Nitrous oxide inhibition of ethylene production in ripening and senescing climacteric fruits

Barbara Gouble [a], Daniel Fath [b], Pierre Soudain [a],*

[a] *Laboratoire de Physiologie des Organes Végétaux après Récolte, CNRS, 4ter Route des Gardes,
92195 Meudon Cedex, France*
[b] *Centre de Recherche Claude-Delorme, SRAM, Air Liquide, Les Loges-en-Josas,
78350 Jouy-en-Josas, France*

Abstract

The anti-ethylene effects of nitrous oxide have been demonstrated for the first time in the ripening and senescence sequences in tomato and avocado fruit. Continuous gas treatment by 80% nitrous oxide plus 20% oxygen had striking inhibitory effects on ethylene production in the two fruit species, though ethylene biosynthesis levels differed greatly.

In preclimacteric treated fruits, nitrous oxide largely extended the lag period, and additively lowered ethylene production rate in tomatoes. In fruits treated at the climacteric stage, nitrous oxide markedly inhibited autocatalytic ethylene evolution. In fruit pretreated with exogenous ethylene, nitrous oxide also reversed induced autocatalysis as an antagonist of ethylene action.

At a later stage, some fruits in air exhibited a second ethylene rise as a signal of ultimate senescence. This was characterised by coincidence of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase activity peak, elevated ACC levels and ACC synthase activity. Nitrous oxide blocked or delayed this senescence ethylene upsurge. Analysis of the metabolic processes involved in the various counteracting actions of nitrous oxide revealed a very significant ethylene antagonist role of the gas which, unlike carbon dioxide, was non-toxic.

Keywords: Ethylene; Nitrous oxide; Tomato; Avocado; Ripening; Senescence

1. Introduction

Ethylene plays a major role in physiological events involving senescence and stress (Abeles et al., 1992). Studies of fruit ripening have increased understanding

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of the ethylene biosynthesis pathway and of genetic control, but little is known of the mechanism of action of the hormone and its receptor (Pech et al., 1993). Anti-ethylene compounds specifically acting on the synthesis and/or action of ethylene have been seen as very promising research tools. Sisler and Wood (1988), for example, discussed the various metabolic interactions observed between carbon dioxide and ethylene and the possibility of competition at binding sites. Davies et al. (1990) explained the lack of ethylene perception after the infiltration of tomato tissue with silver ions in terms of an inhibition of some ripening-related mRNAs.

There are two groups of ethylene antagonists (Abeles et al., 1992): potentially toxic compounds or treatments which may act as metabolic inhibitors (e.g., carbon dioxide at high concentration, ethanol, gamma irradiation), and inhibitors considered to be competitive (e.g., silver ions, norbornadiene and other cyclic olefins). However, the action of most antagonists is complex, as in the case of carbon dioxide (Sisler and Wood, 1988). At low concentrations, carbon dioxide activates the biosynthesis of ethylene via the latter's role as co-factor for 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (Smith and John, 1993). At high concentrations, carbon dioxide may stimulate the activity of ACC oxidase while inhibiting its synthesis (Cheverry et al., 1988). The presumed competitive inhibitors are also toxic and have contrasting effects. Thus while norbornadiene displaces ethylene from binding proteins, silver ions may fail to compete (Abeles et al., 1992) but could prevent ethylene from being recognised.

Characterisation of another gaseous ethylene antagonist, nitrous oxide (N_2O), may offer a new line of research, and could also be of value in extending fruit life, as an addition to present use of carbon dioxide in postharvest technology (Rothan et al., 1990). Nitrous oxide, an anaesthetic, clearly differs from nitrous monoxide and peroxide since it is chemically neutral and non-toxic (Fath et al., 1990). Both nitrous oxide and carbon dioxide have a linear structure (isostery), which confers on them similar physical properties, such as high solubility.

