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REVIEW

Harnessing senescence delaying gases nitric oxide and nitrous oxide: a novel approach to postharvest control of fresh horticultural produce

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Abstract

The effects of ethylene on postharvest horticultural produce are a major limiting factor in their orderly and efficient marketing. Nitric oxide and nitrous oxide have recently been shown to inhibit ethylene action and synthesis in higher plants and their potential for commercial use to extend the storage and marketing life of fruits, vegetables and flowers is reviewed.

Additional key words: autocatalysis, ethylene, nitric oxide synthase, shelf life, stress.

Introduction

Ethylene is metabolised by all horticultural produce and in the confined spaces of postharvest containers or storage chambers it can accumulate to relatively high concentrations. Ethylene can also be derived from a wide range of other sources related to the incomplete combustion of fuels as in automobiles, fork lift vehicles or equipment powered by an internal combustion engine, where such devices are operated in the vicinity of horticultural handling and storage facilities. Ethylene can also occur from cross contamination with ethylene produced from one commodity accumulating around others held in the same storage chamber.

The action of ethylene on horticultural commodities after harvest has many and sometimes contrasting effects (Wills *et al.* 1997). While a beneficial effect is as the

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Abbreviations: ACC - aminocyclopropane-1-carboxylic acid; ACO - ACC oxidase; ACS - ACC synthase; CM - calmodulin; GAS - general adaptation syndrome; HR - hypersensitive response; N_2O - nitrous oxide; NO - nitric oxide; NOS - nitric oxide synthase; W7 - naphthalene sulphonamide.

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initiator of ripening in climacteric fruits, in many postharvest situations it is important to delay ripening in order to allow orderly marketing. All other effects of ethylene are considered deleterious as they promote senescence. The presence of ethylene around postharvest horticultural commodities is therefore considered undesirable. Traditionally, a threshold concentration of 0.1 cm^3 dm^{-3} was considered to be a safe limit of ethylene exposure (Knee *et al.* 1985). However, recent studies in Australia on a wide range of horticultural produce has suggested there is no safe limit of ethylene exposure and that concentrations above 0.005 cm^3 dm^{-3} produce a deleterious response (Wills 1997). These findings have emphasised the benefit that can be obtained from either preventing the accumulation of ethylene around produce or from inhibiting the action of ethylene that does accumulate around produce.

In recent years, the major advances in regulation of ethylene-related postharvest processes in fruits, vegetables and flowers has been by genetic engineering. Much emphasis and effort have been placed on two major biosynthetic pathways: *1*) regulation of ethylene evolution and/or of sensitivity to ethylene and *2*) deceleration of cell wall softening. These processes are not necessarily independent since process *2* involving such enzymes as pectin methylesterase, β-D galactosidase, α-L-arabinosidase and polygalacturonidase may be triggered by process *1* (Guise *et al.* 1997).

Utilizing biotechnological techniques, antisense aminocyclopropane-1-carboxylic acid oxidase (ACO) and/or aminocyclopropane-1-carboxylic acid synthase (ACS) which in certain instances is aided by *Agrobacterium* mediated gene transfer from *Arabidopsis* mutant genes which impart lack of sensitivity to ethylene, several genetically engineered, longer life plant cultivars have been produced. Typical examples are the commercialised Flavor Saver tomato, the *Chanterais* cantaloupe melon, and extended vase-life carnations (*Dianthus caryophyllus*) (Van Altvorst and Bovy 1995). A recent compendium describes the state of the art and on-going research in this area (Kannelis *et al.* 1997).

While current opinion tends to the view that humans or livestock are not at risk by eating transgenic food, there is a certain degree of unease in the general community about the introduction of genetically engineered foods, although less so, in flowers. It is argued that genetic engineering does not always result in predictable modifications to organisms and that interaction between genes can be extremely complex (Morris 1995). For example, insecticidal or herbicidal toxins made by inedible wild type plants and introduced into crops by gene splicing are new food ingredients whose safety to humans should be tested. It is feasible that genetically engineered plants may be induced to produce substances that are more harmful or toxic to humans than the synthetic pesticides or herbicides they are designed to replace.

The wariness of the consumer is reflected in certain supermarkets in the USA who advertise they do *not* sell genetically engineered produce, while in Australia labels of some packaged vegetable produce state "not genetically manipulated". Likewise, while intrinsically appreciating the extended shelf life benefits of genetically manipulated commodities, some consumers are not quite satisfied with their organoleptic qualities.

