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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

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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.

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. The plants were at the

size and maturity that the grower sends to market. 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 fresh produce market. Leaf branches for each produce were randomly distributed into nine units with each weighing about 100 g. In each experiment on the three vegetables, a treatment was applied to three units of produce and 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 μL L^{-1} ethylene at 45 mL min^{-1}. The <0.001 μL L^{-1} concentration was considered ethylene-free as the analytical limit of detection was 0.001 μL L^{-1}, while 0.1 μL 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 μL 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.

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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

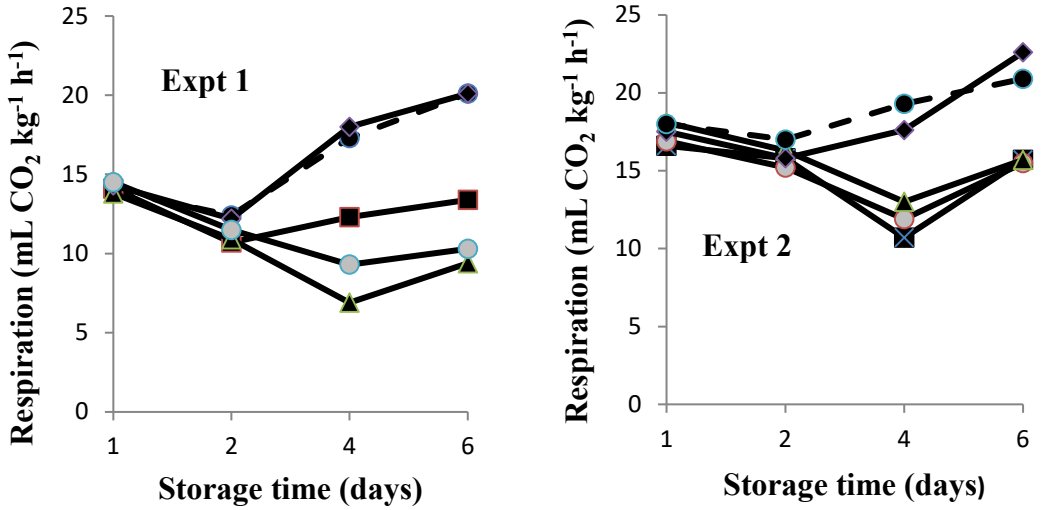

Figure 1. Respiration rate of pak choy leaves sprayed with L-cysteine and D-cysteine solution or fumigated with H_2S during storage at 10°C in 0.1 µL L^{-1} ethylene. Values in each Experiment are the mean of nine readings (three units x three batches of produce).

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.

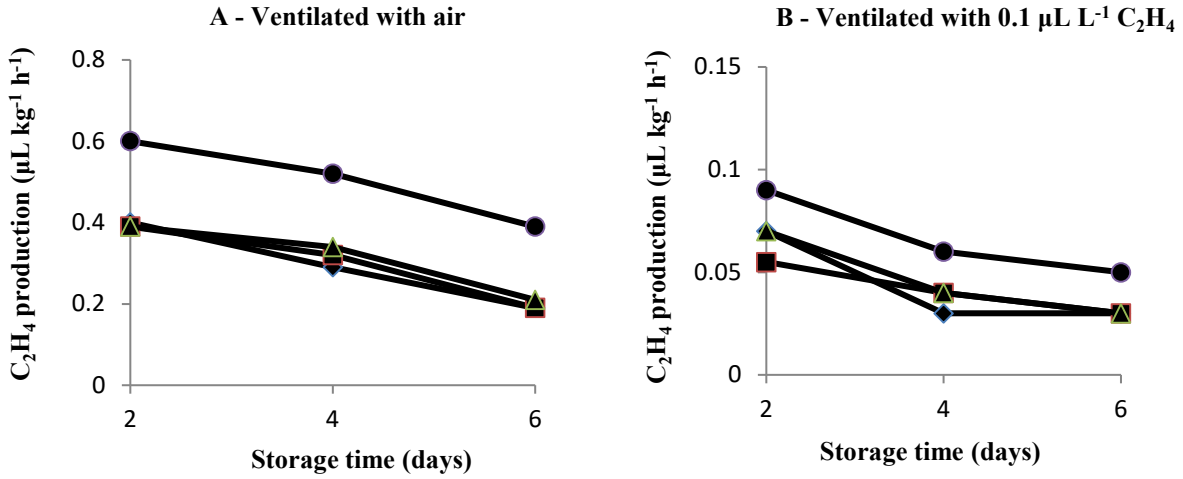

Figure 2. Rate of ethylene production by pak choy leaves sprayed with L-cysteine and D-cysteine solution or fumigated with H_2S during storage at 10°C while ventilated with air (A) and 0.1 µL L^{-1} ethylene (B). Values are the mean of nine readings (three units x three batches of produce).

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.