Little is known of the effects of nitrous oxide on plants. Nitrous oxide is known to bind to lipids and also to proteins, such as cytochrome *c* oxidase. Sowa and Towill (1991) report partial and reversible inhibition of respiration and cytochrome *c* activity in mitochondrial particles isolated from seed, leaf or cellular suspensions. Nitrous oxide also causes changes in chromosome number during cell division, but only when applied at very high pressure, and extends storage survival of some seeds without any toxicity (Sowa and Towill, 1991).

Our initial investigation of the use of nitrous oxide with fruit was prompted by data of Air Liquide (France) on the fungistatic effects of the gas. After establishing the non-toxicity of nitrous oxide in continuous or short-term treatments of tomato fruit (gas concentration, temperature), the study was extended to some characteristic features of ripening patterns. We have shown that nitrous oxide solubility in fruit cells (about 77%) approaches the value for carbon dioxide, and that gas absorption in tissues is entirely reversible (Fath et al., 1990).

The present work is focused on ethylene production in two typical climacteric fruit, tomato and avocado, which are models for metabolic and genetic studies of ethylene. These fruits were selected as they differ greatly in their precursor

ACC levels and in the two related enzyme activities (Hoffman and Yang, 1980). This initial study essentially concerned the effects of nitrous oxide on ethylene production, but published data on the effects of carbon dioxide on ACC synthase (Zamponi et al., 1990) or ACC oxidase (Cheverry et al., 1988) in such fruit species will be used in the discussion of our results.

2. Materials and methods

Plant materials

Tomatoes (*Lycopersicon esculentum* Mill. cv. Capello) grown in rockwool in the greenhouse were obtained at the mature green or turning stages, from the Casay Company in Yonne, France. Preclimacteric avocados (*Persea americana* Mill. cv. Hass) were harvested in Malaga, Spain, and quickly transported. Other avocados were from South Africa and were subject to transport conditions for longer periods.

Gas treatments

Fruits were selected, weighed, stored overnight at experimental temperatures and then placed in a 500-ml or 1-l jar. They were treated under standard conditions with a continuous water-saturated flow of 80% nitrous oxide (N_2O) + 20% oxygen, or flow of air, in the dark. In one experiment, the duration of N_2O treatment was limited to 48 h.

In most experiments, 15°C was used for ripening to increase tissue solubility of N_2O. In some cases, 20°C was used to investigate the effects of N_2O when the senescence process was enhanced by a higher temperature.

Ethylene (20 $\mu l\ l^{-1}$ in air or N_2O mixture) was applied to tomatoes for 48 h.

Nitrous oxide, oxygen and ethylene were purchased from Air Liquide, France.

Ethylene measurement

Ethylene production was followed daily using a Delsi 300 gas chromatograph fitted with a Porapak Q column and FID detector, at 60°C, to separate ethylene and nitrous oxide. Ethylene was measured in 1-ml gas samples and quantified using a 49 $\mu l\ l^{-1}$ standard. Several experiments were replicated and typical results are presented. For each experiment, at least four fruits were used to determine ethylene production levels in whole fruit.

Determination of ACC and ACC synthase activity

ACC content and ACC synthase activity in avocados were analysed. Mesocarp tissue was extracted as described by Bufler (1984) and Su et al. (1984). Frozen tissue was homogenised in a solution (w/w, 1/1.025) containing 100 mM EPPS (pH 7), 3 mM dithiothreitol (DTT), 10 μM pyridoxal phosphate (PLP) and 10% (w/v) PVPP. The homogenate was centrifuged at 15,000 g for 20 min at 4°C, and the supernatant was filtered. An aliquot was used for ACC determination by the method of Lizada and Yang (1979).

The enzyme was purified by passage of supernatant (2.5 ml) through a Sephadex G-25 column previously equilibrated with 2 mM EPPS (pH 8.5) containing 10 μM

PLP. The protein fraction was collected and assayed for ACC synthase activity according to Su et al. (1984).

