) effect on the receptor would almost certainly differ from coordination of hydrogen sulphide and 1-MCP (Pirrung et al., 2008). However, it is well documented that a range of ethylene receptors can be present in horticultural produce with five such receptors having been identified (Lacey and Binder, 2014) and hydrogen sulphide may bind to one or more of these systems. In a recent study on banana ripening, Ge et al. (2017) found that co-fumigation of hydrogen sulphide with ethylene up-regulated expression of ethylene receptor genes MaETR, MaERS1 and MaERS2, while suppressing expression of genes associated with ACC synthase (MaACS1, MaACS2) and ACC oxidase (MaACO1, MaACO2) compared to ethylene alone. In the presence of hydrogen sulphide, these factors were collectively proposed to reduce the ethylene response in bananas.

Whatever the bonding site of hydrogen sulphide, it must be less tightly bound than 1-MCP and can be partially displaced by exogenous ethylene to cater for the greater inhibition by 1-MCP of various aspects of senesence in the presence of ethylene. The lack of any cumulative effect of 1-MCP and hydrogen sulphide could be due to the stronger binding of 1-MCP to a receptor gene or that 1-MCP binds to a receptor that is more central to the action of ethylene. Regardless of the mode of action of hydrogen sulphide and 1-MCP, for pak choy, fumigation with 1-MCP would appear be a more effective commercial option to inhibit senescence than fumigation with hydrogen sulphide.

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## References

Abeles, F.B., Morgan, P.W., Saltveit, M.E., 1992. Ethylene in Plant Biology. Academic Press, New York.
Able, A.J., Wong, L.S., Prasad, A., O'Hare, T.J., 2005. The physiology of senescence in detached pak choy leaves (*Brassica rapa* var *chinensis*) during storage at different temperatures. Postharvest Biol. Technol. 35, 271–278.
Al Ubeed, H.M.S., Wills, R.B.H., Bowyer, M.C., Vuong, Q.V., Golding, J.B., 2017. Interaction of exogenous hydrogen sulphide and ethylene on senescence of green

leafy vegetables. Postharvest Biol. Technol. 133, 81–87.
Fotopoulos, V., Christou, A., Antoniou, C., Manganaris, G.A., 2015. Hydrogen sulphide: a versatile tool for the regulation of growth and defence responses in horticultural crops. J. Hortic. Sci. Biotechnol. 90, 227–234.
Ge, Y., Hu, K.D., Wang, S.S., Hu, L.Y., Chen, X.Y., Li, Y.H., Yang, F., Zhang, H., 2017. Hydrogen sulfide alleviates postharvest ripening and senescence of banana by antagonizing the effect of ethylene. PLoS One 12 (6), e0180113. http://dx.doi.org/10.1371/journal.pone.0180113.
Hancock, J.T., Whiteman, M., 2016. Hydrogen sulfide signaling: interactions with nitric oxide and reactive oxygen species. Annals N.Y. Acad. Sci. 1365, 5–14.
Huque, R., Wills, R.B.H., Pristijono, P., Golding, J.B., 2013. Effect of nitric oxide (NO) and associated control treatments on the metabolism of fresh-cut apple slices in relation to development of surface browning. Postharvest Biol. Technol. 78, 16–23.
Kenigsbuch, D., Chalupoicz, D., Aharon, Z., Maurer, D., Aharoni, N., 2007. The effect of $CO_2$ and 1-methylcyclopropene on the regulation of postharvest senescence of mint, *Mentha longifolia* L. Postharvest Biol. Technol. 43, 165–173.
Lacey, R.F., Binder, B.M., 2014. How plants sense ethylene gas − The ethylene receptors. J. Inorganic Biochem. 133, 58–62.
Li, S.P., Hu, K.D., Hu, Y., Li, Y.H., Jiang, A.M., Xiao, F., Han, Y., Liu, Y.S., Zhang, H., 2014. Hydrogen sulfide alleviates postharvest senescence of broccoli by modulating antioxidant defense and senescence-related gene expression. J. Agric. Food Chem. 62, 1119–1129.
Li, Y., Wills, R.B.H., Golding, J.B., 2017. Interaction of ethylene concentration and storage temperature on postharvest life of the green vegetables pak choi, broccoli, mint, and green bean. J. Hortic. Sci. Biotechnol. 92, 288–293.
Lu, S., 2007. Effect of packaging on shelf-life of minimally processed bok choy (*Brassica chinensis* L.). LWT-Food Sci. Technol. 40, 460–464.
Pirrung, M.C., Bleecker, A.B., Inoue, Y., Rodriguez, F.I., Sugawara, N., Wada, T., Zou, Y., Binder, B.M., 2008. Ethylene receptor antagonists: strained alkenes are necessary but not sufficient. Chem. Biol. 15, 313–321.
Saltveit, M.E., 1999. Effect of ethylene on quality of fresh fruits and vegetables. Postharvest Biol. Technol. 15, 279–292.
E.C. Sisler S.M. Blankenship, (1996) Methods of counteracting an ethylene response in plants US Patent 5.518 988.
Sun, B., Yan, H., Liu, N., Wei, J., Wang, Q., 2012. Effect of 1-MCP treatment on postharvest quality characters, antioxidants and glucosinolates of Chinese kale. Food Chem. 131, 519–526.
Watkins, C.B., 2015. Advances in the use of 1-MCP. In: Wills, R.B.H., Golding, J.B. (Eds.), Advances in Postharvest Fruit and Vegetable Technology. CRC Press, Boca Raton FL, pp. 117–145.
Zhang, H., Hu, S.L., Zhang, Z.J., Hu, L.Y., Jiang, C.X., Wei, Z.J., Liu, J., Wang, H.L., Jiang, S.T., 2011. Hydrogen sulfide acts as a regulator of flower senescence in plants. Postharvest Biol. Technol. 60, 251–257.
Zhao, Y., Biggs, T.D., Xian, M., 2014. Hydrogen sulfide ($H_2S$) releasing agents: chemistry and biological applications. Chem. Communic. 50, 11788–11805.
Zheng, J.L., Hu, L.Y., Hu, K.D., Wu, J., Yang, F., Zhang, H., 2016. Hydrogen sulfide alleviates senescence of fresh-cut apple by regulating antioxidant defense system and senescence-related gene expression. HortScience 51, 152–158.

### 4.4 Interaction of $H_2S$ inhibitor, propargylglycine (PAG) and $H_2S$ on the senescence of pak choy.

H.M.S. Al Ubeed, R.B.H. Wills, M.C. Bowyer, J.B. Golding. Interaction of the hydrogen sulphide inhibitor, propargylglycine (PAG), with hydrogen sulphide on postharvest changes of the green leafy vegetable, pak choy. Accepted for publication in Postharvest Biology and Technology.

**General background**

PAG, as an inhibitor of $H_2S$ synthesis, has been used in many studies to examine the effect of reduced $H_2S$ production on postharvest metabolism. Previous studies in this thesis indicated that $H_2S$ could be acting by both inhibiting ethylene synthesis and the action of ethylene. This study was conducted to explore this dual action hypothesis by examining the interaction of $H_2S$ and PAG on the senescence of pak choy in the presence of exogenous ethylene. Applied treatments were spraying with PAG, fumigation with $H_2S$ and both treatments in sequence and their effect on a range of senescence factors was determined during storage.

**Summary of Results**

Fumigation with $H_2S$ reduced the rate of loss of leaf green colour (i.e. extended market life), respiration rate, ethylene production, ion leakage and enhanced antioxidant activity of pal choy while leaves sprayed with PAG, as expected, showed opposite effects. However, the combined PAG + $H_2S$ treatment showed different effects on the various senescence factors: respiration was found to be similar to the $H_2S$ treatment, loss of green leaf colour was similar to the PAG single treatment and less than the untreated control, antioxidant activity was less than for PAG but higher than for control leaves, and ethylene production and ion leakage were similar to control leaves.

**Conclusion**

If the action of PAG was only by inhibiting $H_2S$ production, subsequent treatment with $H_2S$ should fully negate any effect induced on a senescence factor by PAG and it could be expected that the combined treatment would behave similarly to produce fumigated with $H_2S$ alone. However, respiration was the only senescence-related factor where the PAG + $H_2S$ treatment was not significantly different to leaves fumigated with $H_2S$. The other factors showed the combined PAG + $H_2S$ treatment generated effects that ranged from being similar to the PAG to being similar to control leaves. This indicates that PAG does not exclusively

act as an inhibitor of endogenous $H_2S$ production and thus has effects on metabolism that are not linked to the action of endogenous $H_2S$. The additional actions of PAG could be through blocking the activity of PHP which is a cofactor for numerous enzyme systems.

A copy of the paper submitted for publication in Postharvest Biology and Technology is appended.

# Interaction of the hydrogen sulphide inhibitor, propargylglycine (PAG), with hydrogen sulphide on postharvest changes of the green leafy vegetable, pak choy

**H.M.S. Al Ubeed,[ab] R.B.H. Wills,[a*] M.C. Bowyer, [a] J.B. Golding [ac]**

*Correspondence to: R.B.H. Wills, School of Environmental and Life Sciences, University of Newcastle, Ourimbah, NSW 2258, Australia, E-mail:Ron.Wills@newcastle.edu.au*

*a School of Environmental and Life Sciences, University of Newcastle, Ourimbah NSW 2558, Australia*

*b Department of Life Sciences, Faculty of Science, Al-Muthana University, Al-Muthana, Iraq*

*c New South Wales Department of Primary Industries, Ourimbah, NSW 2258, Australia*

* Corresponding author. Fax +61 2 94994437.

E-mail address: Ron.Wills@newcastle.edu.au (R.B.H. Wills)

## ABSTRACT

Propargylglycine (PAG) is an inhibitor of hydrogen sulphide ($H_2S$) production and has been used to explore the mode of action of $H_2S$ in prolonging storage of horticultural produce but little attention has been given to how PAG and $H_2S$ interact when both are applied to produce. This study examined the effect of sequential application of PAG and $H_2S$ on a range of postharvest senescence factors of the leafy vegetable pak choy (*Brassica rapa* subsp. *Chinensis)* stored at 10 °C. The results showed differential responses between factors when compared to application of PAG or $H_2S$ alone. As expected, fumigation with $H_2S$ reduced the rate of loss of leaf green colour, respiration rate, ethylene production, ion leakage and enhanced antioxidant activity and leaves sprayed with PAG showing converse effects. If PAG acted solely by inhibiting endogenous $H_2S$ production then subsequent treatment with $H_2S$ should fully negate any effect induced by PAG. However, for the combined PAG + $H_2S$ treatment, respiration was similar to untreated leaves, loss of green leaf colour was similar to the PAG single treatment and less than the untreated control, antioxidant activity was less than for PAG but greater than for control leaves, and ethylene production and ion leakage were similar to control leaves. Thus, the concept that PAG is exclusively an inhibitor of endogenous $H_2S$ production was not validated, with PAG having effects on metabolism that

are not linked to the action of endogenous $H_2S$. The additional actions of PAG could be through its inhibition of pyridoxyal-5'-phosphate (PHP) which is a coenzyme for numerous enzyme systems.

*Keywords*: PAG, hydrogen sulphide, senescence; pak choy

## 1. Introduction

Hydrogen sulphide ($H_2S$) was considered a toxic gas, but about 10 years ago was found to be synthesised in mammalian tissues and to have a mediating role in a wide range of cellular physiology functions (Li and Moore, 2008). It is now also known to also act as gaseous plant growth regulator, impacting a diverse range of plant physiological functions such as germination, stomatal movement, root development and flower senescence (Jin and Pei, 2015; Hancock and Whiteman, 2016). A role for $H_2S$ in the regulation of postharvest senescence is quite recent. Zhang et al. (2011) reported delayed senescence in cut flowers and shoot explants treated with solutions of the $H_2S$ donor, sodium hydrogen sulphide (NaHS). Fumigation with $H_2S$ was subsequently extended to a range of postharvest fruit and vegetables including strawberry (Hu et al, 2012), broccoli, (Li et al., 2014), peach (Wang, et al., 2014), mulberry (Hu et al., 2014), and banana (Ge et al., 2017) with an extension in storage life achieved through inhibition of a wide range of senescence characteristics. Work examining the physiological role of $H_2S$ in postharvest produce is limited. Li et al. (2014) and Zheng et al. (2016) showed that exogenous $H_2S$ treatment of broccoli florets and apple slices, respectively, down regulated the expression of genes associated with ethylene biosynthesis. Ge et al. (2017) extended this understanding, showing that $H_2S$ fumigation also upregulated ethylene receptor expression. Further, Al Ubeed et al. (2017) identified that senescence characteristics in the green leafy vegetables, pak choy, basil and kale were delayed by fumigation with $H_2S$ and speculated that the mode of action of $H_2S$ was through inhibition of ethylene production and action.

The ability to inhibit endogenous $H_2S$ production has been a key tool for understanding the physiological actions of $H_2S$ in living systems. A range of inhibitor compounds have been identified including L-propargylglycine which has been widely employed in both animal and plant physiology to probe $H_2S$ activity. García-Mata and Lamattina (2010) utilised DL-propargylglycine (designated in this paper as PAG) to confirm $H_2S$ involvement

in controlling stomatal closure in *Vicia faba* and *Aridopsis thaliana*. More recently, links between $H_2S$ homeostasis and the expression of senescence characteristics in postharvest produce has been investigated using PAG. Li et al. (2014) found the exogenous $H_2S$ treatment of broccoli florets enhanced endogenous $H_2S$ production leading to delayed onset of senescence characteristics and enhanced metabolic activity, while PAG directly lowered LCD and DCD activities, thereby reducing endogenous $H_2S$ levels leading to accelerated senescence. Liu et al. (2017) identified similar effects on daylily with a range of senescence characteristics enhanced by spraying with PAG and inhibited by fumigation with $H_2S$.