The uncertainty surrounding the future impact of genetically engineered foods reinforces the need to continue to develop other novel processes which hold promise of increasing longevity of postharvest horticultural commodities, thereby reducing wastage during handling and storage, and facilitating cost-effective long distance marketing. A major target for delaying senescence and ripening of horticultural produce is through the use of some intervention technique that either reduces the accumulation of ethylene or inhibits the deleterious action of ethylene. This review examines the potential for two gaseous nitrogen oxides to fulfil this function, namely nitric oxide (NO) which is endogenously produced by plants, and nitrous oxide (N_2O), also termed "laughing gas". The promising horticultural use of the ethylene inhibiting and senescence-delaying synthetic gas, 1-methylcyclopropene, has of late been well reviewed elsewhere (Sisler and Serek 1997) and therefore is not here discussed.

Nitric oxide

The NO free radical in recent years has commanded considerable interest and in some circles has been designated "biochemistry's new superstar" (Feldman *et al.* 1993). A recent review of the action of NO in biological systems highlighted the paucity of research, until very recently, relating to the detection of NO in higher plants (Leshem 1996).

Synthesis and function: NO is synthesised by the conversion of L-arginine to L-citrulline, a reaction catalysed by nitric oxide synthase (NOS; EC 1.14.13.39) (Fig. 1) (Kuo *et al.* 1995, Sen and Cheema 1995, Ninneman and Maier 1996, Huang and Knopp 1997). This free radical gas exerts contrasting effects on many physiological and pathological processes in mammalian tissues (Schmidt and Walter

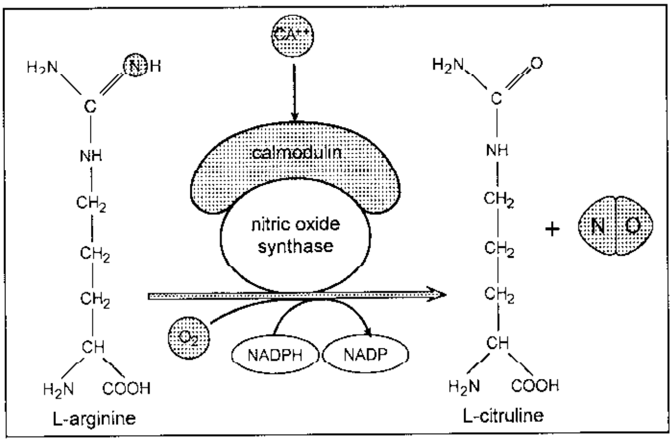

Fig. 1. Mode of NO biosynthesis. A further NOS cofactor is tetrahydrobiopterin.

1994). On one hand, it is a messenger promoting neurotransmission (Bredt and Snyder 1990, Snyder 1992), antitumor and antibacterial activities (Nathan and Hibbs 1991) and vasodilation (Konorev *et al.* 1995). Hence, NO has found therapeutic use to alleviate aschemia, asthma and other pulmonary hypertension complications. On the other hand, NO is involved in induction of neurological diseases (Koprowski *et al.* 1993), nitration of tyrosine (Beckman *et al.* 1994) and reaction with superoxide radical to form peroxynitrite which in turn induces membrane lipid peroxidation (Radi *et al.* 1991). In mammalian systems, hazardously high endogenous NO levels are, *inter alia*, associated with stroke, septic shock and migraines. Moreover, certain NO derivatives such as nitroso-amines may cause DNA breakage. A detailed discussion of the pathophysiological and pharmacological effects of NO and its derivatives may be found elsewhere (Moneada *et al.* 1991).

NOS has been isolated and purified from various vertebrates and invertebrates and the NOS-encoding gene has been cloned from different animal sources (Bredt *et al.* 1991, Geller *et al.* 1993, Regulski and Tully 1995, Sessa *et al.* 1992). NOS actually represents a family of isoforms with some demonstrated to require Ca^{2+} and calmodulin (CM) as co-factors and others not. The latter contention has been challenged by demonstrating that the Ca^{2+}/CM "independent" forms have Ca^{2+} and CM included as integral structural components of the enzyme. Research carried out by Leshem and Haramaty (unpublished data) indicates that the putative plant NOS appears to be Ca^{2+} and CM dependent since NO-promoted plant regulatory processes (to be detailed in a later section) are to a large degree inhibited by the CM blocking compound naphthalene sulphonamide (W7). This NOS and Ca^{2+}/CM interplay may shed further light on the role of Ca^{2+} when impregnated into postharvest fruit in order to improve storage potential (Ferguson and Watkins 1981).

NO characterization and mode of action in higher plants: The existence and characterization of NO in plants was revealed only recently, the emission of NO from pea foliage being detected by Leshem (1996) and Leshem and Haramaty (1996) using a NO-specific probe. Moreover it was found that on a molar basis, pea foliage emitted more NO than ethylene. The presence of the ethylene precursor aminocyclopropane-1-carboxylic acid (ACC), was found to enhance both NO and ethylene emission. It was postulated that ethylene production in growing plants may be regulated by NO (Leshem and Haramaty 1996, Leshem *et al.* 1997). Based on the stoichiometry of endogenously emitted ethylene and NO, it was also concluded that under short term environmental stresses such as drought, heat and salinity, NO emission may act as a natural stress coping agent. Consequently, NO may be a further component of the "general adaptation syndrome (GAS)" reaction in plants whereby the plant employs identical or similar compounds to combat different types of environmental stress (Leshem and Kuiper 1996).