ACC oxidase assay in vivo

Discs of mesocarp (1.5 g) were incubated in a 15-ml vial with 3 ml of reaction mixture containing 0.7 M sorbitol/50 mM MES (pH 6.1), 0.4 mM CHI and 2 mM ACC. Vials were sealed and gently shaken for 2 h at 20°C. Headspace ethylene was measured by gas chromatography (Rothan et al., 1990).

3. Results

Delay of climacteric ethylene rise

Under standard conditions, the ripening in air of preclimacteric tomatoes comprised a lag phase of 1–11 days (Fig. 1). Exposure to 80% nitrous oxide extended this lag phase and delayed the climacteric peak by about eight days. The peak level could be significantly decreased to about 40% of the control value, as observed for two fruits which exhibited a complete ethylene rise during the experiment. N_2O treatment also delayed the appearance of the red stage, in accordance with the close relation between colour change and ethylene production (data not shown).

Preclimacteric avocados in air (Fig. 2) showed less between-fruit variation in lag phase, and a high typical ethylene rise. As in tomatoes, nitrous oxide extended
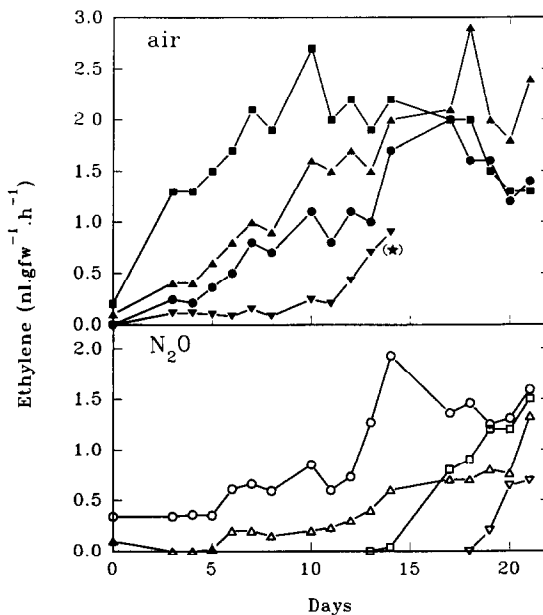

Fig. 1. Effect of nitrous oxide treatment at 15°C on ethylene production (nl gfw^{-1} h^{-1}) of four individual preclimacteric tomatoes compared with air-treated controls at the same temperature. (∗) Fruit discarded because diseased.
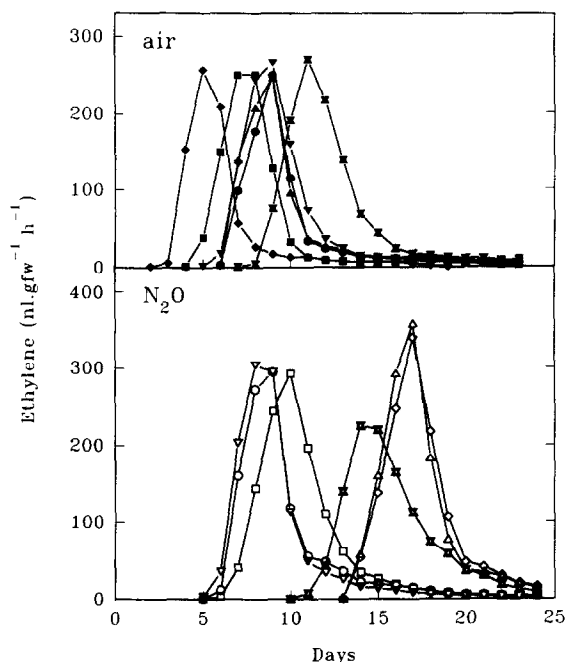
Fig. 2. Effect of nitrous oxide treatment at 15°C on ethylene production (nl gfw^{-1} h^{-1}) of six individual preclimacteric avocados compared with air-treated controls at the same temperature.

the lag period by an average of four days, but did not reduce the intensity of the ethylene rise.