If PAG acted solely on endogenous $H_2S$ production, it could be expected that the addition of exogenous $H_2S$ should negate the inhibitory effect of PAG. The only study where PAG and $H_2S$ have been applied sequentially produce was by Hu et al. (2015) with the leafy vegetable, water spinach (*Ipomea aquatica*). They reported that fumigation with $H_2S$ after spraying with PAG only partially negated the inhibition of $H_2S$ synthesis induced by PAG. In this study we further examined the interaction of PAG and $H_2S$ by measuring a range of postharvest changes of pak choy leaves sequentially treated with PAG and $H_2S$ and comparing these effects to responses of leaves treated with a single application of PAG or $H_2S$.

## 2. Materials and methods:

### *2.1. Produce*

Pak choy plants (*Brassica rapa* subsp. *Chinensis* cv. Shanghai) (also known as bok or pok choy, choi or tsoi)) were sourced from a local farm at Mangrove Mountain, New South Wales and transported to the laboratory within two hours of harvest. Pak choy heads were cut and a specific number of outside leaves (the number varying between different experiments) were selected and gently cleaned with tap water. The leaves from each head were randomly distributed into the required number of treatment units, with each containing 24 leaves that weighed about 400 g. Each treatment unit was placed into a sealable plastic container (4 L) fitted with an inlet and outlet tubes in the lid. All experiments were replicated by obtaining batches of plants on separate occasions with at least two weeks between batches.

### *2.2. Treatments*

Treatments applied to a container of pak choy leaves at 20 °C were:

1. PAG: each side of each leaf in a treatment unit was sprayed with 0.1 mL of an aqueous solution containing 2 mM PAG (Sigma-Aldrich, Australia) then left to air dry at 20 °C. This concentration was shown to give optimum inhibition of LCD and DCD activity in previous studies (Cui et al. 2014, Hu et al. 2015).
2. Control treatments: leaves in a treatment unit remained untreated or were similarly sprayed with water.
3. $H_2S$: treatment units were fumigated for four hours with 250 µL $L^{-1}$ $H_2S$, the optimum concentration reported by Al Ubeed et al. (2017) to inhibit senescence of pak choy. The gas was generated *in situ* by the addition of water to solid NaHS using the method described by Zhao et al. (2014).
4. PAG + $H_2S$: leaves were sprayed with PAG or water and left to dry for three hours then fumigated with $H_2S$ for four hours as per the above treatments.

After seven hours, all containers in an experiment were sealed at 10 °C and ventilated through the inlet tube with air containing 0.1 µL $L^{-1}$ ethylene or ethylene-free air at 45 mL $min^{-1}$.

### *2.3. Physio-chemical assessments*

Leaves in each unit were visually assessed daily for green colour and the time for each unit to develop an unacceptable colour (denoted as the market life) was determined using the scoring scale given below. Respiration rate, as evolved carbon dioxide, and ethylene production were assessed at various times during storage.

#### *2.3.1. Visual leaf colour (market life)*

The change in leaf colour from green to yellow of individual leaves was conducted daily by visual assessment using a scoring scale of 0-5 where 0 = green, 1 = 10%, 2 = 20%, 3 = 30%, 4 = 50% and 5 =>70% loss of original green colour. The mean colour score of all leaves in a treatment unit was calculated daily. An average colour score of 3.0 was considered to be the limit of consumer acceptability and the time for leaves to reach a mean score of 3.0 was designated as the market life of that unit.

### *2.3.2. Respiration rate and ethylene*

After various times in storage, the respiration rate was measured as carbon dioxide evolution. A container containing a treatment unit of pak choy was sealed to allow the accumulation of a measureable concentration of carbon dioxide. A gas sample (5 mL) was collected in a syringe after four hours and the concentration of carbon dioxide in the sample was determined by injecting into a thermal conductivity gas chromatograph as described by Huque et al. (2013). The respiration rate was calculated as μg $CO_2$ $kg^{-1}$ $s^{-1}$.

The concentration of ethylene in atmosphere was determined by a collecting a gas sample (1 mL) and analysing by flame ionization gas chromatography as described by Huque et al. (2013). Samples were obtained from ventilated treatment units just before sealing the container and again three hours after sealing. The difference between readings was used to calculate the rate of ethylene production as ng $kg^{-1}$ $s^{-1}$.

### *2.3.3. Ion leakage*

Ion leakage was determined according to the method described by Lu (2007) after three days storage using five leaves selected from a treatment unit. This involved collecting two disks (50 mm diameter) from each leaf. The disks were immediately immersed in double distilled water (40 mL) in glass vials and incubated for two hours at 25 °C. The conductivity of the solution was then measured with a conductivity meter (Model 4071, Jenway, Staffordshire, UK). The solution was then boiled for 15 min. and after cooling to room temperature, the total conductivity was re-measured. Ion leakage was calculated as the percentage of the initial to final value.

### *2.3.4. Antioxidant activity*

Antioxidant activity was determined after three days of storage using two samples of leaf (each about 4g) that were cut from the top part of five leaves from a treatment unit, ground using a mortar and pestle then mixed with 50% methanol (50 mL). The mixture was placed in an ultrasonic bath (Soniclean, Australia) set at 35 °C and 100 W for 30 min. before being filtered through filter paper (Whatman Grade 5). The filtrate was stored at -20 °C until analysed. Antioxidant activity was determined with 2,2-diphenyl-picrylhydrazine (DPPH), using the method described by Vuong et al. (2013). Briefly, a stock solution of DPPH (24

mg/100 mL methanol) was prepared and stored at −20 °C until required. The working solution was freshly prepared by diluting 10 mL stock solution with 45 mL methanol to obtain an absorbance (λ = 515 nm) of 1.1±0.02. Pak choy extracts (150 µL) were mixed with DPPH solution (2.85 mL) and incubated at room temperature in the dark for one hour. The solution absorbance was then recorded and quantified as mg Trolox equivalent (TE) 100 $g^{-1}$ fresh pak choy using a standard curve of Trolox against absorbance

### *2.4. Statistical analysis*

Data were analysed by two-way analysis of variance (ANOVA) and where a significant difference between treatments was found, the least significant difference (LSD) of the mean values at $P$ = 0.05 was calculated. Statistical procedures were performed using SPSS for Windows, version 22.0 software package (SPSS Chicago, IL).

## 3. Results

### *3.1. Leaf colour (market life)*

The effect of PAG on the change in leaf colour from green to yellow of pak choy, assessed as the time to a fixed colour score (3.0) which is designated as the market life, is shown in Table 1. Spraying leaves with PAG significantly decreased the market life relative to the respective control leaves (water spray or untreated). Fumigation with $H_2S$ significantly increased market life relative to control leaves. When leaves were treated with both PAG and $H_2S$, market life was significantly greater than in leaves sprayed with PAG alone but lower than control leaves. Thus, fumigation with $H_2S$ did not fully negate the inhibitory effect of PAG. Since there was no significant difference between water-sprayed and untreated leaves, in all subsequent experiments, the control treatment was sprayed with water.

### *3.2. Respiration rate*

Figure 1 shows that the respiration rate of pak choy leaves was differentially affected by the treatments. Spraying with PAG resulted in a significantly higher respiration than control (water sprayed) leaves from 2 days of storage, while fumigation with $H_2S$ resulted in a lower respiration rate than control leaves at 4 days. The respiration rate of the combined PAG-$H_2S$ treatment was not significantly different to $H_2S$ alone. Thus, fumigation with $H_2S$ fully reversed the stimulatory effect of PAG on respiration.

### *3.3. Ethylene production*

Figure 2 shows that ethylene production of control leaves ventilated with ethylene-free air decreased during storage. Spraying leaves with PAG significantly increased ethylene production at three and six days storage while fumigation with $H_2S$ significantly decreased ethylene production from one day in storage. The combined PAG-$H_2S$ treatment showed a similar reduction in ethylene production as $H_2S$ alone at one day but at three and six days, ethylene production was similar to control leaves. The data thus indicate that the effect of PAG on ethylene production did not materialise until three days storage whereas $H_2S$ had impacted on ethylene production early in storage.

### *3.4. Ion leakage and antioxidant activiy*

Ion leakage was determined after three days storage and Table 2 shows that ion leakage was significantly increased by PAG and decreased by $H_2S$ compared to control leaves. Application of both PAG and $H_2S$ resulted in an ion leakage that was not significantly different to that of control leaves.

Leaves from the same treatment units used for assessment of ion leakage were also used to determine antioxidant activity. Table 2 shows that there was no significant difference in antioxidant activity between control and PAG-treated leaves but leaves fumigated with $H_2S$ had a significantly higher antioxidant activity. Treatment of leaves with both PAG and $H_2S$ resulted in an antioxidant activity that was significantly lower than $H_2S$ alone but higher than control leaves.

## 4. Discussion

The results show that fumigation of pak choy with $H_2S$ inhibited senescence, as expressed by a reduced rate of loss of leaf green colour (i.e. extended market life), respiration, ethylene production, ion leakage and enhanced antioxidant activity. These effects confirm the findings of Al Ubeed et al. (2017) that postharvest application of $H_2S$ inhibited the senescence of pak choy. This study also showed the converse effect when pak choy leaves sprayed with PAG, accelerated senescence through enhanced green colour loss, respiration, ethylene production and ion leakage, although there was no statistically significant effect on antioxidant activity. These effects are consistent with findings of Hu et al. (2015), Liu et al. (2017) and Li et al. (2014) who found PAG enhanced senescence of water spinach, daylily flowers and broccoli florets respectively.

The synthesis of $H_2S$ in both animals and plants occurs by a variety of enzyme systems which are all pyridoxyal-5'-phosphate (PHP) dependent (Kamoun, 2004; Jin and Pei, 2015). The mode of action of PAG in both animals and plants is attributed to its ability to irreversibly bind to PHP to yield an inactive product (Abeles and Walsh, 1973). This then results in inhibition of $H_2S$ synthesis by blocking the action of the associated PHP cofactor (Guo et al. 2017; Romero et al. 2014). However, PHP is also involved in all cysteine pathways in plants, with many unrelated to $H_2S$ synthesis (Eliot and Kirsch, 2004) with more than 140 enzyme systems identified as being PHP dependent (Richard et al., 2009). If PAG acted solely by inhibiting endogenous $H_2S$ production, it could be expected that treatment with PAG followed by exogenous $H_2S$ would fully negate the diminished endogenous $H_2S$ production induced by PAG. However, respiration was the only senescence-related factor where the PAG + $H_2S$ treatment was not significantly different to that in pak choy fumigated with $H_2S$ alone.

At the other end of the effectiveness scale was loss of green leaf colour, where pak choy treated with PAG + $H_2S$ had a market life that was close to that of the PAG single treatment and was less than the untreated control. This indicates that the exogenous $H_2S$ only weakly nullified the effect of PAG. This is consistent with results presented by Hu et al. (2015) showing that sequential treatment of water spinach with PAG followed by $H_2S$ resulted in only a partial restoration of endogenous $H_2S$ production. Assuming that the loss of green leaf colour was associated with loss of chlorophyll, the effect of PAG on chlorophyll degradation would thus seem to be largely on systems that are independent of $H_2S$ synthesis. Our findings would appear to question conclusions drawn by Li et al. (2014) for broccoli that chlorophyll

retention is predominantly influenced by endogenous $H_2S$ production. It is therefore plausible to assume that as yet unmapped PAG influences outside endogenous $H_2S$ production exist.

The effect on antioxidant activity, as measured by the free radical scavenger DPPH (Okawa et al, 2001) differed again. While PAG alone had no significant effect on antioxidant activity, the PAG + $H_2S$ treatment significantly reduced the increase in antioxidant activity effected by $H_2S$ alone but it was only a partial neutralisation of $H_2S$ as the level was greater than for control leaves. This suggests that for antioxidant activity, the activity of PAG is not solely related to $H_2S$ synthesis. The result is supported by Yu et al. (2013) who speculated that alleviation of inhibited antioxidant capacity in salt stressed cucumber hypocotyls and radicles by exogenous $H_2S$ may be linked to $H_2S$-induced stimulation of glutathione synthesis, a known antioxidant metabolite in plants (Riemenschneider et al., 2005).

The effect of the PAG + $H_2S$ treatment had a different effect on the rate of ethylene production during storage. After one day of storage, ethylene production of the combined treatment was not significantly different to that generated by $H_2S$ alone but at three and six days, the rate of production was not significantly different to control leaves. This could be explained by $H_2S$ having a rapid effect on ethylene production which is seen at one day but the effect of PAG on ethylene production is not generated until three days when it negates the effect of $H_2S$ and thus has a similar production rate to control leaves. Ion leakage showed a similar response with the PAG + $H_2S$ treatment being not significantly different to control leaves but was greater than $H_2S$ and lower than PAG. Thus, the beneficial effect of $H_2S$ would seem to largely neutralise the detrimental action of PAG on ethylene production and ion leakage.

Thus, the concept that PAG is exclusively an inhibitor of endogenous $H_2S$ production would seem to be an oversimplification, with PAG potentially having effects on pak choy metabolism that are not linked to the action of endogenous $H_2S$. The additional actions of PAG would seem to be linked to its inhibition of PHP which is a coenzyme for numerous enzyme systems. It can therefore be misleading to conclude that effects from the application of PAG are entirely due to effect on endogenous $H_2S$ production. However, it is recognised that we applied a single transient exposure to $H_2S$ and continuous exposure to exogenous $H_2S$ may generate different responses. Notwithstanding, it could be expected that leaves absorbed sufficient $H_2S$ during the four hour exposure to maintain its effect beyond the exposure period.

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**References**

Abeles, R.H., Walsh, C.T. 1973. Acetylenic enzyme inactivators. Inactivation of gamma-cystathionase, in vitro and in vivo by propargylglycine. *J. Amer. Chem. Soc. 95,* 6124-6125.

Al Ubeed, H.M.S., Wills, R.B.H., Bowyer, M.C., Vuong, Q.V., Golding, J.B, 2017. Interaction of exogenous hydrogen sulphide and ethylene on senescence of green leafy vegetables. Postharvest Biol. Technol. 133, 81-87.

Cui, W.T., Chen, H.P., Zhu, K.K., Jin, Q.J., Xie, Y.J., Cui, J., Xia, Y., Zhang, J., Shen, W.B., 2014. Cadmium-induced hydrogen sulfide synthesis is involved in cadmium tolerance in *Medicago sativa* by reestablishment of reduced (Homo) glutathione and reactive oxygen species homeostasis. PLoS One 9, e109669.

