Factors Influencing Ethylene-induced Isocoumarin Formation and Increased Respiration in Carrots

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Abstract. Ethylene-induced formation of isocoumarin was characterized in relation to ethylene-enhanced respiration in whole or cut carrots (*Daucus carota* L.). Ethylene concentrations (0.1 to 5 ppm) and temperatures (1 to 15C) that increased respiration also favored a more rapid formation of isocoumarin (8-hydroxy-3-methyl-6-methoxy-3,4-dihydro-isocoumarin). Exposing mature carrots to 0.5 ppm C_2H_4 for 14 days at 1 or 5C resulted in isocoumarin contents of 20 and 40 mg/100 g peel, respectively. These levels were easily detected as a bitter flavor in the intact carrot roots. Immature carrots formed higher levels of isocoumarin than mature carrots; 180 mg/100 g peel were detected in young carrots stored 14 days at 5C in air containing 0.5 ppm C_2H_4. Freshly harvested carrots exposed to 5 ppm C_2H_4 accumulated 4-fold higher isocoumarin levels than those formed by carrots stored 30 days at 5C before exposure to C_2H_4. An atmosphere of 100% O_2 potentiated the effect of C_2H_4 on isocoumarin formation, resulting in a 5-fold increase over that found in carrots treated with C_2H_4 in air. A storage atmosphere of 0.5 ppm C_2H_4 in 1% O_2 resulted in isocoumarin levels about one-half those attained in 0.5 ppm C_2H_4 in air. Sliced, cut, or dropped carrots exposed to C_2H_4 showed greater isocoumarin accumulation rates than intact uninjured carrots. Peeled baby carrots, however, had little capacity to form isocoumarin. In general, the more rapid the respiratory rise in response to C_2H_4, the more rapidly isocoumarin accumulated. The greater the respiratory response to ethylene, the higher the level of isocoumarin formed.

The formation of isocoumarin (8-hydroxy-3-methyl-6-methoxy-3,4-dihydro-isocoumarin) in carrot roots is induced by ethylene (Sarkar and Phan, 1979). This compound, associated with bitterness in carrots (Carlton et al., 1961; Simon, 1985), was first isolated by Sondheimer (1957) and later identified as a phytoalexin by Condon and Kuc (1960). Similar to other phytoalexins induced in nonphotosynthetic plant storage tissues under stress conditions, isocoumarin is synthesized from acetyl CoA (Kurosaki and Nishi, 1988; Uritani and Asahi, 1980). The proposed sequence for isocoumarin synthesis is the condensation of 1 acetyl CoA and 4 malonyl CoA molecules, with subsequent reduction and methylation (Kurosaki and Nishi, 1988). The mechanism by which ethylene brings about the formation of isocoumarin in carrots is not completely elucidated. Sarkar and Phan (1979) proposed that *de novo* synthesis of an enzyme is necessary since cycloheximide completely inhibited its formation if added before, but not after, ethylene treatment. Kurosaki and Nishi (1988) partially characterized an ethylene-inducible methyltransferase involved in the final step of isocoumarin synthesis.

Sarkar and Phan (1979) also proposed that ethylene stimulated glycolysis, providing excess acetyl CoA, which could be shunted to isocoumarin synthesis. Stitt et al. (1986) demonstrated that ethylene leads to an increase in glycolytic intermediates and activity of fructose-6-phosphate 2-kinase in carrots. Oxygen is

necessary for ethylene action in plant tissues (Burg and Burg, 1967), and Carlton et al. (1961) confirmed its requirement for ethylene-induced isocoumarin formation. Theologis and Laties (1982) demonstrated that oxygen potentiates the respiratory rise induced by ethylene in several plant tissues. Nichols and Laties (1985) showed that this respiratory rise is not obligatorily linked to ethylene induction of gene expression in carrots. Nevertheless, ethylene stimulates a respiratory rise and induces isocoumarin formation in carrots.

Sarkar and Phan (1979) reported that, under ethylene, carrot peel respired at higher rates than internal tissues. Although they did not analyze the distribution of isocoumarin in carrots, Atkin (1956) had previously shown that most of it accumulated in the high-respiring epidermal tissues. The concentrated distribution of isocoumarin in the peel of carrot roots is similar to the distribution of total phenols (Yan, 1989). In addition, there is a longitudinal gradient, with higher levels of phenols present at the stem end. Within the pulp of carrots, the level of phenols is low and is similar in the cortex and vascular cylinder (Yan, 1989).

In this study, the time courses of the respiratory rise and isocoumarin formation induced by different ethylene concentrations and postharvest storage conditions were compared. The relationship between these ethylene-induced phenomena were examined under conditions known to affect ethylene action including temperature, water stress, oxygen levels, and the physiological state of the tissue. The isocoumarin-forming potential of cut carrots was evaluated in relation to commercial handling practices and to intact carrots.