Inhibition of autocatalytic ethylene production

In pink tomatoes, ethylene production in air was 2 nl g^{-1} h^{-1} and decreased progressively to 1.15 nl g^{-1} h^{-1} at the dark red stage (Fig. 3). Continuous N_2O treatment applied at this climacteric phase decreased ethylene production by about 60% with respect to the initial rate in air.

During 48-h N_2O treatment, ethylene production was measured precisely, and decreased rapidly over 8–10 h. After transfer to air, no residual effect was noticed and ethylene returned to control levels.

At this pink stage, either a 48-h pulse or continuous N_2O treatments did not retard fruit colour development (data not shown).

Inhibition of exogenous C_2H_4-induced changes in ethylene rise

Pretreatment of preclimacteric tomatoes with 20 μl l^{-1} C_2H_4 in air accelerated and synchronised the onset of the peak subsequently exhibited in air (Fig. 4). Almost all fruits also showed an increase in ethylene production (cf. Fig. 1), with a bimodal profile during ripening and senescence.

When pretreated with C_2H_4 in N_2O and stored in air, the fruits exhibited a typical
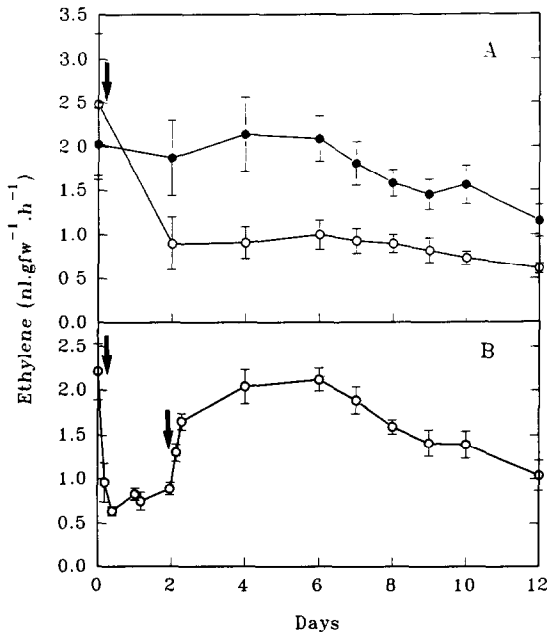
Fig. 3. Effect of continuous (A) or 48-h (B) nitrous oxide treatments (○) at 15°C on ethylene production (nl gfw^{-1} h^{-1}) of climacteric tomatoes compared with air-treated controls (●). Arrows indicate the start and end of N_2O flushing. Results are means for five fruits ± SD.

profile, with a significant lag phase, and in most cases low ethylene production.

When tomatoes were stored in N_2O after pretreatment with C_2H_4, in air or N_2O, ethylene production was drastically lowered. Stimulation of ripening by exogenous C_2H_4 was overcome by storage under N_2O and fruits exhibited low levels of ethylene, like fruits not pretreated with C_2H_4 (Fig. 1).

Inhibition of the senescence ethylene rise

Tomatoes ripened more quickly in air at 20°C (Fig. 5) than at 15°C (cf. Fig. 1), but the climacteric peak rate was quite similar. In some fruits, we observed a second rise at the dark red stage which was often more intense (occasionally very much so) than the initial climacteric rise.

Storage under N_2O, even at 20°C, slowed ripening and suppressed, or greatly deferred, the senescence ethylene rise of the red tomatoes.

Early postclimacteric avocados (darkening of pericarp occurred at day 2) also exhibited a second ethylene peak after 6–8 days at 20°C in air (Fig. 6). Treatment with N_2O delayed (by about five days) and approximately halved ethylene production in overripe fruits. After 12 and 15 days, the organoleptic qualities of avocados under N_2O were superior to the controls (data not shown).