Eliot, A.C., Kirsch, J.F., 2004. Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. Annu. Rev. Biochem. 73, 383–415.

García-Mata, C., Lamattina, L., 2010. Hydrogen sulphide, a novel gasotransmitter involved in guard cell signalling. New Phytol. 188, 977-984.

Ge, Y., Hu, K.D., Wang, S.S., Hu, L.Y., Chen, X.Y., Li, Y.H., Yang, Y., Yang, F., Zhang, H. 2017. Hydrogen sulfide alleviates postharvest ripening and senescence of banana by antagonizing the effect of ethylene. PloS One, 12, e0180113.

Guo, H., Zhou, H., Zhang, J., Guan, W., Xu, S., Shen, W., Xu, G., Xie, Y., Foyer, C.H., 2017. L-cysteine desulfhydrase-related $H_2S$ production is involved in OsSE5- promoted ammonium tolerance in roots of *Oryza sativa*. Plant Cell Environ. 40, 1777- 1790.

Hancock, J.T., Whiteman, M., 2016. Hydrogen sulfide signaling: interactions with nitric oxide and reactive oxygen species. Annal New York Acad. Sci. 1365, 5–14.

Hu, L.Y., Hu, S.L., Wu, J., Li, Y.H., Zheng, J.L., Wei, Z.J., Liu, J., Wang, H.L., Liu, Y.S., Zhang, H., 2012. Hydrogen sulfide prolongs postharvest shelf life of strawberry and plays an antioxidative role in fruits. J. Agric. Food Chem. 60, 8684–8693.

Hu, H., Liu, D., Li, P., Shen, W., 2015. Hydrogen sulfide delays leaf yellowing of stored water spinach (*Ipomoea aquatica*) during dark-induced senescence by delaying chlorophyll breakdown, maintaining energy status and increasing antioxidative capacity. Postharvest Biol. Technol. 108, 8-20.

Hu, H., Shen, W., Li, P., 2014. Effects of hydrogen sulphide on quality and antioxidant capacity of mulberry fruit. Internat. J. Food Sci. Technol. 49, 399-409.

Huque, R., Wills, R.B.H., Pristijono, P., Golding, J.B., 2013. Effect of nitric oxide (NO) and associated control treatments on the metabolism of fresh-cut apple slices in relation to development of surface browning. Postharvest Biol. Technol. 78, 16-23.

Jin, Z., Pei, Y., 2015. Physiological implications of hydrogen sulfide in plants: pleasant exploration behind its unpleasant odour. Oxidative Med. Cellular Longevity doi: 10.1155/2015/397502.

Kamoun, P., 2004. Endogenous hydrogen sulfide production in mammals. Amino Acids, 26, 243-254.

Li, S.P., Hu, K.D., Hu, L.Y., Li, Y.H., Jiang, A.M., Xiao, F., Han, Y., Liu, Y.S., Zhang, H., 2014. Hydrogen sulfide alleviates postharvest senescence of broccoli by modulating antioxidant defense and senescence-related gene expression. J. Agric. Food Chem. 62, 1119-1129.

Li, L. Moore, P.K. 2008. Putative biological roles of hydrogen sulfide in health and disease: a breath of not so fresh air? Trends Pharmacol. Sci. 29, 84-90.

Liu, D., Xu, S., Hu, H., Pan,J., Li, P., Shen, W., 2017. Endogenous hydrogen sulfide homeostasis is responsible for the alleviation of senescence of postharvest daylily flower via increasing antioxidant capacity and maintained energy status. J. Agric. Food Chem. 65, 718-726.

Lu, S., 2007. Effect of packaging on shelf-life of minimally processed bok choy (*Brassica chinensis* L.). LWT-Food Sci. Technol. 40, 460-464.

Okawa, M. Kinjo, J., Nohara, T., Ono, M., 2001. DPPH (1,1-Diphenyl-2-Picrylhydrazyl) radical scavenging activity of flavonoids obtained from some medicinal plants. Biol. Pharmacol. Bull. 24, 1202–1205.

Richard, J.P., Amyes, T.L., Crugeiras, J., Rios, A., . 2009 Pyridoxal 5'-phosphate: electrophilic catalyst extraordinaire Current Opinion Chem. Biol. 13, 468–476.

Riemenschneider, A., Nikiforova, V., Hoefgen, R., de Kok, L,. Papenbrock, J., 2005. Impact of elevated $H_2S$ on metabolite levels, activity of enzymes and expression of genes involved in cysteine metabolism. Plant Physiol. Biochem., 43, 473-483.

Romero, L.C., Aroca, A.M., Laureano-Marín A.M., Moreno, I., García, I., Gotor, C., 2014. Cysteine and cysteine-related signaling pathways in *Arabidopsis thaliana*. Molecular Plant 7, 264–276.

Vuong, Q.V., Hirun, S., Roach, P.D., Bowyer, M.C., Phillips, P.A., Scarlett, C.J., 2013. Effect of extraction conditions on total phenolic compounds and antioxidant activities of *Carica papaya* leaf aqueous extracts. J. Herbal Med. 3, 104-111.

Wang, W., Shen, Y.G., Zhu, L.Q., Zhang, W., Wu, S.F., Du, H.Y., 2014. Effects of exogenous $H_2S$ on preservation of peaches and reactive oxygen metabolism. J. Fruit Sci. 31, 302–307.

Yu, L., Zhang, C., Shang, H., Wang X., Wei M., Yang, F., Shi, Q., 2013. Exogenous hydrogen sulfide enhanced antioxidant capacity, amylase activities and salt tolerance of cucumber hypocotyls and radicles, J. Internat. Agric., 12, 445-456.

Zhang, H., Hu, S.L., Zhang, Z.J., Hu, L.Y., Jiang, C.X., Wei, Z.J., Liu, J., Wang, H.L. Jiang, S.T., 2011. Hydrogen sulfide acts as a regulator of flower senescence in plants. Postharvest Biol. Technol. 60, 251–257.

Zheng, J.L., Hu, L.Y., Hu, K.D., Wu, J., Yang, F., Zhang, H., 2016. Hydrogen sulfide alleviates senescence of fresh-cut apple by regulating antioxidant defense system and senescence-related gene expression. HortScience, 51, 152-158.

Zhao, Y., Biggs, T.D., Xian, M,, 2014. Hydrogen sulfide ($H_2S$) releasing agents: chemistry and biological applications. Chem. Commun. 50, 11788–11805.

**Table 1**.

Market life of pak choy sprayed with PAG and/or fumigated with $H_2S$ during storage at 10 °C and ventilation with air containing 0.1 µL $L^{-1}$ ethylene.

| Treatment | Market life (days) |
|---|---|
| PAG | 7.2[a] |
| PAG + $H_2S$ | 7.8[b] |
| Water | 8.4[c] |
| Untreated | 8.6[c] |
| Water + $H_2S$ | 10.3[d] |
| *LSD* | *0.44* |

Each value is the mean of 30 assessments (10 batches of produce x 3 units). LSD values are the least significant difference between means at $P = 0.05$.

**Table 2.**

Ion leakage and antioxidant activity of pak choy leaves that had been sprayed with PAG and/or fumigated with $H_2S$ after three days storage at 10 °C and ventilated with air containing 0.1 µL $L^{-1}$ ethylene.

| Treatment | Ion leakage (%) | Antioxidant activity (mg TE 100 $g^{-1}$) |
|---|---|---|
| PAG | 73.6[c] | 0.0578[a] |
| Water | 44.5[b] | 0.0583[a] |
| PAG + $H_2S$ | 37.5[b] | 0.0653[b] |
| $H_2S$ | 28.8[a] | 0.0704[c] |
| *LSD* | *8.7* | *0.005* |

Each value is the mean of 12 assessments (4 batches of produce x 3 units). LSD values are the least significant difference between means at $P = 0.05$.

**Figure 1.**

Respiration rate (µg $CO_2$ .$kg^{-1}$ $s^{-1}$) of pak choy sprayed with PAG and/or fumigated with $H_2S$ during storage at 10 °C and ventilation with air containing 0.1 µL $L^{-1}$ ethylene.

Treatments: ♦ PAG, ▲ control (water spray), ○ $H_2S$, ■ PAG + $H_2S$.

Each value is the mean of 15 readings (5 batches of produce x 3 units). LSD between means was 1.15 at $P = 0.05$.

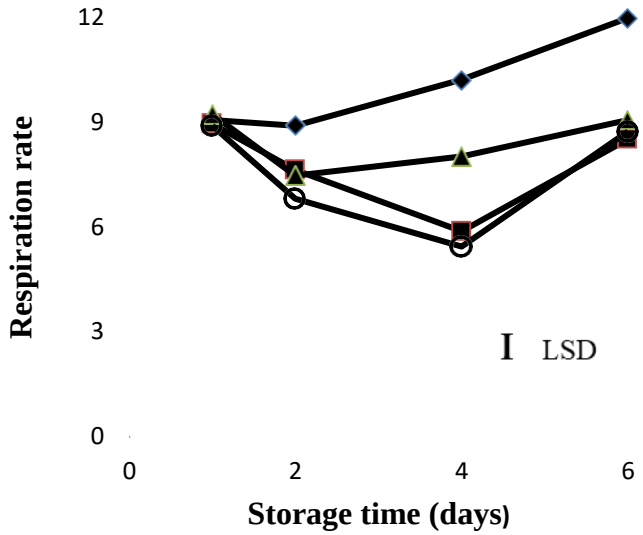
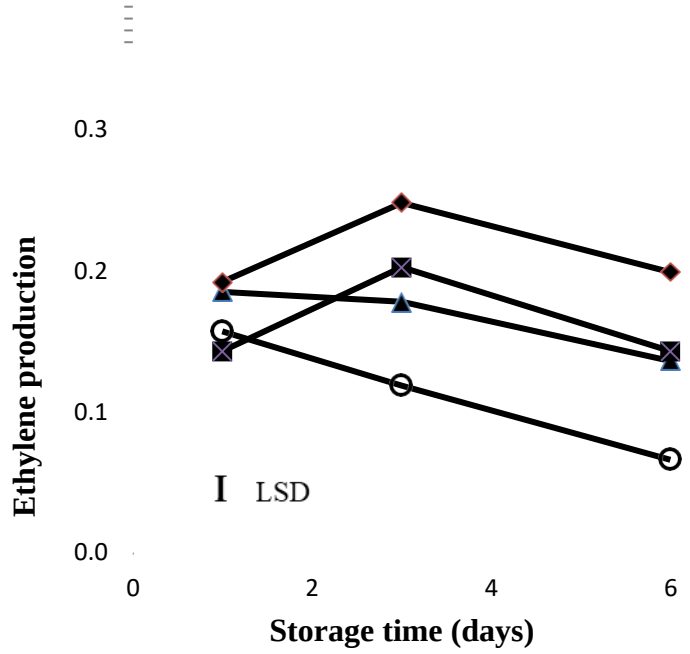


**Figure 2.**

Ethylene production (ng $kg^{-1}$ $s^{-1}$) of pak choy sprayed with PAG and/or fumigated with $H_2S$ during storage at 10 °C and ventilation with ethylene-free air.

Treatments: ♦ PAG, ▲ control (water spray), ○ $H_2S$, ■ PAG + $H_2S$.

Each value is the mean of 9 readings (3 batches of produce x 3 units). LSD between means was 0.025 at $P = 0.05$.



### 4.5 Use of cysteine to capture the beneficial effect of $H_2S$ on senescence of leafy vegetables

H.M.S. Al Ubeed, R.B.H. Wills, M.C. Bowyer, J.B. Golding. Inhibition of postharvest senescence of green leafy vegetables, by exogenous D-cysteine and L-cysteine as precursors of hydrogen sulphide. Journal of Horticultural Science and Biotechnology (submitted)

**General background**

While $H_2S$ inhibits senescence, it presents a range of logistical, safety and regulatory issues that would impinge on gaining regulatory approved for commercial treatment. D-cysteine and L-cysteine are metabolised to $H_2S$ by plant tissues albeit by different pathways and being naturally synthesised amino acids have the potential to be an alternate method to capture the benefit of $H_2S$. A few studies have examined the effects of L-cysteine on postharvest produce but with fresh-cut products due to cysteine being well known as an anti-browning agent. There are no reported studies with D- and DL-cysteine. The effect of spraying pak choy with the cysteines on senescence was examined during storage in the presence of 0.1 µL/L ethylene.

**Summary of Results**

Pak choy leaves were sprayed with a range of concentrations of D-cysteine, L- cysteine or DL-cysteine and a concentration of 10 mmol was found to be optimal to extend market life and reduce respiration rate and ethylene production. The benefits achieved with the cysteines were similar to those achieved by fumigation with 250 µL/L $H_2S$. L-cysteine sprays were also evaluated on parsley and peppermint leaves with both showing enhanced market life and reduced respiration compared to untreated leaves.

**Conclusion**

The similar level of inhibition of senescence achieved with the cysteines and $H_2S$ indicates that cysteine applied by spraying with is readily absorbed into active metabolic sites in leaf tissue. The small amount of cysteine applied to leaves is well below the no-observed-adverse-effect level (NOAEL) for both D- and L-cysteine and thus appears to not have any potential adverse health effects. This study is the first report on applying D- or DL-cysteine to fruit and vegetables.

A copy of the paper submitted for publication in Science of Horticultural Science and Biotechnology is appended.

# Inhibition of postharvest senescence of green leafy vegetables by exogenous D-cysteine and L-cysteine as precursors of hydrogen sulphide

H. M. S. Al Ubeed[ab], R. B. H. Wills[a] , M. C. Bowyer[a] and J.B. Golding[ac]

[a] School of Environmental and Life Sciences, University of Newcastle, Ourimbah NSW 2558, Australia, [b]Department of Life Sciences, Faculty of Science, Al-Muthana University, Al-Muthana, Iraq, [c]New South Wales Department of Primary Industries, Ourimbah, NSW 2258, Australia.