A particularly interesting and promising lead as to the possibility of a further and pivotal role of NO in higher plants relates to resistance to pathogenic infection and in particular to the nature of the "hypersensitive response" (HR). The HR reaction is characterised by the rapid death of plant cells, normally within 24 h, at the site of pathogen penetration. As a result, the pathogen is localised at the penetration site and thus prevented from spreading to other parts of the plant (Goodman and Novacky 1994). In postharvest physiology, besides general interest in phytosanity, this is of particular consequence in leafy vegetables. Employing a combination of the arginine/citrulline assay, specific compounds which either release NO or inhibit NOS, and NOS antibodies, elegant experimentation has demonstrated active NO participation in invoking the HR response in tobacco when challenged with *Ralstonia solanacearum* (syn. *Pseudomonas solanacearum*) (Huang and Knopp 1997).

All of the above essentially promotive effects were obtained at low NO concentrations. However, at higher concentrations exceeding the 10^{-5} M to 10^{-4} M range which are prevalent under prolonged environmental stress or at industrially polluted sites, NO is inhibitory (Leshem *et al.* 1997). We here mention that to date, all concentrations of NO experimentally applied and found to be horticulturally effective in the following reported trials were in the range of three orders of magnitude less than those found in the "hazard zone".

Kinetics of NO evolution in ripening postharvest fruits: To determine endogenous NO content, a series of trials carried out in the authors' laboratories employed a 200 mM tipped microprobe of a differentially sensitive NO sensor (*World Precision Instruments*, USA) either inserted directly into the flesh of unripe green produce and of the same produce when ripe, or held 1 cm above the fruit, vegetable or flower exteriors. In the latter case care was taken during the brief measurement duration (usually < 2 min) to forestall $NO \rightarrow NO_2$ conversion.

Such observations carried out on a wide spectrum of fruits, vegetables and flowers (both climacteric and non-climacteric) and cutting across a wide array of botanical families, revealed that emission of endogenous NO in all cases was considerably higher (usually two to fivefold) in the unripe fruit than in the ripe fruit and that with ripening or senescence, NO decrease goes hand-in-hand with ethylene increase (Leshem, Wills, Veng-Va Ku and Wade, unpublished results). Tested horticultural produce included avocado (*Persea americana*), bananas (*Musa sapientum* var. *cavendishi*), *Citrus* spp., broccoli (*Brassica oleracea*), kiwi (*Actinidia chinensis*), cherry tomatoes (*Lycopersicum esculentum*), persimmons (*Diospyros kaki*) and the flower species — carnations (*Dianthus caryophyllus*) and 'Waratah' (*Telopea speciosissima*), an indigenous Australian proteaceous ornamental. Concerning ethylene, it is well documented that emission of this gas increases with maturation of these and related species (Wills *et al.* 1997); thus an ethylene/NO stoichiometry is apparent.

Further experimentation with grapefruit (*Citrus paradisi*) and other citrus species indicated that in peel tissue, the coloured flavedo contained approximately four times more NO than the white albedo. The role of NO in maintenance of peel chlorophyll retention was further explored by comparing a green peel fruit (sweet lime, *Citrus auranthifolia*) to a fruit whose peel is protected from light by dense brown trichome pubescence (kiwifruit, *Actinidia chinensis*). Results obtained in the laboratory of one of the authors (Y.Y.L.) showed that in unripe fruit, iradiation increased NO emission in both fruit types. However, in the green immature fruit the NO increase was far greater while in mature fruits iradiation had only a marginal effect on NO levels.

Collectively, the above observations indicate that a decrease of NO emission is directly related to fruit ripening and that in those species with green peel a major site of NO emission is chlorophyll related.

Horticultural application of NO: The findings reported in the above section suggest that exogenous application of NO could delay the fruit ripening process in climacteric and non-climacteric fruit and furthermore, could inhibit onset of general senescence. A problem faced in the application of NO is that in air, NO rapidly converts to NO_2 with half life of *ca.* 5 - 12 s (Snyder 1992). The use of exogenous NO thus necessitates fumigation with NO in an oxygen-depleted atmosphere. This was achieved in a series of experiments with a wide range of climacteric and non-climacteric fruit and vegetables by initially displacing oxygen with nitrogen in the atmosphere around produce followed by the inclusion of low concentrations of NO into the nitrogen atmosphere for 2 - 24 h. It appears that optimal effective NO concentrations and fumigation duration vary between different commodities.