Materials and Methods

Mature 'Packer 83' carrot roots used for fresh market were harvested from commercial plantings in Cuyama, Calif., or were obtained from a local distributor ('Imperator-58'). Immature carrot roots ('Caropak') used for commercial preparation of baby carrots were obtained on the day of harvest from a carrot processor in Bakersfield, Calif. Carrots were held at 0 to1C and washed with

water containing 0.01% to 0.02% sodium hypochlorite before treatment. Typically, roots were held in glass jars ventilated with a continuous flow of humidified (about 95% relative humidity) air or humidified air with different ethylene concentrations prepared by dilution from a 5000 ppm stock. Ethylene concentrations were monitored by a gas chromatograph with a flame ionization detector, and held within 10% of the stated concentration. Ethylene levels in air controls were below the limit of detection (0.005 ppm). Three replicates per treatment were typically used, and temperatures were held at ±0.5C of the specified temperature.

After excising 1 cm from the stem and basal ends, sliced carrots were prepared by cutting intact roots in half and then slicing the sections longitudinally into quarters. The 5-cm segments were prepared from the same lot of freshly harvested carrots abrasively peeled and polished commercially for baby carrot samples. Water-stressed carrots were prepared by holding intact carrots at 5C for 48 h to induce a total weight loss of 8%. To simulate physical handling stress, carrot segments were dropped vertically on each cut end through a column 60 cm high.

The concentration of CO_2 was determined by injecting 1-ml samples from the storage container headspace into an infrared gas analyzer (model PIR-200; Horiba). Respiration rates were calculated from the difference in CO_2 concentrations between the inlet (<0.04%) and outlet air streams using a 0.5% CO_2 standard for calibration. Flow rates were selected to maintain CO_2 levels in the container between 0.25% and 0.5%.

Isocoumarin content was determined on a 3-g portion of carrot peel or 6-g portion of carrot pulp (cortex plus vascular tissues) extracted overnight in a 25-ml screw-cap glass vial with 15 ml of spectrophotometric grade hexane at ambient temperature. The solution was decanted and isocoumarin reextracted with an equal volume of 80% ethanol. Isocoumarin content was calculated from the absorbance of the ethanol layer at 267 nm using a molar absorptivity of 14800 (Sondheimer, 1957) and a partition recovery factor of 84% ± 2%.

The characteristic ultraviolet absorption of isocoumarin shows a peak at 267 and 302 nm, with a resulting peak height ratio of 2.47 (Sondheimer, 1957). When a noncharacteristic isocoumarin spectrum was observed, or the ratio of the 267- and 302-nm peaks was different from that expected for isocoumarin (2.47) due to the presence of interfering compounds, a further purification was carried out by thin layer chromatography (TLC). Aliquots of the hexane extracts were spotted on fluorescent 250-μm silica gel plates and developed with 60 toluene : 35 methylacetate : 7 formic acid (by volume). The fluorescent isocoumarin spot was removed and suspended in 95% ethanol for the spectrophotometric determination.

Although the partition procedure eliminated interfering compounds, a residual absorption noncharacteristic of isocoumarin, as revealed after purification by TLC, was detected in intact carrots stored in air. However, this never exceeded the equivalent of 4 to 10 mg of isocoumarin/100 g peel and is reported as air controls. The partition method was used for routine analysis, but frequently was corroborated by TLC purification. Direct light was avoided during extraction and analysis, and extracts were held at –20C if not analyzed immediately. Carrot peel was routinely analyzed since about 70% to 80% of the isocoumarin formed in roots was found in the peel. A minimum of one carrot from each of three replicates per treatment per sample period was analyzed.

Harshness and bitterness are two common off-flavors reported in carrots (Simon, 1985). Harshness is a strong, burning, turpentine-like flavor due to terpenoids in carrots at harvest; bitterness is a lingering disagreeable flavor detected on the back of the tongue and is not detected in freshly harvested carrots. The occurrence of isocoumarin generally coincides with bitter flavor, although other phenolic compounds may also contribute to bitter flavor (Simon, 1985). Bitterness was detected by a four-member panel using a hedonic scale of 1 to 5, where 1 = no bitterness detected; 2 = slight bitterness, distinguishable from harsh flavor; 3 = moderate bitterness, carrots noticeably bitter; 4 = carrots were very bitter; and 5 = carrots were extremely bitter, unpalatable sample. The correlation coefficient (based on 58 observations) between isocoumarin content and the hedonic scale was 0.78. A bitter flavor (score of 2) was detected in carrots with about 20 mg isocoumarin/100 g peel. Isocoumarin levels of about 50 to 150 mg/100 g peel typically resulted in scores of 2 to 3; scores of >3 were associated with variable but high isocoumarin levels.