Corresponding author: R. B. H. Wills, ron.wills@newcastle.edu.au

**Word count:** 4023

**KEYWORDS:** Cysteine, hydrogen sulphide, postharvest senescence, pak choy, green vegetables

## Abstract

Hydrogen sulphide ($H_2S$) inhibits senescence in harvested fruit and vegetables but presents logistical, safety and regulatory issues to become a commercial treatment. D-cysteine and L-cysteine are semi-essential amino acids that are metabolised to hydrogen sulphide by plant tissues albeit by different pathways. This paper examines the effect of cysteine on postharvest senescence of three green leafy vegetables. Spraying pak choy leaves with 10 mmol D-cysteine, L-cysteine or DL-cysteine inhibited leaf senescence through a delayed loss of green colour expressed as market life, reduced respiration rate and reduced ethylene production. The beneficial effects of cysteine were similar to those achieved by fumigation with hydrogen sulphide. L-cysteine sprays on parsley and peppermint leaves also showed reduced leaf colour loss and respiration compared to untreated leaves. Cysteine, either as the racemate or individual enantiomers, is considered to have commercial potential for green leafy vegetables as it provides the beneficial effect of hydrogen sulphide but should be easier to register for commercial use due to the GRAS status of L-cysteine.

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## Introduction

Fumigation with hydrogen sulphide gas has been shown to extend the storage life of many horticultural commodities such as strawberry (Chang, Shi, Zhu, Li & Wang, 2014), broccoli (Li et al., 2014), water spinach (Hu, Liu, Li & Shen, 2015), and grape (Ni et al., 2016). However, any commercial use of hydrogen sulphide ($H_2S$) gas poses logistical and safety issues in applying a fumigation treatment to foods and it is unclear who would manage the required regulatory approval without having patented exclusivity of future sales. An alternate option is to utilise a metabolic precursor of $H_2S$ that would be more readily accepted as a postharvest treatment for fruit and vegetables.

Cysteine (2-amino-3-sulphanylpropanoic acid) exists as L- and D- enantiomers with both forms synthesised in plants (Li, 2013) and animals (Prabhakar, 2012) and both linked to $H_2S$ metabolism. Cysteine is considered the primary source of endogenous $H_2S$ in plants (Wang, 2012). Cysteine desulfhydrases (CDes) utilise D- and L-cysteine separately to generate $H_2S$ albeit by different enzyme systems. L-cysteine decomposition is catalysed by L-cysteine desulfhydrase (L-CD) (Papenbrock,, Riemenschneider, Kamp, Schulz-Vogt & Schmidt, 2007; Alvarez, Calo, Romero, García & Goto, 2010) while D-cysteine is metabolised by D- cysteine desulfhydrase (D-CD) (Nagasawa, Takafumi, Hidehiko & Hideaki, 1985; Jin & Pei, 2015). In addition, a third enzyme, D-cysteine desulfhydrase 2 (D-CD2) has been reported to degrade both cysteine enantiomers simultaneously (Jin & Pei, 2015). The potential for cysteine as a postharvest treatment relates to L-cysteine being a semi-essential amino acid and having Generally Regarded As Safe (GRAS) status. In addition, L-cysteine is approved as an additive to baking flour (USDA, 2017). D-cysteine is a naturally occurring non- proteogenic amino acid and while it does not currently have GRAS status it has a similar toxicological profile as L-cysteine (Shibui, Sakai, Manabe & Masuyama, 2017) which should not present a problem for postharvest use.

The few studies investigating the effects of L-cysteine on postharvest produce have focused mainly on its use on fresh-cut products due to cysteine being well known as an anti-browning agent through inhibiting peroxidase and polyphenol oxidase activity (Sharma & Rao, 2013). L-cysteine has been found to inhibit the development of browning in a range of produce including avocado, pear, mango, apple and banana (Ali, Khan & Malik, 2016). Ali et al. (2016) also reported that L-cysteine added to whole litchi fruit inhibited the development of tissue browning. To the best of our knowledge, there have been no reported studies involving exogenous application of D-cysteine or the DL-racemate to postharvest produce.

Al Ubeed, Wills, Bowyer, Vuong & Golding (2017) recently identified that the Asian leafy green vegetable, pak choy (*Brassica rapa* subsp. *Chinensis* - also known as bok or pok choy, choi or tsoi), when fumigated with $H_2S$, reduced endogenous ethylene production, and it was speculated that this could be causally linked to delayed chlorophyll degradation - a characteristic previously linked to $H_2S$ fumigation (Li et al., 2014; Hu et al., 2015)- and the beneficial impact on a range of physicochemical factors associated with senescence. In the present study, we investigate whether the application of cysteine may similarly act to delay postharvest senescence. L- and D-cysteine and the DL racemate were independently applied as an aqueous spray to pak choy leaves that were then stored at 10°C in the presence of a controlled level of ethylene to simulate likely commercial conditions. The impact of these treatments on loss of green colour (expressed in terms of consumer market life), respiration (as a measure of general metabolism) and endogenous ethylene production was then compared to $H_2S$ fumigated pak choy stored under identical conditions. The effect of L- cysteine was also evaluated on colour change and respiration of peppermint (*Mentha* × *piperita*) and parsley (*Petroselinum crispum*) leaves.

## Experimental

### *Produce*

Pak choy plants (cv. ‘Shanghai’) were harvested from a local commercial farm at Mangrove Mountain, NSW and transported to the laboratory within two hours. All plants selected for

each experiment were of uniform size (about 10 cm length) and colour and without damage to leaves or stem. Pak choy heads were cut and four outside leaves were selected and cleaned with tap water. The leaves from each head were randomly distributed into the required number of treatment units, each containing the same weight of produce. For the first two experiments there were 24 leaves with a total average weight of about 400 g and in subsequent experiments there were 15 leaves of total average weight about 200 g. Parsley and peppermint plants were obtained from a local market. Leaf branches for each produce were randomly distributed into nine units with each weighing about 100 g. In each experiment, a treatment was applied to three units of produce. All experiments were replicated with batches of plants obtained on three separate occasions, with at least two weeks between batches.

***Treatments***

Cysteine was applied by spraying each side of each leaf in a treatment unit with 0.1 mL of an aqueous solution containing the hydrochloride monohydrate of L-cysteine, D-cysteine (Sigma-Aldrich, Steinheim, Germany) or DL-cysteine (Tokyo Chemical Industry, Tokyo). Control treatments were similarly sprayed with water or fumigated for four hours with 250 $\mu L\ L^{-1}$ $H_2S$, the optimum concentration reported by Al Ubeed et al. (2017), that was generated *in situ* by the addition of water to solid sodium hydrogen sulphide (NaHS) using the method described by Zhao, Biggs & Xian (2014).

Sprayed produce was allowed to dry in ambient air for four hours at 20°C. Each treatment unit was then placed into a 4 L plastic container that was fitted with inlet and outlet ports in the lid. Containers were placed into a temperature controlled cabinet at 10°C and polyethylene tubing (5 mm diam.) was connected to the inlet port through which flowed humidified air containing <0.001 or 0.1 $\mu L\ L^{-1}$ ethylene at 45 mL $min^{-1}$. The <0.001 $\mu L\ L^{-1}$ concentration was considered ethylene-free as the analytical limit of detection was 0.001 $\mu L\ L^{-1}$, while 0.1 $\mu L\ L^{-1}$ is an ethylene concentration commonly present in commercial consignments (Li, Wills & Golding, 2017). The ethylene-free air was obtained by passing atmospheric air through a tube filled with potassium permanganate adsorbed onto alumina pellets and 0.1 $\mu L\ L^{-1}$ ethylene was obtained by mixing the ethylene-free air with a regulated flow of ethylene from a cylinder (1 mL $L^{-1}$ ethylene in air, BOC Gases, Sydney). The gas mixtures were humidified by bubbling through water in a tall 2 L glass jar (225 cm height) to ensure a high humidity of 97-99 % RH was maintained in the gas stream to minimise water loss.

***Physio-chemical assessments***

Leaves in each unit were visually assessed daily for green colour and the time for each unit to develop an unacceptable colour (denoted as the market life) was determined using the scoring scale given below. The respiration rate as evolved carbon dioxide and ethylene production were also was assessed at various times during storage.

*Visual leaf colour (market life)*

Each leaf in a unit was visually assessed daily for leaf colour of pak choy and parsley from green to yellow and assigned a colour score using a 0-5 scale where 0 = green, 1 = 10%, 2 = 20%, 3 = 30%, 4 = 50% and 5 =>70% loss of original green colour (Li et al., 2017). For peppermint, leaves developed brown patches and the extent of browning on each leaf was scored on a similar 0-5 scale. The mean colour score of all leaves in a treatment unit was calculated daily. An average colour score of 3.0 was considered to be the limit of consumer acceptability and the time for leaves to reach a mean score of 3.0 was designated as the market life of that unit. Assessment of a unit was terminated when a mean score of 3.0 was attained.

*Respiration rate and ethylene*

Respiration was measured as carbon dioxide production. A container containing a treatment unit of pak choy was sealed for four hours when a gas sample (5 mL) was collected in a syringe and the concentration of carbon dioxide in the sample was determined by injecting into a thermal conductivity gas chromatograph as described by Huque, Wills, Pristijon & Golding (2013). The respiration rate was calculated as mL $kg^{-1}$ $h^{-1}$.

The concentration of ethylene in the atmosphere around produce was determined by a collecting a gas sample (1 mL) and analysing by flame ionization gas chromatography as described by Huque et al. (2013). Samples were collected at frequent intervals from the ventilating gas streams to ensure the desired ethylene concentration was maintained. Samples were also obtained from ventilated treatment units just before sealing the container and again three hours after sealing. The difference between readings was used to calculate the rate of ethylene production as µL $kg^{-1}$ $h^{-1}$.

***Statistical analysis***

Data were analysed by two-way analysis of variance (ANOVA) and where a significant difference between treatments was found the least significant difference (LSD) of the mean values at P=0.05 was calculated. Statistical procedures were performed using SPSS for Microsoft version 22.0 software package (SPSS Chicago, IL).

**Results**

***Effect of L-cysteine and D-cysteine on market life and respiration of pak choy***

The market life, as assessed by the change in leaf colour from green to yellow to a fixed colour score (3.0), and the respiration rate during storage at 10 °C were determined on pak choy leaves that were sprayed with a solution containing L-cysteine or D-cysteine in separate experiments. The effects were compared to a control (water spray) treatment and leaves fumigated with 250 µL $L^{-1}$ $H_2S$.

*Market life*

The market life of pak choy leaves was found to be significantly different between treatments in both experiments (Table 1). Experiment 1 compared the effect of different concentrations of L-cysteine and showed that the longest market life occurred with pak choy fumigated with $H_2S$. However, the optimum concentration of L-cysteine which significantly extended the market life of leaves over control leaves was 10 mmol. Leaves sprayed with 25 mmol L-cysteine had the shortest market life, which was about half that of the water control.

Experiment 2 showed the effect of D-cysteine but also included the optimal 10 mmol L-cysteine treatment from Experiment 1. Table 1 shows that a spray application of 10 mmol D-cysteine was equally effective as $H_2S$ fumigation in extending market life. As expected from Experiment 1, the application of L-cysteine resulted in a significant extension in market life over the water control but was significantly lower than that achieved by D-cysteine. However, leaves sprayed with 25 mmol D-cysteine had the shortest market life of about half that of the water control.

*Respiration rate*

The respiration rate during storage showed a significant effect of treatment but there was also a significant interaction between storage time and treatment at *($P < 0.001$).* Figure 1 shows that in Experiment 1 respiration rates were not significantly different between treatments after one and two days at 10°C but after four and six days storage, leaves treated with 5 and 10 mmol L-cysteine had a significantly lower respiration than the water control and were not significantly different to leaves fumigated with $H_2S$.

Figure 1 also shows that in Experiment 2 the respiration rate was not significantly different in all treatments after one and two days at 10°C but after four and six days, leaves treated with 10 mmol D-cysteine and 10 mmol L-cysteine had a significantly lower respiration rate than the water control but were not significantly different to leaves fumigated with $H_2S$. In both experiments, leaves sprayed with 25 mmol L-cysteine or D-cysteine were not significantly different in respiration rate to the respective water controls.

***Effect of L-cysteine and D-cysteine on ethylene production of pak choy during storage in air and ethylene***

Pak choy leaves were sprayed with 10 mmol L-cysteine and D-cysteine - the optimal concentration determined in the previous experiments. Leaves were also fumigated with 250 µL $L^{-1}$ $H_2S$ and two control treatments were included, i.e. leaves sprayed with water (as a control for the cysteine treatments) and untreated leaves (as a control for the $H_2S$ fumigation treatment). All treatments were stored at 10°C in containers ventilated with ethylene-free air or air containing 0.1 µL $L^{-1}$ ethylene. Al Ubeed et al. (2017) showed endogenous ethylene production of pak choy was greatly reduced in the presence of exogenous ethylene. Hence, ventilation with air was included to ensure the study could better observe the effect of cysteine on endogenous ethylene production. The colour score was also determined after six days storage to confirm that the cysteines were inhibiting colour loss.

There was a significant effect of treatment, storage time and the presence of ethylene on the rate of production of ethylene, but there was no significant interaction between these factors (Figure 2). When containers were ventilated with 0.1 µL $L^{-1}$ ethylene, the rate of endogenous ethylene production was reduced to about one-eighth the rate compared to those ventilated with ethylene-free air. However, within each ventilating regime, the rate of

ethylene production relative to control leaves was reduced by L-cysteine, D-cysteine and $H_2S$ with no significant difference between the three treatments. The effect of treatments and ethylene ventilation were fully established at the first measurement, which was after two days at 10°C and while the absolute rate of ethylene production decreased during storage, the relativity between treatments remained constant. As there was no significant difference between the control treatments (sprayed with water or unsprayed), a single value for control is presented in Figure 2.

The colour score of pak choy leaves after six days storage at 10°C showed a significant effect of both treatment and the presence of ethylene but there was no significant interaction between treatment and ethylene (Table 2). The effectiveness of the treatments for inhibiting the loss of green colour (lower colour score) was D-cysteine > L-cysteine = $H_2S$, which all had a lower colour score than control treatments. As expected, loss of green colour was also significantly enhanced by the presence of exogenous ethylene.