Even at 15°C, such overripe avocados in air could exhibit a second ethylene rise after ten days (Table 1), while those under N_2O produced a lower and constant
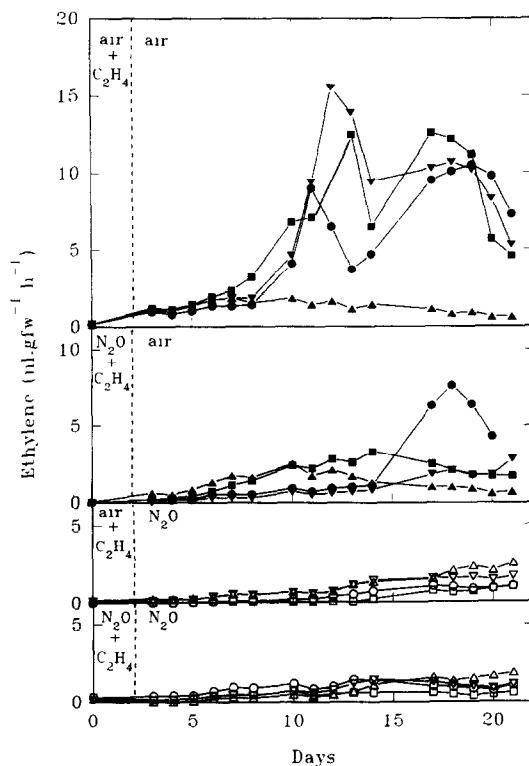
Fig. 4. Effect of 48-h C_2H_4 pretreatment (20 $\mu l \, l^{-1}$) in air or in nitrous oxide on ethylene production (nl $gfw^{-1} \, h^{-1}$) of preclimacteric tomatoes transferred to air (filled symbols) or post-treated with nitrous oxide (open symbols) at 15°C. For each treatment, four individual fruits were selected.
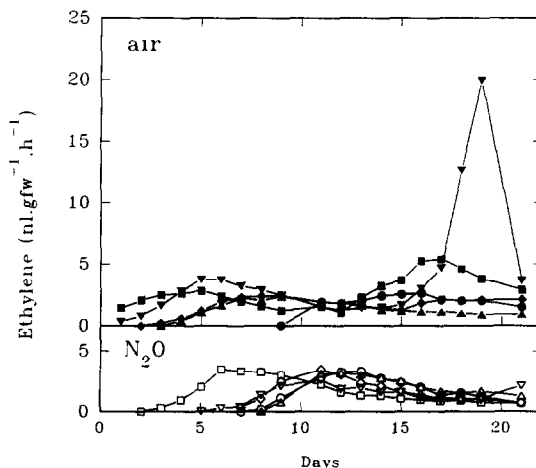

Fig. 5. Effect of nitrous oxide treatment at 20°C on ethylene production (nl $gfw^{-1} \, h^{-1}$) of five individual tomatoes at the climacteric and postclimacteric stages, compared with air-treated controls at the same temperature.
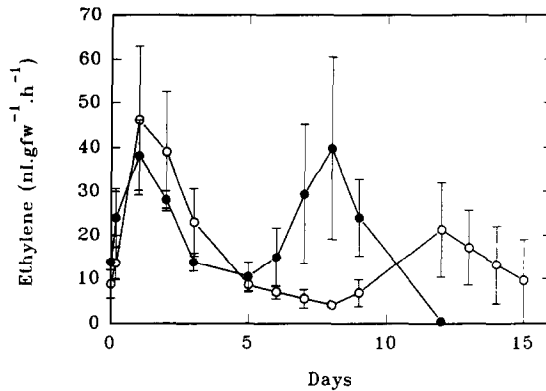
Fig. 6. Effect of nitrous oxide treatment (○) at 20°C on ethylene production (nl gfw^{-1} h^{-1}) of early postclimacteric avocados compared with air-treated controls (●). Results are means for four fruits ± SD.