Results

Effect of temperature and ethylene concentration on respiration rates and isocoumarin formation. To estimate the threshold conditions needed for ethylene-induced respiratory response and isocoumarin formation, the effects of low ethylene concentrations at low temperatures were studied (Fig. 1). Carrots exposed to 0.1 ppm ethylene at 1 or 5C showed negligible to small increases in respiration rates as well as in isocoumarin levels over 30 days, and bitterness was barely detectable in only a few carrots. Treating carrots with 0.5 ppm ethylene induced lower isocoumarin levels at 1 than at 5C. Respiration rates were low,
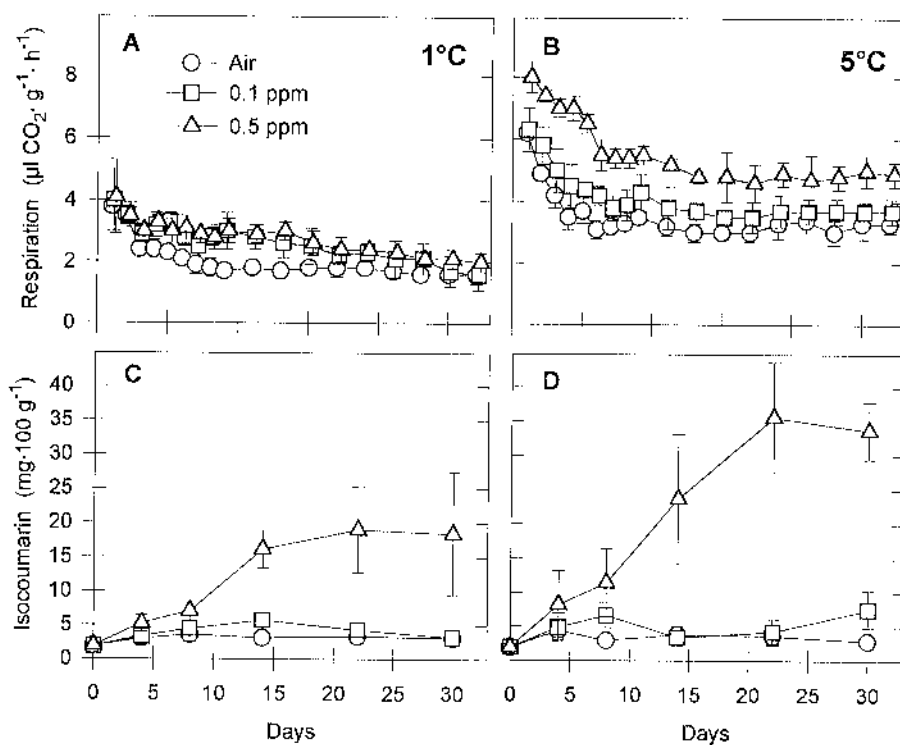

Fig. 1. Respiration rates (**A** and **B**) of intact carrots and isocoumarin levels (**C** and **D**) in the peel of mature carrots during storage at 1 or 5C in air or 0.1 or 0.5 ppm ethylene.
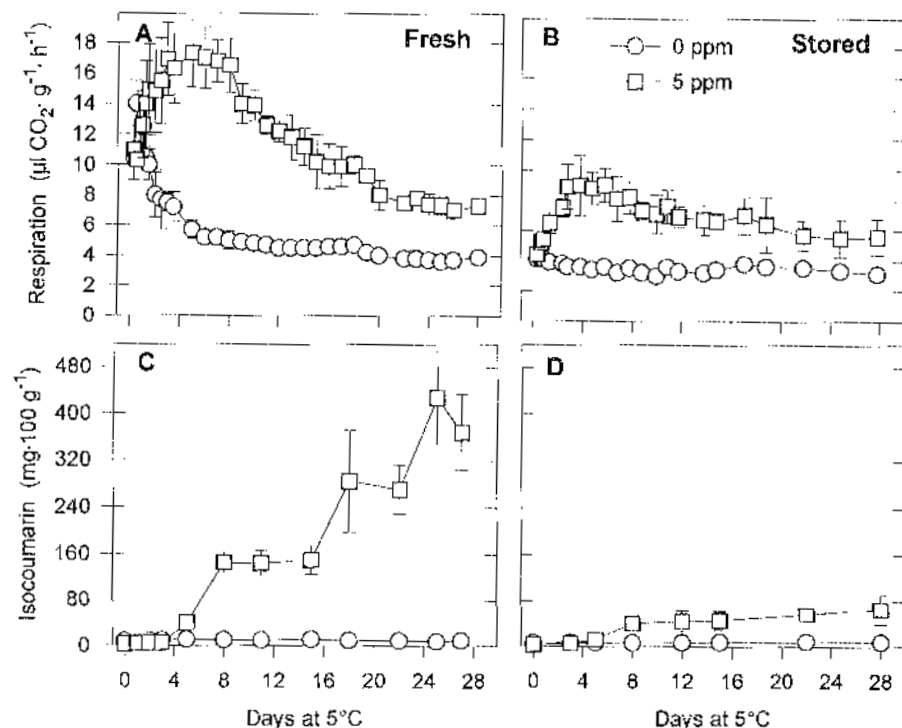
Fig. 2. Respiration rates and isocoumarin levels of fresh (**A** and **C**) and stored (**B** and **D**) mature carrots during storage in air or 5 ppm ethylene at 5C. Stored carrots were those that had been previously stored for 30 days at 5C in air.