### *Effect of DL-cysteine on market life and respiration of pak choy*

Pak choy leaves were sprayed with 10 mmol L-cysteine, D-cysteine and DL-cysteine and market life and respiration rate were determined. All three cysteine treatments increased the market life of pak choy relative to the control leaves, with no significant difference between the cysteines (Table 3). However, the increase in market life of the cysteine treatments was not as great as that achieved by fumigation with $H_2S$. Table 3 also shows that in comparison to the respiration rate of the control leaves, the respiration in the D-cysteine, L-cysteine and $H_2S$ treatments was significantly suppressed to a similar extent. Spraying with DL-cysteine also resulted in a significant reduction in respiration compared to control but this effect was not as great as $H_2S$ and the single cysteine compounds.

### *Effect of L-cysteine on market life and respiration of parsley and peppermint*

Parsley and peppermint leaves were sprayed with solutions of L-cysteine and assessed for market life (based on the change in leaf colour from green to yellow for parsley and appearance of brown areas for peppermint) and respiration rate during storage at 10°C. Table 4 shows that for parsley and peppermint, spraying leaves with 10 mmol L-cysteine resulted in the greatest reduction in respiration rate and extension in market life. The 5 mmol L-cysteine spray was also beneficial but but generally less effective than the 10 mmol spray. The market life of parsley and peppermint of leaves sprayed with 5 mmol L-cysteine was significantly greater than control but significantly less than leaves sprayed with 10 mmol L-cysteine. The respiration rates of parsley and peppermint were significantly less than control leaves but for parsley respiration was greater than for leaves sprayed with 10 mmol L-cysteine while for peppermint, 5 and 10 mmol sprays were not significantly different.

## Discussion

Spraying pak choy leaves with D-cysteine, L-cysteine and DL-cysteine all resulted in an extension in market life and reduction in respiration compared to the control leaves. While there was some variation in relative effectiveness of the three cysteine treatments between experiments, in general, they can be considered to be effective in inhibiting senescence to a

similar extent as $H_2S$. The reduction in endogenous ethylene production by both D- and L-cysteine occurred early in storage before effects on respiration and colour change were observed and were similar in magnitude to that achieved by $H_2S$ fumigation. This suggests that the inhbition of senesence by the cysteines could be due to their rapid conversion to $H_2S$ which Al Ubeed et al. (2017) concluded was acting by inhibiting endogenous ethylene production or action. While L-cysteine has been studied for its ability to inhibit browning of fresh-cut fruit (Sharma & Rao, 2013), this is the first report on postharvest effects of D- cysteine and DL-cysteine.

The beneficial effect achieved by spraying leaves with an aqueous solution of cysteine, indicates that cysteine is readily absorbed into active metabolic sites in the leaf tissue. The ready absorption into the leaf was further attested by the inhibition of senescence being achieved without the inclusion of a wetting agent in the dip solution. At the optimal solution concentration of 10 mmol cysteine and 0.2 mL of solution sprayed onto pak choy leaves with an average weight of 14 g, the amount of cysteine added was equivalent to the addition of about 17 mg $kg^{-1}$ of pak choy. This is well below the no-observed-adverse-effect level (NOAEL) of 500 mg $kg^{-1}$ $day^{-1}$ for both D- and L-cysteine determined on rats by Shibui et al. (2017). Thus, commercial usage of D-cysteine, L-cysteine or DL-cysteine would appear to not have any potential adverse health effects.

The beneficial effect of cysteine on delaying senescence of parsley and peppermint leaves in addition to pak choy suggests the possibility of commercial potential with leafy vegetables in general. Cysteine capitalises on the beneficial effect of hydrogen sulphide but should be more amenable to register for commercial use as it does not have the safety issues associated with hydrogen sulphide. Use of cysteine as a spray or dip treatment would also be adaptable to existing packinghouse infrastructure and a faster treatment to apply than batch fumigation for four hours. Studies would also seem warranted to evaluate the effect of postharvest addition of cysteine on other types of fruit and vegetables. However, the uptake of cysteine into non-leafy vegetables may be more difficult to achieve and require development of an appropriate dipping technique. If the three forms of cysteine continue to offer similar postharvest benefits, the commercial potential would depend on the relative costs of the compounds. At present, L-cysteine has the lowest cost but this could change if a marked increase in product volume from commercial usage stimulated, for example, production of the racemate by non-stereospecific chemical synthesis.

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## Conflict of interest

The authors certify they have no affiliation with or involvement in any organisation or entity with an interest in the subject matter or materials discussed in this manuscript.

## References

Al Ubeed, H.M.S., Wills, R.B.H., Bowyer, M.C, Vuong, Q.V., & Golding, J.B. (2017). Interaction of exogenous hydrogen sulphide and ethylene on senescence of green leafy vegetables. *Postharvest Biology and Technology, 133*, 81-87.

Ali, S., Khan, A.S., & Malik, A.U. (2016). Postharvest L -cysteine application delayed pericarp browning, suppressed lipid peroxidation and maintained antioxidative activities of litchi fruit. *Postharvest Biology and Technology, 121*, 135–142.

Alvarez, C., Calo, L., Romero, L.C., García, I., & Gotor, C. (2010). An *O*-acetylserine(thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis., *Plant Physiology, 152*, 656–669,

Chang, Z., Shi, J., Zhu, L., Li, C., & Wang, Q., (2014). Cooperative effects of hydrogen sulfide and nitric oxide on delaying softening and decay of strawberry. *International Journal of Agricultural and Biological Engineering, 7*, 114–122.

Hu, H., Liu, D., Li, P., & Shen, W. (2015). Hydrogen sulfide delays leaf yellowing of stored water spinach (*Ipomoea aquatica*) during dark-induced senescence by delaying chlorophyll breakdown, maintaining energy status and increasing antioxidative capacity. *Postharvest Biology and Technology, 108*, 8-20.

Huque, R., Wills, R.B.H., Pristijono, P., & Golding, J.B. (2013). Effect of nitric oxide (NO) and associated control treatments on the metabolism of fresh-cut apple slices in relation to development of surface browning. *Postharvest Biology and Technology, 78*, 16-23.

Jin, Z., & Pei, Y. (2015). Physiological implications of hydrogen sulfide in plants: pleasant exploration behind its unpleasant odour. *Oxidative Medicine and. Cellular Longevity,* Article 397502. doi.org/10.1155/2015/397502

Li, S.P., Hu, K.D., Hu, Y., Li, Y.H., Jiang, A.M., Xiao, F., Han, Y., Liu, Y.S., & Zhang, H. (2014). Hydrogen sulfide alleviates postharvest senescence of broccoli by modulating antioxidant defense and senescence-related gene expression. *Journal of Agricultural and Food Chemistry, 62*, 1119-1129.

Li, Y., Wills, R.B.H., & Golding, J.B. (2017). Interaction of ethylene concentration and storage temperature on postharvest life of the green vegetables, pak choi, broccoli, mint and green bean. *Journal of Horticultural Science and Biotechnology, 92*, 288-293.

Li, Z.G. (2013). Hydrogen sulfide: A multifunctional gaseous molecule in plants. *Russian Journal of Plant Physiology, 60***,** 733–740.

Nagasawa, T., Takafumi, I., Hidehiko, K., & Hideaki, Y. (1985). D-Cysteine desulfhydrase of *Escherichia coli* purifaction and characterization. *European Journal of Biochemistry, 153*, 541-551.

Ni, Z.J., Hu, K.D., Song, C.B., Ma, R.H., Li, Z.R., Zheng, J.L., & Zhang, H. (2016). Hydrogen sulfide alleviates postharvest senescence of grape by modulating the antioxidant defenses. *Oxidative Medicine and Cellular Longevity,* Article 4715651. doi.org/10.1155/2016/4715651

Papenbrock, J., Riemenschneider, A., Kamp, A., Schulz-Vogt, H.N., & Schmidt, A. (2007). Characterization of cysteine-degrading and $H_2S$-releasing enzymes of higher plants - from the field to the test tube and back. *Plant Biology, 9*, 582–588.

Prabhakar, N.R. (2012). Carbon monoxide (CO) & hydrogen sulfide ($H_2S$) in hypoxic sensing by the carotid body. *Respiratory Physiology and Neurobiology, 184*,165-169.

Sharma, S., & Rao, T.V.R. (2013). Effect of honey and L-cysteine as antioxidants on the quality attributes of fresh-cut carambola (*Averrhoa carambola* L.) stored at two different temperatures. *International Journal of Postharvest Technology and Innovation, 3*, 362-381.

Shibui, Y., Sakai, R., Manabe, Y., & Masuyama, T. (2017). Comparisons of L-cysteine and D-cysteine toxicity in 4-week repeated dose toxicity studies of rats receiving daily oral administration. *Journal of Toxicologic Pathology, 30,* 217-229.

USDA (2017). Code of Federal Regulations. Title 21, Vol 3. (Revised April 1, 2017) [21CFR184.1271]. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1271.

Wang, R. (2012). Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Physiological Reviews, 92,* 791–896.

Zhao, Y., Biggs, T.D., & Xian, M. (2014). Hydrogen sulfide ($H_2S$) releasing agents: chemistry and biological applications. *Chemical Communications, 50,* 11788–11805.

**Table 1.** Market life of pak choy leaves sprayed with L-cysteine (Experiment 1) and D-cysteine (Experiment 2), or fumigated with $H_2S$ and stored at 10°C in 0.1 µL $L^{-1}$ ethylene.

| Expt | Treatment | Market life (days) |
|---|---|---|
| 1 | Control (water spray) | $8.4^{c}$ |
| | 5 mmol L-cysteine | $8.8^{bc}$ |
| | 10 mmol L-cysteine | $9.3^{b}$ |
| | 25 mmol L-cysteine | $4.0^{d}$ |
| | 250 µL $L^{-1}$ $H_2S$ | $10.0^{a}$ |
| | *LSD* | *0.66* |
| 2 | Control (water spray) | $8.1^{c}$ |
| | 10 mmol L-cysteine | $9.3^{b}$ |
| | 10 mmol D-cysteine | $11.0^{a}$ |
| | 25 mmol D-cysteine | $4.2^{d}$ |
| | 250 µL $L^{-1}$ $H_2S$ | $11.3^{a}$ |
| | *LSD* | *0.60* |

Values in each experiment are the mean of nine assessments (three units x three batches of produce). LSD values are the least significant difference between means at *P*=0.05

**Table 2.** Colour score of pak choy leaves sprayed with 10 mmol L-cysteine and D-cysteine solution or fumigated with $H_2S$ after six days storage at 10°C while ventilated with air or 0.1 µL $L^{-1}$ ethylene.

| Treatment | Leaf colour score | | |
|---|---|---|---|
| | Ventilating gas | | |
| | Air | Ethylene | Mean |
| Control(water spray) | 2.6 | 2.7 | $2.6^{c}$ |
| Control(no spray) | 2.6 | 2.8 | $2.7^{c}$ |
| L-Cysteine | 1.7 | 2.1 | $1.9^{b}$ |
| D-Cysteine | 1.3 | 1.6 | $1.4^{a}$ |
| $H_2S$ | 1.6 | 2.0 | $1.8^{b}$ |
| Mean | $1.9^{a}$ | $2.2^{b}$ | |
| *LSD* | *0.22* | | *0.34* |

Values are the mean of nine readings (three units x three batches of produce). LSD values not sharing the same superscript in the same row or column are significantly different at *P*=0.05.

**Table 3**. Market life and respiration rate of pak choy leaves sprayed with cysteine enantiomers or fumigated with $H_2S$ during storage at 10°C in 0.1 µL $L^{-1}$ ethylene.

| Treatment | Market life (days) | Respiration (mL $kg^{-1}$ $h^{-1}$ $CO_2$) | | | |
|---|---|---|---|---|---|
| | | 2 days | 4 days | 6 days | Mean |
| Control (water) | 7.3[a] | 14.2 | 15.5 | 17.8 | 15.8[c] |
| 250 µL $L^{-1}$ $H_2S$ | 11.9[c] | 10.2 | 8.3 | 10.0 | 9.5[a] |
| 10 mmol L-cysteine | 9.8[b] | 10.4 | 8.9 | 10.7 | 10.0[a] |
| 10 mmol D-cysteine | 9.9[b] | 10.2 | 8.0 | 10.0 | 9.4[a] |
| 10 mmol DL-cysteine | 9.6[b] | 11.4 | 13.3 | 14.7 | 13.2[b] |
| *LSD* | *0.87* | | *2.5* | | *1.4* |

Values at each storage time are the mean of nine assessments (three units x three batches of produce). LSD values not sharing the same superscript in the same column are significantly different at *P*=0.05.