Table 1
Effect of nitrous oxide treatment at 15°C on the ethylene production, ACC content and ACC oxidase and ACC synthase activities of early postclimacteric avocados

Time (days)	C$_2$H$_4$ (nl gfw^{-1} h^{-1})		ACC oxidase (nl gfw^{-1} h^{-1})		ACC (nmol gfw^{-1})		ACC synthase (nmol gfw^{-1} h^{-1})	
	Air	N$_2$O	Air	N$_2$O	Air	N$_2$O	Air	N$_2$O
1	9.1	15.4	1.6	2.2	0.9	3.0	1.1	2.6
3	10.2	17.3	4.0	4.9	0.9	1.3	0.6	1.8
8	6.7	9.0	7.7	6.7	0.7	0.9	0.8	1.4
10	21.3	9.2	13.0	8.0	3.5	1.3	1.4	0.6
13	41.6	9.0	14.4	13.4	13.0	1.4	7.2	1.0
15	12.0	10.3	9.9	4.8	28.0	3.3	7.7	2.3

Values are means for two fruits.

amount of ethylene. Similar changes in ACC oxidase activities were observed for the two treatments, with maxima at day 10 in air and day 13 in N$_2$O. More significantly, ACC contents and ACC synthase activities of fruits in air remained low for ten days and then increased with the senescence ethylene rise. Avocados under N$_2$O did not exhibit this phenomenon.

4. Discussion

Our results show that nitrous oxide may strongly modify ethylene production by tomatoes and avocados at various physiological stages, and allow analysis of the antagonist action of this gas.

Nitrous oxide extended the lag phase which precedes the ethylene rise and delayed colour change in preclimacteric fruits of tomatoes and avocados (Figs. 1

and 2). The length of this lag phase varied considerably among individual tomatoes, but the difference in trends between treated and control fruit series was highly significant. In mature-green tissues, ACC synthase is known as the rate-limiting enzyme, whereas the ACC oxidase system already exists, even if its activity is low (Sitrit et al., 1986). The mode of action of nitrous oxide may be similar to that of carbon dioxide during the ripening initiation period (Bufler, 1984; Zamponi et al., 1990). In particular, it may inhibit ACC synthase synthesis and activity, delaying ACC accumulation and possible conversion into ethylene, as also observed in our experiment on postclimacteric avocados (Table 1).

Nitrous oxide also lowered ethylene production rate in tomatoes treated at the preclimacteric or climacteric stages (Figs. 1 and 3). In climacteric material, the marked effect of nitrous oxide on the autocatalytic process occurred rapidly and, by analogy with carbon dioxide, could preferentially involve inhibition of ACC oxidase synthesis (Cheverry et al., 1988). With the short-term treatment (48 h), no residual effect was observed after removal of nitrous oxide. With carbon dioxide application, a residual effect dependent on temperature and the initial developmental stage of the fruit was observed (Marcellin and Chaves, 1983). Compared to other such experiments with carbon dioxide (Buescher, 1979), nitrous oxide did not influence the colour of climacteric treated tomatoes, suggesting that it became less effective when pigment synthesis was initiated, or that colour change was not always strictly correlated with ethylene action.

In contrast to the results for tomatoes, nitrous oxide did not reduce ethylene production rate in treated preclimacteric avocados (Fig. 2). This may be due to marked between-species differences in ethylene production and biosynthesis. Nitrous oxide could have a threshold effect, which is probably limited by higher ACC levels in avocado tissues (Hoffman and Yang, 1980) and higher ACC synthase and ACC oxidase activities (Sitrit et al., 1986).