Effect of oxygen levels on respiration rates and isocoumarin formation. To investigate the effect of oxygen on isocoumarin formation, whole carrots were held at 15C for 9 days in air or 100% O_2 in the absence or presence of 5 ppm ethylene. Carrots held in pure O_2 (Fig. 3b) showed a more variable and slightly higher respiratory rate than carrots held in air (Fig. 3a). For carrots exposed to ethylene in 21% O_2, maximum respiration rates occurred after 1 day, whereas respiration of carrots treated with ethylene in 100% O_2 continued increasing up to 3 days, and resulted in maximal rates twice those produced by carrots treated with ethylene in air.

Isocoumarin levels in ethylene-treated carrots held under air and O_2 were similar after 2 days (Fig. 3 c and d). By day 3, however, a 2-fold difference was observed, and after 5 days the isocoumarin levels in carrots treated with ethylene in O_2 were 5-fold higher than those of carrots treated with ethylene in air. These results clearly demonstrate that isocoumarin formation was preceded by increased respiratory activity. It was also apparent that dramatic differences in accumulated isocoumarin levels occurred only after similarly large differences in respiration rates were observed.

Isocoumarin levels in peel of carrots exposed to 0.5 ppm ethylene in 1% O_2 were about one-half the levels detected in carrots

and although there was only a slight increase in respiration rates of carrots treated with 0.5 ppm ethylene at 1C, a substantial respiratory increase was observed at 5C.

Effect of storage period of carrots on respiration and isocoumarin formation. Freshly harvested carrots and carrots that had been stored for 30 days at 5C under a continuous flow of humidified air were used to study the response of fresh and aged carrots to ethylene. Initial respiration of aged carrots treated with ethylene at 5C was lower than that of corresponding fresh carrots (Fig. 2 a and b). Isocoumarin levels found in aged carrots exposed to ethylene (Fig. 2d) were markedly lower than those in fresh ethylene-exposed carrots (Fig. 2c).
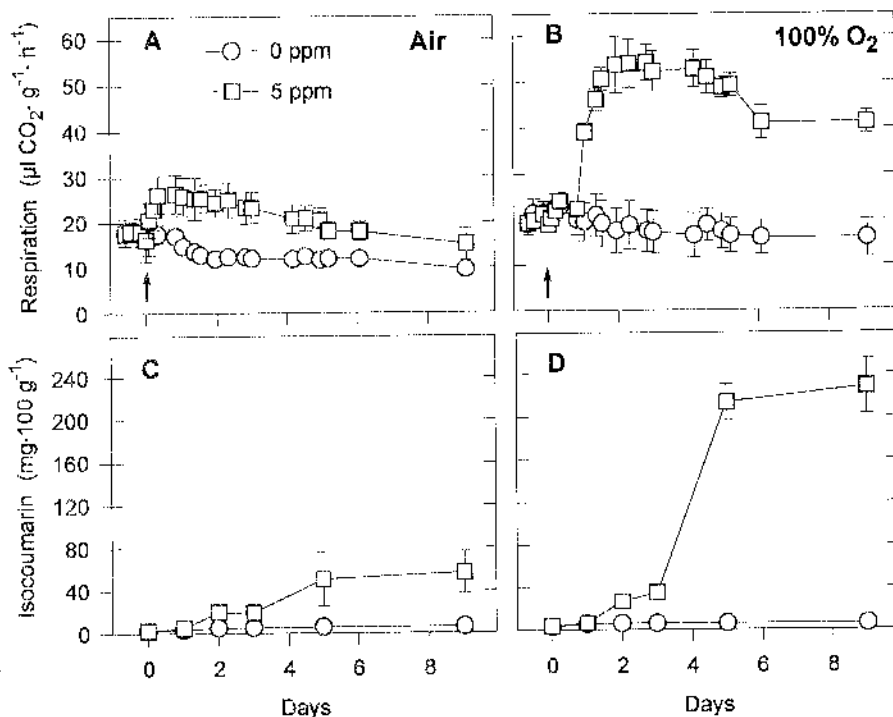
The diminished capacity of aged carrots to respond to ethylene treatment was further confirmed with carrots stored for 2 months in air or 5 ppm ethylene at 5C. Isocoumarin levels in ethylene-treated carrots stored 2 months were less than those produced by carrots stored 1 month (data not shown). Additionally, 100 ppm ethylene applied to carrots previously treated with 5 ppm ethylene failed to induce a significant increase in isocoumarin levels. Changes in respiration rates of these carrots were also negligible (data not shown), further demonstrating the decreased responsiveness of stored carrot tissue to ethylene action.