**Table4**. Leaf colour and respiration rate of parsley and mint leaves sprayed with L-cysteine during subsequent storage at 10°C in 0.1 µL $L^{-1}$ ethylene.

| Treatment | Respiration (mL $kg^{-1}$ $h^{-1}$ $CO_2$) | | Market life (days) | |
|---|---|---|---|---|
| | Parsley | Peppermint | Parsley | Peppermint |
| Control | 29.7[c] | 26.1[b] | 6.3[a] | 6.3[a] |
| 10 mmol L-cysteine | 11.9[a] | 10.3[a] | 7.9[c] | 7.3[c] |
| 5 mmol L-cysteine | 20.3[b] | 14.8[a] | 6.9[b] | 6.8[b] |
| *LSD* | *8.3* | *5.9* | *0.6* | *0.5* |

Values are the mean of nine assessments (three units x three batches of produce). LSD values not sharing the same superscript in the same column are significantly different at *P*=0.05

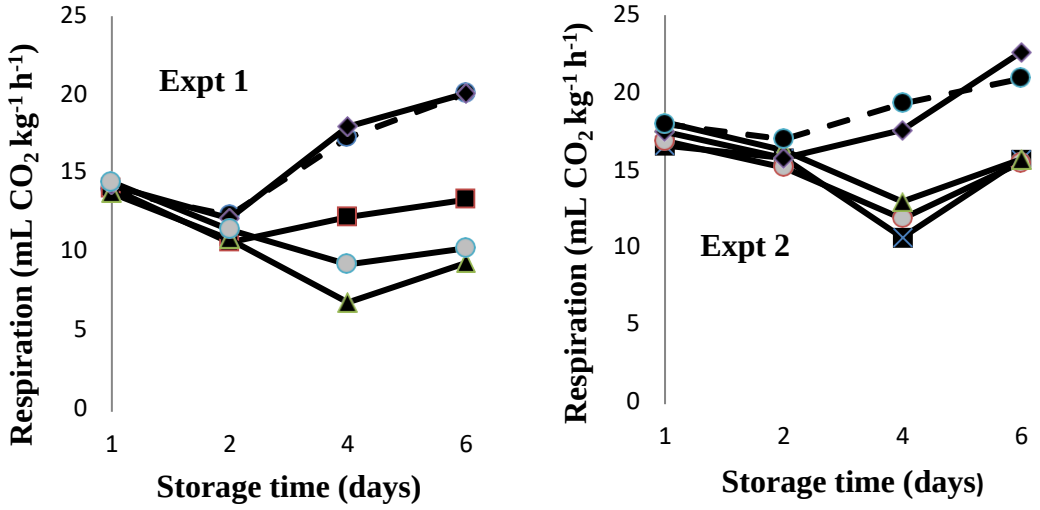


**Figure 1.** Respiration rate of pak choy leaves sprayed with L-cysteine and D-cysteine solution or fumigated with $H_2S$ during storage at 10°C in 0.1 µL $L^{-1}$ ethylene. Values in each Experiment are the mean of nine readings (three units x three batches of produce).

Treatments in Experiment 1 are water control (-●-), L-cysteine at 5 (■), 10 (▲) and 25 (♦) mmol, and $H_2S$ (○). LSD at $P$ = 0.05 between individual values is 6.5.

Treatments in Experiment 2 are water control (-●-), L-cysteine at 10 mmol (▲), D-cysteine at 10 (■) and 25 (♦) mmol, and $H_2S$ (○). LSD at $P$ = 0.05 between individual values is 3.3.

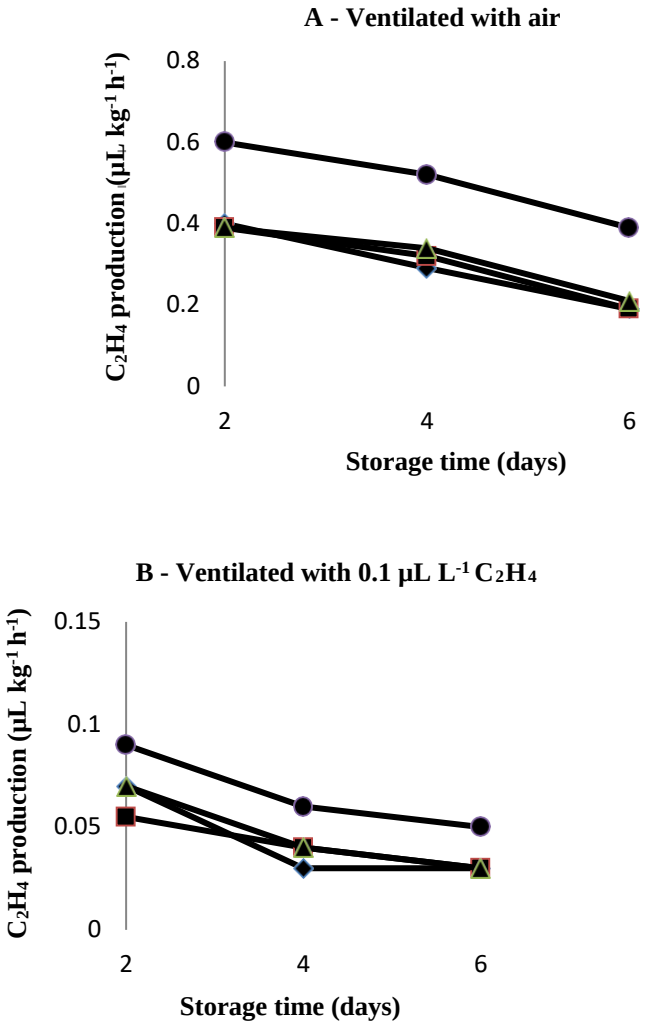


**Figure 2.** Rate of ethylene production by pak choy leaves sprayed with L-cysteine and D-cysteine solution or fumigated with $H_2S$ during storage at 10°C while ventilated with air (A) and 0.1 µL $L^{-1}$ ethylene (B). Values are the mean of nine readings (three units x three batches of produce).

Treatments are control (●), L-cysteine (■), D-cysteine (▲) and $H_2S$ (♦). LSD at $P$ = 0.05 between individual values is 0.07 for pak choy ventilated with air and 0.03 when ventilated with 0.01 µL $L^{-1}$ ethylene.

## 5. References


Able, A., Wong, L., Prasad, A., & O'Hare, T. (2003). The effects of 1-methylcyclopropene on the shelf life of minimally processed leafy Asian vegetables. *Postharvest Biology and Technology,* 27, 157-161.

Able, A. J., Wong, L. S., Prasad, A., & O'Hare, T. J. (2005). The physiology of senescence in detached pak choy leaves (*Brassica rapa var. Chinensis*) during storage at different temperatures. *Postharvest Biology and Technology,* 35, 271-278.

Adams, D., & Yang, S. (1979). Ethylene biosynthesis: identification of 1- aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proceedings of the National Academy of Sciences,* 76, 170- 174.

Ali, S., Khan, A. S., & Malik, A. U. (2016). Postharvest l-cysteine application delayed pericarp browning, suppressed lipid peroxidation and maintained antioxidative activities of litchi fruit. *Postharvest Biology and Technology,* 121, 135-142.

Aneja, V. P., Aneja, A. P., & Adams, D. F. (1982). Biogenic sulfur compounds and the global sulfur cycle. *Journal of the Air Pollution Control Association,* 32, 803-807.

Azzolini, M., Jacomino, A. P., Bron, I. U., Kluge, R. A., & Schiavinato, M. A. (2005). Ripening of" Pedro Sato" guava: study on its climacteric or non-climacteric nature. *Brazilian Journal of Plant Physiology,* 17, 299-306.

Beyer, E. M. (1976). A potent inhibitor of ethylene action in plants. *Plant Physiology,* 58, 268-271.

Bhatia, M. (2005). Hydrogen sulfide as a vasodilator. *International Union of Biochemistry and Molecular Biology life,* 57, 603-606.

Bico, S., Raposo, M., Morais, R., & Morais, A. (2009). Combined effects of chemical dip and/or carrageenan coating and/or controlled atmosphere on quality of fresh-cut banana. *Food Control,* 20, 508-514.

Bleecker, A. B., Estelle, M. A., Somerville, C., & Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in Arabidopsis thaliana. *Science,* 241, 1086-1090.

Buchanan-Wollaston, V. (1997). The molecular biology of leaf senescence. *Journal of Experimental Botany*, 48, 181-199.

Caliendo, G., Cirino, G., Santagada, V., & Wallace, J. L. (2010). Synthesis and biological effects of hydrogen sulfide ($H_2S$): development of $H_2S$-releasing drugs as pharmaceuticals. *Journal of Medicinal Chemistry*, 53, 6275-6286.

Chang, C., Kwok, S. F., Bleecker, A. B., & Meyerowitz, E. M. (1993). Arabidopsis ethylene-response gene ETR1: Similarity of product to two-component regulators. *Science*, 262, 539-544.

Chen, J., Wu, F.-H., Wang, W.-H., Zheng, C.-J., Lin, G.-H., Dong, J.-X., He, Z.-M. P., Zheng, H.-L. (2011). Hydrogen Sulphide enhances photosynthesis through promoting chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification in Spinacia oleracea seedlings. *Journal of Experimental Botany*, 62, 4481-4493.

Churchill, K., & Klepper, L. (1979). Effects of ametryn [2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-s-triazine] on nitrate reductase activity and nitrite content of wheat (Triticum aestivum L.). *Pesticide Biochemistry and Physiology*, 12, 156-162.

Colelli, G., Castillo García, S., Colantuono, F., & Amodio, M. (2013). Application of antioxidant compounds to preserve fresh-cut peaches quality. Paper presented at the VIII International Peach Symposium. *Acta Horticulturae*, 2**,** 1084.

Daldal, H., Beder, B., Serin, S., & Sungurtekin, H. (2010). Hydrogen sulfide toxicity in a thermal spring: a fatal outcome. *Clinical Toxicology, 48*, 755-756.

Dangl, J. L., & Jones, J. D. (2001). Plant pathogens and integrated defence responses to infection. *Nature, 411*, 826.

Davies, J. N., Hobson, G. E., & McGlasson, W. (1981). The constituents of tomato fruit the influence of environment, nutrition, and genotype. *Critical Reviews in Food Science & Nutrition*, 15, 205-280.

di Salvo, M. L., Contestabile, R., & Safo, M. K. (2011). Vitamin B6 salvage enzymes: mechanism, structure and regulation. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1814, 1597-1608.

Dorantes-Alvarez, L., Parada-Dorantes, L., Ortiz-Moreno, A., Santiago-Pineda, T., Chiralt-Boix, A., & Barbosa-Cánovas, G. (1998). Effect of anti-browning compounds on the quality of minimally processed avocados/Efecto de inhibidores del pardeamiento en la calidad de aguacates mínimamente procesados. *Food Science and Technology International*, 4, 107-113.

Finney, E. E. (1973). Elementary concepts of rheology relevant to food texture studies. In A. Kramer, A. S. Szczesniak (Ed.), *Texture Measurements of Foods* (1st ed.,pp. 33-51). Dordrecht, Holland: D. Reidel.

Fiorucci, S., Antonelli, E., Distrutti, E., Rizzo, G., Mencarelli, A., Orlandi, S.,Zanadro,R., Renga,B., Di Santa,M., Morelli, A., Cirino, G., Wallace, J. (2005). Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. *Gastroenterology*, 129, 1210-1224.

Flagan, R. C., & Seinfeld, J. H. (2012). *Fundamentals of air pollution engineering* (1st ed.).Mineola, N Y: Dover, pp 174.

Gane, R. (1934). Production of ethylene by some ripening fruits. *Nature*, 134, 1008.

García-Mata, C., & Lamattina, L. (2010). Hydrogen sulphide, a novel gasotransmitter involved in guard cell signalling. *New Phytologist*, 188, 977-984.

Ge, Y., Hu, K.-D., Wang, S.-S., Hu, L.-Y., Chen, X.-Y., Li, Y.-H.,Yang. H., Yang, F., Zhang, H. (2017). Hydrogen sulfide alleviates postharvest ripening and senescence of banana by antagonizing the effect of ethylene. *PloS One*, 12, e0180113.

Golding, J., Shearer, D., Wyllie, S., & McGlasson, W. (1998). Application of 1-MCP and propylene to identify ethylene-dependent ripening processes in mature banana fruit. *Postharvest Biology and Technology*, 14, 87-98.

Gorny, J. R., Hess-Pierce, B., Cifuentes, R. A., & Kader, A. A. (2002). Quality changes in fresh-cut pear slices as affected by controlled atmospheres and chemical preservatives. *Postharvest Biology and Technology*, 24, 271-278.

Grommes, J., & Soehnlein, O. (2011). Contribution of neutrophils to acute lung injury. *Molecular Medicine*, 17, 293.

Guerrero-Beltrán, J. A., Swanson, B. G., & Barbosa-Cánovas, G. V. (2005). Inhibition of polyphenoloxidase in mango puree with 4-hexylresorcinol, cysteine and ascorbic acid. *Lebensmittel-Wissenschaft & Technologies - Food Science and Technology,* 38, 625-630.

Hartle, M. D., & Pluth, M. D. (2016). A practical guide to working with $H_2S$ at the interface of chemistry and biology. *Chemical Society Reviews, 45*, 6108-6117.

Hershkovitz, V., Saguy, S. I., & Pesis, E. (2005). Postharvest application of 1-MCP to improve the quality of various avocado cultivars. *Postharvest Biology and Technology,* 37, 252-264.

Hicks, E.W. (1957). Rail transport vehicles used for perishable produce in Australia. In: Second Conference of *Fruit Storage Investigators*, Sydney, 30-31 July 1956. CSIRO, Melbourne, 72-74.

Hodgkinson, A. (1970). Determination of oxalic acid in biological material. *Clinical Chemistry*, 16, 547-557.

Hu, H., Liu, D., Li, P., & Shen, W. (2015). Hydrogen sulfide delays leaf yellowing of stored water spinach (*Ipomoea aquatica*) during dark-induced senescence by delaying chlorophyll breakdown, maintaining energy status and increasing antioxidative capacity. *Postharvest Biology and Technology*, 108, 8-20.

Hu, K.-D., Wang, Q., Hu, L.-Y., Gao, S.-P., Wu, J., Li, Y.-H.,Zheng, J-L., Han,Y., Liu, Y-S., Zhang, H. (2014). Hydrogen sulfide prolongs postharvest storage of fresh-cut pears (*Pyrus pyrifolia*) by alleviation of oxidative damage and inhibition of fungal growth. *PloS One,* 9, e85524.

Hu, L.-Y., Hu, S.-L., Wu, J., Li, Y.-H., Zheng, J.-L., Wei, Z.-J.,Zheng,J-L., Han,Y., Liu,Y-S., Zhang, H. (2012). Hydrogen sulfide prolongs postharvest shelf life of strawberry and plays an antioxidative role in fruits. *Journal of Agricultural and Food Chemistry,* 60, 8684-8693.

Hu, Y., Vandenbussche, F., & Van Der Straeten, D. (2013). Chemical genetics as a tool to study ethylene biology in plants. *Plant Chemical Biology*, 184-201.

Huang, J., Niknahad, H., Khan, S., & O'Brien, P. J. (1998). Hepatocyte-catalysed detoxification of cyanide by L-and D-cysteine. *Biochemical Pharmacology*, 55, 1983-1990.