This technique, as opposed to those in common use employing "controlled atmospheres" viz. enhancing CO_2 concentration or reducing O_2 concentration, has the advantage of being of short duration allows produce to be stored and marketed without the need to employ any further treatment. For the proposed horticultural treatment, NO may be procured commercially; alternatively this free radical gas may be generated chemically (Leshem and Haramaty 1996).

Taken altogether the above observations suggest a novel means of postharvest control to maximize the shelf life of fruits, vegetables and flowers, the principles and the practical details of which are described elsewhere (Leshem and Wills 1977). We here mention that in most of the above-tested commodities and in others, including strawberries (*Fragaria ananassa*), as compared to non-treated controls, shelf life duration increment was in the range of 50 - 150 %. Experiments are under way, testing a wide variety of commodities both climacteric and non-climacteric. A further advantage is that NO is an endogenously produced gaseous plant compound and not a synthetic chemical.

Nitrous oxide

N_2O is a naturally occurring atmospheric gas, its primary source soil containing aerobic denitrifying bacteria (Anderson and Levine 1986, Firestone and Davidson 1989). Soil N_2O emission can be increased by addition of nitrite fertilizer (Shepherd *et al.* 1991) and irrigation of dry fields may increase N_2O evolution to the extent of three orders of magnitude (Davidson 1992). The topic of N_2O and NO emissions from soils has been extensively reviewed by Williams *et al.* (1992). At room temperatures N_2O is inert and stable and moreover, it is chemically neutral and like carbon dioxide has an isoteric linear structure which confers on both molecules similar physical properties such as relative stability and high solubility. This biophysical similarity of N_2O to CO_2 may be pertinent to the control of ethylene in the controlled atmosphere storage of postharvest climacteric fruit. On a per molecule basis, N_2O is approximately 200 × more potent than carbon dioxide with regard to atmospheric warming, however, because of its much lower atmospheric concentrations it contributes only 6 % to total global warming (Williams *et al.* 1992).

In medical practice, N_2O is used as an analgesic and an anaesthetic, and at high concentrations is a narcotic. The endogenous occurrence of N_2O in higher plants has not been reported. Nevertheless, in trials with application of exogenous N_2O, respiration of mitochondria from *Distichlis spicata* leaves and seeds was partially and reversibly inhibited. Moreover, storage of seed life of this species was extended (Sowa and Towill 1991).

N_2O effects on ripening and senescing climacteric fruits: Trials on horticultural produce to date with N_2O have only been reported for the climacteric fruits, tomato (*Lycopersicum esculentum* cv. Cappelo) and avocado (*Persea americana* cv. Hass) (Gouble *et al.* 1995). These were conducted by continuous gas treatment while placing the fruit in a 80 % N_2O + 20 % oxygen atmosphere. With tomatoes, N_2O inhibited the onset of ethylene synthesis which ordinarily occurs at the preclimacteric stage of ripening and lowered the rate of ethylene production when ripening commenced. In fruits treated at the later climacteric stage, N_2O inhibited ethylene autocatalysis — the importance of the latter process in other climacteric species being well discussed by Sfakiotakis *et al.* (1997). Parallel trials carried out on avocado produced essentially similar results with a 4-d delay in the normal ethylene/climacteric lag period, but there was no reduced rate of the ethylene rise when it did occur. Overall results suggest that N_2O is a potent antagonist of ethylene production as well of its action (Fath *et al.* 1990). Still required are further data extending the number of fruit and vegetables that may respond to N_2O. Moreover, information is still forthcoming on its efficiency in non-climacteric species. Further short term trials at considerably lower gas concentrations are also warranted.

Conclusions

The demonstrated effects of NO and N_2O in extending the postharvest life of fresh horticultural produce through inhibiting ethylene production and action will considerably advance the understanding of ethylene biotechnology and are a meaningful contribution towards the empirical understanding of the mode of interaction of N-containing gases with ethylene in higher plants. Their use has obvious immense potential commercial value in the storage and marketing of fruits, vegetables and flowers although considerable work is still required to demonstrate a cost-effective benefit to specific produce. In this regard, the use of NO with its initial short term fumigation application at low concentration would appear to have some advantage over N_2O where a longer term exposure at much higher concentration is required. This advantage can be a more economical use of gas as well as a reduced occupational health and safety issue. However, the need for NO to be applied in an oxygen-free atmosphere will require some innovation in the design and operation of the fumigation process.

Finally, despite what is stated in the Introduction, we do not rule out the possibility of prospective future use–parallel to that of senescence delaying gases and other techniques–of NOS-encoding-gene bioengineering in order to increase shelf life duration of horticultural produce and also possibly to improve pathogen resistance.

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