We were interested in determining if isocoumarin levels would decrease if carrots were removed from the ethylene atmosphere. To examine this, 'Packer 83' carrots were transferred to air after 10 days of treatment with 5 ppm ethylene at 5 or 15C. Although analyses every 3 days over 2 weeks showed highly variable levels, no significant differences in isocoumarin levels compared to those found in carrots continuously treated with ethylene were evident (data not shown).

Fig. 3. Respiration rates and isocoumarin levels of mature carrots during storage at 15C in air or 5 ppm ethylene in air (**A** and **C**) or in 100% O_2 (**B** and **D**). The arrow indicates the beginning of ethylene treatment.

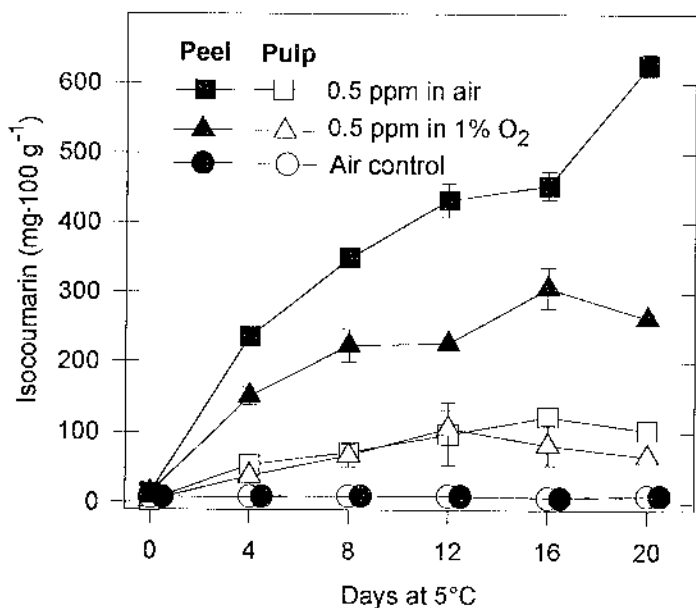J. Amer. Soc. Hort. Sci. 121(3):537–542. 1996.

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Fig. 4. Isocoumarin levels in the pulp and peel of immature carrots stored at 5C in air, 0.5 ppm ethylene in air, or 0.5 ppm ethylene in 1% O_2.

treated with the same ethylene concentration in air (Fig. 4). Much lower isocoumarin levels were formed in the pulp and did not differ between ethylene provided in 1% or 21% O_2. An interesting ancillary observation was that carrots (with peel) stored in 1% O_2 were consistently sweeter than those stored in air (based on observations during sensory analysis for bitterness).

Effect of physical stress on respiration and isocoumarin formation. The effect of ethylene treatment on whole unstressed, sliced and water-stressed carrots stored at 5C was compared. Under air, sliced carrots showed a substantial increase in respiration rate which subsequently declined but did not return to levels found in unstressed carrots (Fig. 5). Under 0.5 ppm ethylene, sliced carrots showed a rapid increase in respiration rate, reaching a maximum after 4 days. Although the respiration rate subsequently declined, it remained higher than rates of unstressed carrots exposed to ethylene throughout the 4-week storage period. Water-stressed carrots stored in air had slightly higher respiration rates than unstressed carrots; respiration rates of nonstressed and water-stressed carrots were similar when exposed to 0.5 ppm ethylene (data not shown).

The stress caused by slicing carrots was sufficient to induce detectable levels of isocoumarin up to 10 mg/100 g in carrots held in air (Fig. 5d), which were similar to those formed by intact carrots exposed to 0.1 ppm ethylene (Fig. 1). Stress caused by 8% water loss did not result in detectable increases of isocoumarin in carrots held in air. Sliced carrots exposed to 0.5 ppm ethylene accumulated isocoumarin levels three times those of intact carrots after 22 days (Fig. 5 c and d). Ethylene-induced isocoumarin formation in water-stressed carrots was not different from that induced in unstressed intact carrots (data not shown).