Jiang, Y., Joyce, D. C., & Terry, L. A. (2001). 1-Methylcyclopropene treatment affects strawberry fruit decay. *Postharvest Biology and Technology*, 23, 227-232.

Jin, Z., & Pei, Y. (2015). Physiological implications of hydrogen sulfide in plants: pleasant exploration behind its unpleasant odour. *Oxidative Medicine and Cellular Longevity*. 6, Article ID 397502.

Jin, Z., Shen, J., Qiao, Z., Yang, G., Wang, R., & Pei, Y. (2011). Hydrogen sulfide improves drought resistance in *Arabidopsis thaliana*. *Biochemical and Biophysical Research Communications*, 414, 481-486.

Kader, A. A. (1985). Ethylene-induced senescence and physiological disorders in harvested horticultural crops. *HortScience*, 20, 54-57.

Kader, A. A. (2002). Quality parameters of fresh-cut fruit and vegetable products. In O. Lamikanra (Ed.), *Fresh- Cut Fruit and Vegetables* (pp.11-20). Boca Raton, FL: CRC Press.

Kader, A. A., & Saltveit, M. E. (2003). Respiration and gas exchange.In J. A. Bartz, J. K. Brecht (Ed.), *Postharvest Physiology and Pathology of Vegetables*, (2nd ed., vol.2, pp .7-29). New York, U.S.A: Marcel Dekker.

Katz, E., Lagunes, P. M., Riov, J., Weiss, D., & Goldschmidt, E. E. (2004). Molecular and physiological evidence suggests the existence of a system II-like pathway of ethylene production in non-climacteric Citrus fruit. *Planta*, 219, 243-252.

Kimura, Y., Toyofuku, Y., Koike, S., Shibuya, N., Nagahara, N., Lefer, D.,Ogasawara,Y.,Kimura, H. (2015). Identification of $H_2S_3$ and $H_2S$ produced by 3-mercaptopyruvate sulfurtransferase in the brain. *Scientific Reports*, 5, 14774.

Klee, H. J. (2002). Control of ethylene-mediated processes in tomato at the level of receptors. *Journal of Experimental Botany*, 53, 2057-2063.

Kruszyna, H., Kruszyna, R., & Smith, R. P. (1985). Cyanide and sulfide interact with nitrogenous compounds to influence the relaxation of various smooth muscles.

Proceedings of the Society for Experimental Biology and Medicine, 179, 44-49.

Lai, D., Mao, Y., Zhou, H., Li, F., Wu, M., Zhang, J.,He,Z.,Cui,W.,Xie, Y. (2014). Endogenous hydrogen sulfide enhances salt tolerance by coupling the reestablishment of redox homeostasis and preventing salt-induced $K^+$ loss in seedlings of *Medicago sativa*. *Plant Science,* 225, 117-129.

Lee, Z. W., Zhou, J., Chen, C.-S., Zhao, Y., Tan, C.-H., Li, L., Moore, P, k., Deng, L.-W. (2011). The Slow-Releasing hydrogen sulfide donor, GYY4137, exhibits novel anti-cancer effects in vitro and in vivo. *PloS One,* 6, e21077.

Leshem, Y. Y., & Haramaty, E. (1996). Plant aging: the emission of NO and ethylene and effect of NO-releasing compounds on growth of pea (*Pisum sativum*) foliage. *Journal Plant Physiol,* 148, 258-263.

Leshem, Y. Y., Wills, R. B., & Ku, V. V. V. (1998). Evidence for the function of the free radical gas nitric oxide (NO•) as an endogenous maturation and senescence regulating factor in higher plants. *Plant Physiology and Biochemistry,* 36, 825-833.

Li, D., Limwachiranon, J., Li, L., Du, R., & Luo, Z. (2016). Involvement of energy metabolism to chilling tolerance induced by hydrogen sulfide in cold-stored banana fruit. *Food Chemistry,* 208, 272-278.

Li, S.-P., Hu, K.-D., Hu, L.-Y., Li, Y.-H., Jiang, A.-M., Xiao, F.,Han,Y., Liu,Y.-S.,Zhang, H. (2014). Hydrogen sulfide alleviates postharvest senescence of broccoli by modulating antioxidant defense and senescence-related gene expression. *Journal of Agricultural and Food Chemistry,* 62, 1119-1129.

Li, Y., Wills, R., & Golding, J. (2017). Interaction of ethylene concentration and storage temperature on postharvest life of the green vegetables pak choi, broccoli, mint, and green bean. *The Journal of Horticultural Science and Biotechnology,* 92, 288-293.

Lin, Z., Arciga-Reyes, L., Zhong, S., Alexander, L., Hackett, R., Wilson, I., & Grierson, D. (2008). SlTPR1, a tomato tetratricopeptide repeat protein, interacts with the ethylene receptors NR and LeETR1, modulating ethylene and auxin responses and development. *Journal of Experimental Botany,* 59, 4271-4287.

Linden, D. R. (2014). Hydrogen sulfide signaling in the gastrointestinal tract. *Antioxidants & Redox Signaling,* 20, 818-830.

Lisjak, M., Srivastava, N., Teklic, T., Civale, L., Lewandowski, K., Wilson, I.,Wood,M.E.,Whiteman,M.,Hancock, J. T. (2010). A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation. *Plant Physiology and Biochemistry,* 48, 931-935.

Lisjak, M., Teklić, T., Wilson, I. D., Wood, M., Whiteman, M., & Hancock, J. T. (2011). Hydrogen sulfide effects on stomatal apertures. *Plant Signaling & Behavior,* 6, 1444-1446.

Liu, D., Xu, S., Hu, H., Pan, J., Li, P., & Shen, W. (2017). Endogenous hydrogen sulfide homeostasis is responsible for the alleviation of senescence of postharvest daylily flower via increasing antioxidant capacity and maintained energy status. *Journal of Agricultural and Food Chemistry,* 65, 718-726.

Luo, Y. (2016). Bok choy. *The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks, Agriculture Handbook,* 66, 243.

Martínez-Romero, D., Bailen, G., Serrano, M., Guillén, F., Valverde, J. M., Zapata, P., Castillo, S.,Valero, D. (2007). Tools to maintain postharvest fruit and vegetable quality through the inhibition of ethylene action: a review. *Critical Reviews in Food Science and Nutrition,* 47, 543-560.

Mayer, B., & Hemmens, B. (1997). Biosynthesis and action of nitric oxide in mammalian cells. *Trends in Biochemical Sciences,* 22, 477-481.

Millar, A. H., & Day, D. A. (1996). Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. *Federation of European Biochemical Societies Letters,* 398, 155-158.

Mita, S., Kawamura, S., Yamawaki, K., Nakamura, K., & Hyodo, H. (1998). Differential expression of genes involved in the biosynthesis and perception of ethylene during ripening of passion fruit (*Passiflora edulis Sims*). *Plant and Cell Physiology,* 39, 1209-1217.

Miyazaki, J. H., & Yang, S. F. (1987). The methionine salvage pathway in relation to ethylene and polyamine biosynthesis. *Physiologia Plantarum,* 69, 366-370.

Morgan, P. W., & Drew, M. C. (1997). Ethylene and plant responses to stress. *Physiologia Plantarum,* 100, 620-630.

Morgott, D. A. (2015). Anthropogenic and biogenic sources of Ethylene and the potential for human exposure: A literature review. *Chemico-Biological Interactions,* 241, 10-22.

Ni, Z.-J., Hu, K.-D., Song, C.-B., Ma, R.-H., Li, Z.-R., Zheng, J.-L., Fu, L.-H., Wei, Z.-J., Zhang, H. (2016). Hydrogen sulfide alleviates postharvest senescence of grape by modulating the antioxidant defenses. *Oxidative Medicine and Cellular Longevity.* 14, Article ID 4715651.

Nip, W. K., Nollet, L. M., Paliyath, G., & Simpson, B. K. (2008). *Food Biochemistry an Introduction. In Y.H. Hui (Ed.), Food Biochemistry and Food Processing* (2nd. Ed) Ames, Iowa: Black well, PP (3-24).

Olafsdottir, S., Gardarsson, S., & Andradottir, H. (2014). Natural near field sinks of hydrogen sulfide from two geothermal power plants in Iceland. *Atmospheric Environment,* 96, 236-244.

Olivier, C., Halseth, D. E., Mizubuti, E. S., & Loria, R. (1998). Postharvest application of organic and inorganic salts for suppression of silver scurf on potato tubers. *Plant Disease*, 82, 213-217.

Olson, K. R. (2011). The therapeutic potential of hydrogen sulfide: separating hype from hope. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 301, R297-R312.

Paliyath, G., Murr, D. P., Handa, A. K., & Lurie, S. (2009). *Postharvest Biology and Technology of fruits, Vegetables, and Flowers. Ames,* Iowa : Wiley-Blackwell, 28-33.

Papenbrock, J., Riemenschneider, A., Kamp, A., Schulz-Vogt, H. N., & Schmidt, A. (2007). Characterization of cysteine-degrading and $H_2S$-releasing enzymes of higher plants-from the field to the test tube and back. *Plant Biology,* 9, 582-588.

Pech, J.-C., Latché, A., & Bouzayen, M. (2010). Ethylene Biosynthesis. In *Plant Hormones.* Springer, 115-136.

Prabhakar, N. R. (2012). Carbon monoxide (CO) and hydrogen sulfide ($H_2S$) in hypoxic sensing by the carotid body. *Respiratory Physiology & Neurobiology, 184,* 165-169.

Pratt, H. K., & Goeschl, J. D. (1969). Physiological roles of ethylene in plants. *Annual Review of Plant Physiology*, 20, 541-5.

Qu, K., Chen, C. P., Halliwell, B., Moore, P. K., & Wong, P. T.-H. (2006). Hydrogen sulfide is a mediator of cerebral ischemic damage. *Stroke*, 37, 889-893.

Rennenberg, H., Arabatzis, N., & Grundel, I. (1987). Cysteine desulphydrase activity in higher plants: evidence for the action of L-and D-cysteine specific enzymes. *Phytochemistry*, 26, 1583-1589.

Rodrıguez, F. I., Esch, J. J., Hall, A. E., Binder, B. M., Schaller, G. E., & Bleecker, A. B. (1999). A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. *Science*, 283, 996-998.

Rojas-Grau, M. A., Sobrino-Lopez, A., Tapia, M. S., & Martin-Belloso, O. (2006). Browning inhibition in fresh-cut 'Fuji' apple slices by natural antibrowning agents. *Journal of Food Science*. issue 1, S59-S65.

Romero, L. C., Aroca, M. Á., Laureano-Marín, A. M., Moreno, I., García, I., & Gotor, C. (2014). Cysteine and cysteine-related signaling pathways in *Arabidopsis thaliana*. *Molecular* plant, *7*, 264-276.

Rose, P., Dymock, B. W., & Moore, P. K. (2015). GYY4137, a novel water-soluble, $H_2S$-releasing molecule. In E. Cadenas, L. Packer (Eds.), *Methods in Enzymology* ($1^{st}$ .ed., vol.554, pp.143-167). Burlington,Vermont: Academic Press.

Roshchina, V. V., & Roshchina, V. D. (2013). *Ozone and plant cell.* Dordrecht, Netherlands: Springer Science & Business Media,181-182.

Sakai, H., Hua, J., Chen, Q. G., Chang, C., Medrano, L. J., Bleecker, A. B., & Meyerowitz, E. M. (1998). ETR2 is an ETR1-like gene involved in ethylene signaling in Arabidopsis. *Proceedings of the National Academy of Sciences*, 95, 5812-5817.

Sandhya. (2010). Modified atmosphere packaging of fresh produce: Current status and future needs. *Lebensmittel-Wissenschaft & Technologies -Food Science and Technology*, 43, 381-392.

Scott, K., McGlasson, W., & Roberts, E. (1970). Potassium permanganate as an ethylene absorbent in polyethylene bags to delay ripening of bananas during storage. *Animal Production Science,* 10, 237-240.

Sharma, S., & Rao, T. R. (2013). Effect of honey and L-cysteine as antioxidants on the quality attributes of fresh-cut carambola (*Averrhoa carambola L.*) stored at two different temperatures. *International Journal of Postharvest Technology and Innovation* 24*, 3,* 362-381.

El-Sharkawy, I., Jones, B., Li, Z. G., Lelièvre, J. M., Pech, J. C., & Latché, A. (2003). Isolation and characterization of four ethylene perception elements and their expression during ripening in pears (*Pyrus communis L.*) with/without cold requirement. *Journal of Experimental Botany*, 54, 1615-1625.

Shibui, Y., Sakai, R., Manabe, Y., & Masuyama, T. (2017). Comparisons of l-cysteine and d-cysteine toxicity in 4-week repeated-dose toxicity studies of rats receiving daily oral administration. *Journal of Toxicologic Pathology*, 30, 217-229.

Sisler, E. C., & Blankenship, S. M. (1996). Method of counteracting an ethylene response in plants. *U.S. Patent No. 5,518,988*. Washington, DC: U.S. Patent and Trademark Office.

Sisler, E. C., Reid, M. S., & Fujino, D. W. (1983). *Investigation of the mode of action of ethylene in carnation senescence.* Paper presented at the *II International Symposium on Carnation Culture. Acta Horticulturae*, *141*, 229-234.

Skog, & CL Chu, L. (2001). Effect of ozone on qualities of fruits and vegetables in cold storage. *Canadian Journal of Plant Science*, 81, 773-778.

Snowdon, A. L. (2010). *Post-Harvest Diseases and Disorders of Fruits and Vegetable* (vol. 2). London, UK: Manson.

Soegiarto, L., & Wills, R. (2004). Short term fumigation with nitric oxide gas in air to extend the postharvest life of broccoli, green bean, and bok choy. *HortTechnology*, *14*, 538-540.

Stipanuk, M. H. (2004). Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Annual Review of Nutrition ., spell out* 24, 539-577.