Brief exposure to exogenous ethylene results in accelerated and more uniform ripening of various climacteric fruit species (Abeles et al., 1992). This process is strongly correlated with stimulation of ACC oxidase (Liu et al., 1985). Accumulation of the ACC oxidase mRNA is detected within a few hours of fruit treatment (Pech et al., 1993). In general, no increase in ACC synthase activity or ACC content was observed, and therefore a constant ethylene production rate is found for tomato or avocado (Liu et al., 1985; Starrett and Laties, 1991). In our experiments (Fig. 4), mature-green tomatoes ripening normally in air after the ethylene pulse exhibited earlier and highly stimulated ethylene production. This unusual response could be due to application of exogenous ethylene to fruits with non-limiting ACC contents. This stimulation was only promoted after some delay and may indicate that the initial ethylene pulse-related activation of ACC oxidase is still occurring.

When exogenous ethylene was applied to tomatoes under nitrous oxide, before ripening in air, stimulation of ethylene production was eliminated by nitrous oxide, as expected (Fig. 4). This inhibition may affect ACC oxidase synthesis, as with carbon dioxide (Cheverry et al., 1988). As no residual action of nitrous oxide was seen under our experimental conditions, ripening tomatoes after treatment showed an ethylene trend similar to that of untreated fruit (Fig. 1). When exposure

to exogenous ethylene in air or nitrous oxide was followed by application of nitrous oxide, ethylene production during ripening stabilised at a low level. This is consistent with the hypothesis of a strong antagonist action of nitrous oxide both on ACC oxidase and ACC synthase synthesis.

A second ethylene rise was seen in some tomatoes ripening and senescing in air up to the postclimacteric stage under standard conditions, or after an ethylene pulse (Figs. 4 and 5). The increase varied among individual fruits. In avocados, it appeared more regularly, especially at elevated temperature, which accelerated senescence (Fig. 6). Ethylene production was also higher than in tomatoes, as during the climacteric rise. This second ethylene peak could be interpreted as a signal accompanying the ultimate developmental stage, identical to the ethylene burst previously observed in flowers (Trippi and Paulin, 1984).

Hoffman and Yang (1980) were the first to describe a renewed rise in ACC levels in overripe avocado, tomato and banana, but did not observe a corresponding increase in ethylene emission. This lack of production led them to postulate suppression of ACC oxidase activity linked to membrane deterioration in senescing tissues. More recent work revealed that most of the enzyme is not membrane-bound but rather located in the apoplasm (Pech et al., 1993), which may explain our results. Some stabilisation of membrane integrity is still necessary for ACC oxidase activity, since few postclimacteric fruit exhibited an ethylene rise, depending on heterogeneous resistance to senescence. Our results (Table 1) indicate that senescence ethylene production is normally correlated with increases in ACC and in ACC synthase and ACC oxidase activities. The peaking of ethylene production and ACC oxidase activity may distinguish the phenomenon from pathogen-induced ethylene emission (Pech et al., 1993), which involves sustained and substantial ethylene production (without a sharp maximum), as we occasionally noted in some infected fruits.

Nitrous oxide treatment also inhibited the senescence ethylene rise. Tomatoes at 15°C (after ethylene pulse) or at 20°C, and avocados at 15°C, did not exhibit any senescence ethylene upsurge (Figs. 4 and 5, Table 1). In avocados at 20°C, nitrous oxide was less efficient, but the second increases were delayed and markedly reduced (Fig. 6).

As observed with the initiation, ripening and senescence periods of fruit development, our overall results demonstrate that nitrous oxide is a potent antagonist of ethylene production and action. However, fruit response to treatment may sometimes be heterogeneous, depending on physiological factors, as seen with carbon dioxide. Although the mode of action of carbon dioxide is partly elucidated, analysis of the carbon dioxide–ethylene interaction remains complex (Sisler and Wood, 1988), and treatment efficiency varies with species, physiological stage and experimental conditions, such as temperature (Marcellin and Chaves, 1983). Unlike carbon dioxide, nitrous oxide is not toxic and its use in further experiments should enable the exact conditions for its antagonist action on ethylene to be established. Further detailed experiments on the effects of nitrous oxide on ethylene biosynthesis, and notably on threshold enzymatic activities, should clarify its mode of action.

Acknowledgements

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