In Fig. 6, isocoumarin levels of whole carrots and 5-cm pieces are compared. Exposure to 0.25 ppm ethylene resulted in similar low levels of isocoumarin formation. However, exposure to 0.5 ppm resulted in higher rates of accumulation of isocoumarin in cut pieces than in the whole carrots. With both intact and cut pieces, exposure to 0.125 ppm ethylene resulted in isocoumarin levels approaching those of carrot pieces given the 0.25 ppm treatment (data not shown). After 16 days, whole carrots treated with 0.25 and 0.5 ppm were noticeably bitter (average score >3).
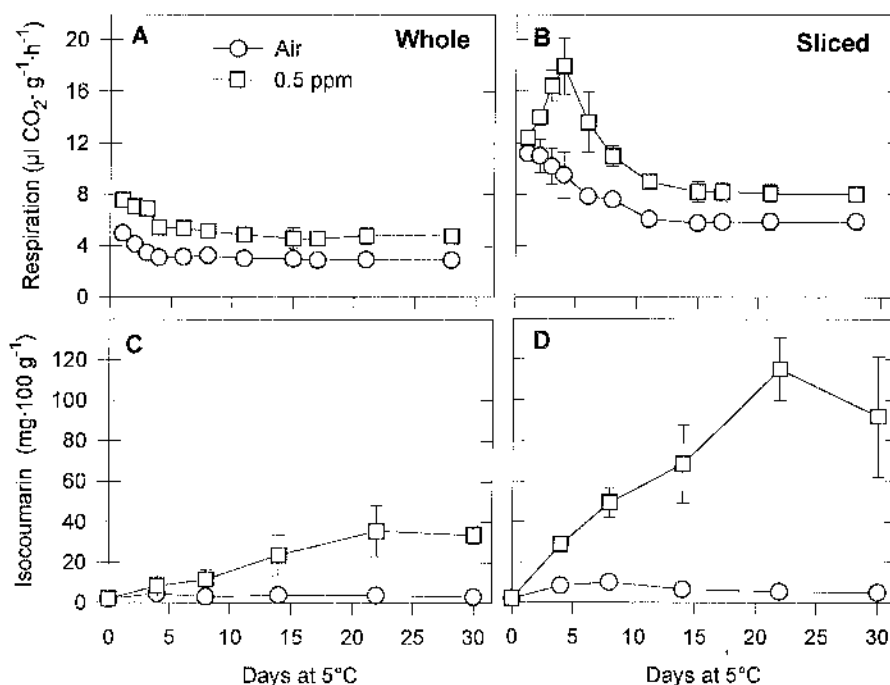
Most of the isocoumarin formed in the carrot root occurs in the peel, although substantial levels were induced in the pulp (Fig. 7). In cut carrot pieces, isocoumarin levels were about 20% of those found in the peel (Fig. 4). Higher levels of isocoumarin were found in the pulp of cut pieces than in the pulp of intact carrots or prepared baby carrots. No significant accumulation of isocoumarin occurred in baby carrots exposed to C_2H_4, and these consistently received a hedonic score of <2 for bitterness.

Dropping carrot pieces from a height of 60 cm on each end resulted in significant increases in the respiration rates during subsequent storage in air or 0.5 ppm ethylene (Fig. 8a). Respiration rates of dropped carrots stored in air were similar to those of control pieces stored in 0.5 ppm ethylene. Dropping increased the initial rate of isocoumarin formation in ethylene-exposed carrots (Fig. 8b). After 32 days, however, no differences in isocoumarin levels were observed between the dropped and control carrots.

Effect of maturity at harvest on respiration rates and isocoumarin formation. The respiration rates of immature carrots were 30% to 50% greater than those of mature carrots stored in air (data not shown). Both immature and mature roots showed about a 40% stimulation in respiration rate in response to 0.5 ppm C_2H_4.

Younger carrots also formed higher levels of isocoumarin than mature carrots. The maximum levels of isocoumarin formed in response to 0.5 ppm C_2H_4 at 5C were 40 and 400 mg/100 g peel for mature and immature carrots, respectively (Figs. 1d and 6). Higher

Fig. 5. Respiration rates (**A** and **B**) and isocoumarin levels (**C** and **D**) in the peel of unstressed mature whole carrots (**A** and **C**) or carrot pieces (**B** and **D**) during storage at 5C in air or 0.5 ppm ethylene. Pieces were prepared from halved and then quartered roots.
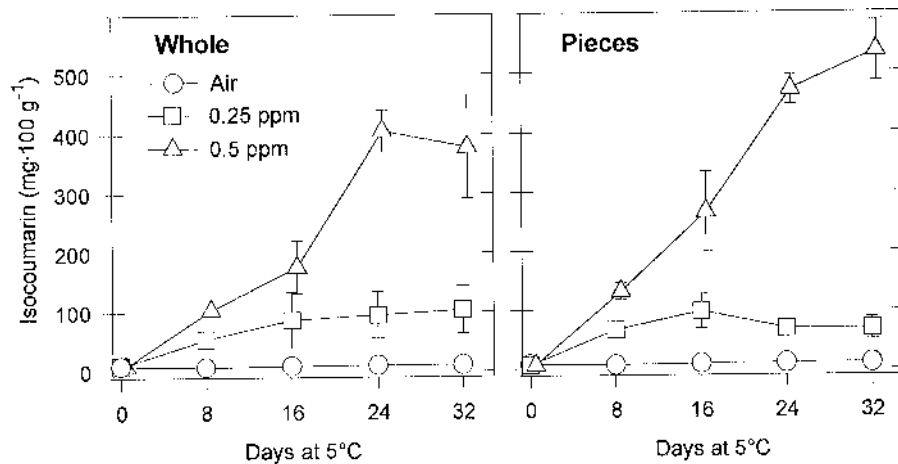
Fig. 6. Isocoumarin levels in the peel of immature whole carrots or 5-cm pieces during storage at 5C in air or 0.25 or 0.5 ppm ethylene.