Sun, Q., Collins, R., Huang, S., Holmberg-Schiavone, L., Anand, G. S., Tan, C.-H., Van-den-Berg, S., Deng, L.-W., Moore,P.k., Karlberg, T. (2009). Structural basis for the inhibition mechanism of human cystathionine γ-lyase, an enzyme responsible for the production of $H_2S$. *Journal of Biological Chemistry*, 284, 3076-3085.

Sun, Y., Zhang, W., Zeng, T., Nie, Q., Zhang, F., & Zhu, L. (2015). Hydrogen sulfide inhibits enzymatic browning of fresh-cut lotus root slices by regulating phenolic metabolism. *Food Chemistry,* 177, 376-381.

Takahashi, H., Kopriva, S., Giordano, M., Saito, K., & Hell, R. (2011). Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. *Annual Review of Plant Biology*, 62, 157-184.

Tas, C. E., Hendessi, S., Baysal, M., Unal, S., Cebeci, F. C., Menceloglu, Y. Z., & Unal, H. (2017). Halloysite nanotubes/polyethylene nanocomposites for active food packaging materials with ethylene scavenging and gas barrier properties. *Food and Bioprocess Technology,* 10, 789-798.

Thompson, C. R., & Kats, G. (1978). Effects of continuous hydrogen sulfide fumigation on crop and forest plants. *Environmental Science & Technology,* 12, 550-553.

Tiwari, B., & Muthukumarappan, K. (2012). Ozone in fruit and vegetable processing. *Ozone in Food Processing*, 55-80.

USDA, Code of Federal Regulations. Title 21, Vol 3. (Revised April 1, 2017) [21CFR184.1271].Retrieved from https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1271

Wallace, J. L. (2007). Hydrogen sulfide-releasing anti-inflammatory drugs. *Trends in Pharmacological Sciences*, 28, 501-505.

Wang, R. (2002). Two's company, three'sa crowd: can $H_2S$ be the third endogenous gaseous transmitter? *The Federation of American Societies for Experimental Biology Journal,* 16, 1792-1798.

Wang, T.-T. (2010). Application of Postharvest Technologies for Vegetable Crops in Taiwan. In Technology on Reducing Post-harvest Losses and Maintaining Quality of Fruits and Vegetables (pp. 12-18): Proceedings of AARDO Workshop.

Warton, M., Wills, R., & Ku, V. (2000). Ethylene levels associated with fruit and vegetables during marketing. *Australian Journal of Experimental Agriculture,* 40, 465-470.

Watkins, C. B. (2002). Ethylene synthesis, mode of action consequences and control. In M. Knee (Eds.), *Fruit Quality and Its Biological Basis (*pp.180-224). Sheffield, UK: Sheffield Academic press.

Watkins, C. B. (2015). Advances in the use of 1-MCP. In R. B. H. Wills & J. B. Golding (Eds.), *Advances in Postharvest Fruit and Vegetable Technology* (pp.117-145). Boca Raton, FL: CRC press.

Wills, R. B. H. (2015). Low ethylene technology in non-optimal storage temperatures. In R. B. H. Wills & J. B. Golding (Eds.), *Advances in Postharvest Fruit and Vegetable Technology* (pp. 167-190). Beco Raton, FL: CRC Press.

Wills, R., & Golding, J. (2016). *Postharvest: an Introduction to the Physiology and Handling of Fruit and Vegetables* (6th.ed.). Sydney, NSW: UNSW press.

Wills, R. B. H., Harris, D. R., & Seberry, J. A. (2012). Use of ventilation with ambient air to inhibit ripening of banana during long distance transport. *Food Australia,* 64, 38-44.

Wills, R., McGlasson, B., Graham, D., Joyce, D., & Rushing, J. W. (1999). Postharvest: An Introduction to the Physiology and Handling of Fruit,Vegetables and Ornamentals. *Journal of Vegetable Crop Production, 4*, 83-84.

Wills, R. B. H., Ku, V. V. V., & Leshem, Y. Y. (2000). Fumigation with nitric oxide to extend the postharvest life of strawberries. *Postharvest Biology and Technology, 18*, 75-79.

Yahia, E., Ornelas-Paz, J. D. J., & Elansari, A. (2011). Postharvest technologies to maintain the quality of tropical and subtropical fruits. In E. M. Yahia *Postharvest Biology and Technology of Tropical and Subtropical Fruits(*1st.ed.,vol.1*):* Fundamental Issues, 142-195e. Philadelphia, Pennsylvania: Woodhead.

Yamasaki, S., Fujii, N., & Takahashi, H. (2000). The ethylene-regulated expression of CS-ETR2 and CS-ERS genes in cucumber plants and their possible involvement with sex expression in flowers. *Plant and Cell Physiology,* 41, 608-616.

Yang, S. F., & Hoffman, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology,* 35, 155-189.

Zaharah, S., & Singh, Z. (2011). Postharvest nitric oxide fumigation alleviates chilling injury, delays fruit ripening and maintains quality in cold-stored 'Kensington Pride'mango. *Postharvest Biology and Technology,* 60, 202-210.

Zhao Y, Biggs TD and Xian M, (2014). Hydrogen sulfide ($H_2S$) releasing agents: chemistry and biological applications. *Chemical Communications* 50**:** 11788–11805

Zhang, H., Hu, L.-Y., Li, P., Hu, K.-D., Jiang, C.-X., & Luo, J.-P. (2010). Hydrogen sulfide alleviated chromium toxicity in wheat. *Biologia Plantarum, 54,* 743-747.

Zhang, H., Hu, L. Y., Hu, K. D., He, Y. D., Wang, S. H., & Luo, J. P. (2008). Hydrogen sulfide promotes wheat seed germination and alleviates oxidative damage against copper stress. *Journal of Integrative Plant Biology, 50,* 1518-1529.

Zhang, H., Hu, S.-L., Zhang, Z.-J., Hu, L.-Y., Jiang, C.-X., Wei, Z.-J., Liu, J., Wang ,H.-L., Jiang, S.-T. (2011). Hydrogen sulfide acts as a regulator of flower senescence in plants. *Postharvest Biology and Technology,* 60, 251-257.

Zheng, J.-L., Hu, L.-Y., Hu, K.-D., Wu, J., Yang, F., & Zhang, H. (2016). Hydrogen sulfide alleviates senescence of fresh-cut apple by regulating antioxidant defense system and senescence-related gene expression. *HortScience,* 51, 152-158.

Zhu, L., Wang, W., Shi, J., Zhang, W., Shen, Y., Du, H., & Wu, S. (2014). Hydrogen sulfide extends the postharvest life and enhances antioxidant activity of kiwifruit during storage. *Journal of the Science of Food and Agriculture, 94,* 2699-2704.

## 6. Appendix 1

Statement of the contribution of the candidate and other authors

This is to confirm that Hebah Muhsien Sabiah Al Ubeed has contributed to the series of research which are submitted as a part of her PhD thesis as shown in each paper below.

| | 5/06/2018 | | 5/06/2018 |
|---|---|---|---|
| …… | …… | …… | …… |
| Prof R.B.H. Wills | Date | Dr J.B. Golding | Date |

| | 5/06/2018 | | 5/06/2018 |
|---|---|---|---|
| …… | …… | …… | …… |
| A/Prof M.C. Bowyer | Date | Dr Q.V. Vuong | Date |

Endorsed by

…… …20/06/2018……

Prof F. Martin, Assistant Dean (Research Training) Date

1. **H.M.S. Al Ubeed**, R.B.H. Wills, M.C. Bowyer, Q.V. Vuong, J.B. Golding. Interaction of exogenous hydrogen sulphide and ethylene on senescence of green leafy vegetables. Postharvest Biology and Technology (2017), 133, 81-87, https://doi.org/10.1016/j.postharvbio.2017.07.010.

| Contributor | Statement of Contribution |
|---|---|
| H.M.S. Al Ubeed (Candidate) | Designed experiments (40%)<br>Performed experiments (100%)<br>Analysed data (80%)<br>Wrote the manuscript (50%) |
| R.B.H. Wills | Designed experiments (40%)<br>Analysed data (10%)<br>Wrote the manuscript (50%) |
| M.C. Bowyer | Designed experiments (10%)<br>Critically reviewed the manuscript (70%) |
| J.B. Golding | Designed experiments (10%)<br>Critically reviewed the manuscript (20%) |
| Q.V. Vuong | Critically reviewed the manuscript (10%)<br>Analysed data (10%) |

2. **H.M.S. Al Ubeed**, R.B.H. Wills, M.C. Bowyer, Q.V. Vuong, J.B. Golding. Effect of hydrogen sulphide, nitric oxide and ethylene on the postharvest deterioration of Pak choy International Society for Horticultural Science (ISHS) VI Postharvest Unlimited Conference. Madrid, Spain October 2017. (Oral presentation) Accepted for publication in Acta Horticulture.

| Contributor | Statement of Contribution |
|---|---|
| H.M.S. Al Ubeed (Candidate) | Designed experiments (50%)<br>Performed experiments (100%)<br>Analysed data (80%)<br>Wrote the manuscript (60%) |
| R.B.H. Wills | Designed experiments (50%)<br>Analysed data (20%)<br>Critically reviewed the manuscript (40%) |
| M.C. Bowyer | Critically reviewed the manuscript (30%) |
| J.B. Golding | Wrote the manuscript (40%) |
| Q.V. Vuong | Critically reviewed the manuscript (30%) |

3. **H.M.S. Al Ubeed**, R.B.H. Wills, M.C. Bowyer, J.B. Golding. Comparison of hydrogen sulphide with 1-methylcyclopropene (1-MCP) to inhibit senescence of the leafy vegetable, pak choy. Postharvest Biology and Technology (2018), 137, 129–133, https://doi.org/10.1016/j.postharvbio.2017.11.020.

| | |
|---|---|
| H.M.S. Al Ubeed (Candidate) | Designed experiments (40%)<br>Performed experiments (100%)<br>Analysed data (80%)<br>Wrote the manuscript (50%) |
| R.B.H. Wills | Designed experiments (40%)<br>Analysed data (20%)<br>Wrote the manuscript (50%) |
| M.C. Bowyer | Designed experiments (5%)<br>Critically reviewed the manuscript (70%) |
| J.B. Golding | Designed experiments (15%)<br>Critically reviewed the manuscript (30%) |

4. **H.M.S. Al Ubeed**, R.B.H. Wills, M.C. Bowyer, J.B. Golding. Interaction of the hydrogen sulphide inhibitor, propargylglycine (PAG), with hydrogen sulphide on postharvest changes of the green leafy vegetable, pak choy. Accepted for publication in Postharvest Biology and Technology.

| Contributor | Statement of Contribution |
|---|---|
| H.M.S. Al Ubeed (Candidate) | Designed experiments (30%)<br>Performed experiments (100%)<br>Analysed data (80%)<br>Wrote the manuscript (30%) |
| R.B.H. Wills | Designed experiments (30%)<br>Analysed data (20%)<br>Wrote the manuscript (50%) |
| M.C. Bowyer | Designed experiments (30%)<br>Critically reviewed the manuscript (75%)<br>Wrote the manuscript (20%) |
| J.B. Golding | Designed experiments (10%)<br>Critically reviewed the manuscript (25%) |

**5. H.M.S. Al Ubeed**, R.B.H. Wills, M.C. Bowyer, J.B. Golding. Inhibition of postharvest senescence of green leafy vegetables, by exogenous D-cysteine and L-cysteine as precursors of hydrogen sulphide. Journal of Horticultural Science and Biotechnology (submitted)

| Contributor | Statement of Contribution |
|---|---|
| H.M.S. Al Ubeed (Candidate) | Designed experiments (40%)<br>Performed experiments (100%)<br>Analysed data (80%)<br>Wrote the manuscript (40%) |
| R.B.H. Wills | Designed experiments (40%)<br>Analysed data (20%)<br>Wrote the manuscript (50%) |
| M.C. Bowyer | Designed experiments (10%)<br>Critically reviewed the manuscript (50%)<br>Wrote the manuscript (10%) |
| J.B. Golding | Designed experiments (10%)<br>Critically reviewed the manuscript (50%) |

# Appendix 2

## List of Abbreviations

ABA Abscisic acid

ACC 1-aminocyclopropane-1-carboxylic acid

ACS 1-aminocyclopropane-1-carboxylic acid synthase

ACO 1-aminocyclopropane-1-carboxylic acid oxidase

AOA Amino-oxyacetic acid (Carboxy methoxyamine)

AVG Aminoethyloxyvinylglycine

CAT Catalase

CAT Cysteine aminotransferase

CBS Cystathionine –β- Synthase

$C_2H_4$ Ethylene

CSE Cystathionine–γ-lyase

$CO_2$ Carbon dioxide

CTR Constitutive triple response complex

DAO D-Amino acid oxidase

DPPH 2,2-diphenyl-1-picrylhydrazyl

EDRF Endothelium derived relaxing factor

ER Endoplasmic reticulum

D-CD D-Cysteine desulphhydrase

GRAS Generally recognised as Safe Status

GYY4137 4-Methoxyphenylmorpholino-phosphinodithioate acid

HNTs Halloysite nanotubes

$H_2S$ Hydrogen sulphide

$KMnO_4$ Potassium permanganate

Lawesson’s reagent 4-Methoxyphenyl-1,2,3,4-dithia di phosphetane-2,4,disulfide

L-CD L-Cysteine desulfhydrase

3-MAT 3-Mercaptopyruvate sulfurtransferase

1-MCP Methylcyclopropene

mRNA Messenger ribonucleic acid

MST-SSH Mercapto pyruvate sulfurtransferase-hydropersulfide

$Na_2S$ Sodium sulphide

NaHS Sodium hydrosulphide

NO Nitric oxide

OAS O-acetyl L-serine

$O_2$ Oxygen

$O_3$ Ozone

$O^{\cdot}$ Oxygen radicals

OAS-TL O-acetyl-L-serine (thiol) lyase

PAG L- Propargylglycine

PLP Pyridoxal -5-phosphate

POD Peroxidase

PPO Polyphenol oxidase

SAM S-adenosyl-L-methionine

SAT Serine acetyltransferase

SOD Superoxide dismutase

TA Titratable acidity

TPC Total phenolic content

TSS Total soluble solids