levels of isocoumarin were consistently found in the peel of immature carrots (Figs. 4, 6, and 8) compared to the levels found in mature carrots (Figs. 1, 2, 3 and 5).

Discussion

Ethylene stimulates many processes in plant tissues, including respiration and phytoalexin formation (Solomos, 1988; Uritani and Asahi, 1980). Factors that would favor an increase in ethylene-enhanced respiratory activity in carrots could also favor an increase in the capacity to synthesize isocoumarin. In the present investigation, this hypothesis was tested using a range of postharvest conditions.

At the lowest ethylene concentration used (0.1 ppm), a marginal increase in respiration of ethylene-treated carrots held at 5C corresponded to a negligible increase in isocoumarin. At higher ethylene concentrations (0.5 ppm) respiration and isocoumarin formation rates were higher. Relatively high ethylene concentrations (50 ppm) and high temperatures (1C) further increased respiration and isocoumarin formation in carrots (Lafuente et al., 1989). These results are consistent with those previously reported by Sarkar and Phan (1979), who found that the initial rate of isocoumarin formation increased with increasing ethylene concentration and temperature. The more rapid initial accumulation was probably due to the influence of temperature on the rate of de novo enzyme synthesis (Sarkar and Phan, 1979). With time, however, levels of isocoumarin reached similar plateau concentrations over a range of storage temperatures at a given ethylene concentration (Lafuente et al., 1989; Sarkar and Phan, 1979).

Pure O_2 potentiated ethylene-enhanced respiration of carrots and resulted in a large increase in isocoumarin. In addition, it was clearly demonstrated that the synthesis of isocoumarin was preceded by a respiratory increase. The rate of isocoumarin formation under ethylene in air or O_2 was initially similar, but subsequently there was a dramatic increase in isocoumarin content of carrots treated with ethylene in O_2. This could indicate that the initial rate of synthesis of the isocoumarin-forming enzymes depends on ethylene concentration when O_2 is not limiting. After this induction period, levels of isocoumarin correlated with rises in respiration, conceivably because of increased availability of ATP and precursors necessary for isocoumarin synthesis.

When O_2 is limiting, respiration is reduced and a slower rate of isocoumarin formation could be expected. The isocoumarin content of carrot pieces exposed to 0.5 ppm ethylene in 1% O_2 was about one-half that of carrots treated with ethylene in air; respiration of carrots in 1% O_2 were reduced 30% to 35%. A nitrogen

pretreatment was found to prevent the formation of isocoumarin in carrots subsequently stored in ethylene (Carlton et al., 1961).

Changes in the physiological state of carrots could be expected to affect their capacity to respond to ethylene. Immature carrots in air respire at rates 30% to 40% higher than mature carrots. In response to ethylene, a similar percentage stimulation of respiration occurred in immature and mature carrots. However, the isocoumarin forming capacity of the more metabolically active immature carrots was dramatically greater than that of mature carrots. Freshly harvested carrots produced substantially higher amounts of isocoumarin than carrots stored 30 days before exposure to ethylene. An increased storage period was also found to decrease the formation of isocoumarin in *Botrytis cinerea*-inoculated carrots (Goodliffe and Heale, 1978). Ethylene enhanced respiration of freshly harvested carrots was double that of stored carrots.

We have shown that low O_2, developmental stage at harvest, and postharvest storage can reduce the responsiveness of carrots when exposed to ethylene. Other techniques, such as temperature pretreatments (MacDonald and Dekock, 1958), modify the metabolic activity of carrots, and nitrogen pretreatment has been shown to inhibit isocoumarin formation (Carlton et al., 1961). High CO_2 (30%) can compete with ethylene and retard the synthesis of all phenols in carrots (Yan, 1989) and, therefore, could affect isocoumarin synthesis. A food processing technique such as blanching, which reduces enzymatic activity, diminishes formation of isocoumarin and other phenolic compounds (Howard et al., 1994).

Our study has also shown that stress conditions that favored ethylene-enhanced respiration rate also favored ethylene-induced
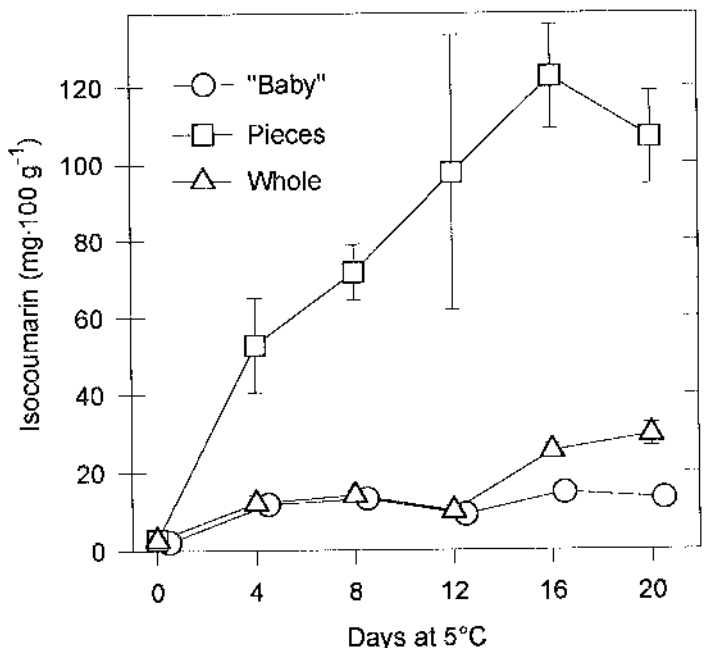

Fig. 7. Isocoumarin levels in the pulp of immature whole carrots, cut pieces, or baby carrots during storage at 5C in 0.5 ppm ethylene.
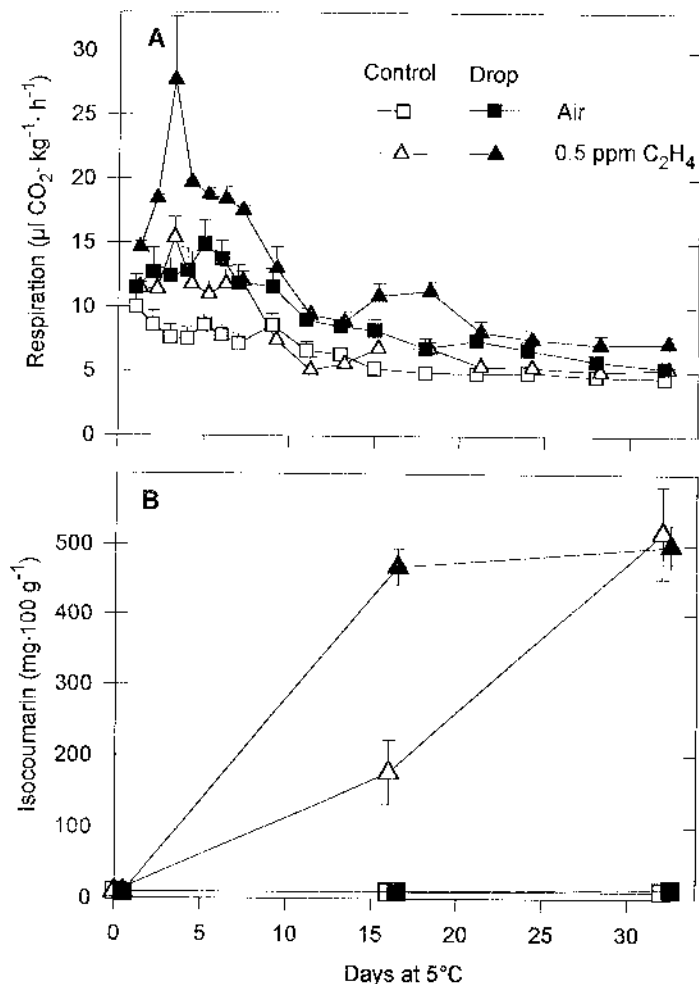
Fig. 8. Respiration rates and isocoumarin levels of 5-cm pieces stored at 5C in air or 0.5 ppm ethylene. Pieces were prepared from immature carrots; injured segments were dropped from a height of 60 cm on both ends.

isocoumarin formation. The respiratory activities of sliced carrots held under ethylene or air at 5C were higher than those of unstressed carrots. Although the effect of slicing was greater under ethylene, the high initial respiration of sliced carrots under air should be noted. This temporal increase in respiration could be associated with formation of low levels of isocoumarin in the absence of exogenous ethylene. Chalutz et al. (1969) reported that carrot disks in air at 20C produced ethylene at a rate that increased from 0.04 to 0.1 $\mu l \cdot g^{-1} \cdot h^{-1}$ and subsequently declined over 24 h. In the present study, sliced carrots at 5C did not produce ethylene at rates >0.01 $\mu l \cdot g^{-1} \cdot h^{-1}$. It is possible that the intracellular concentration of ethylene in sliced or stressed carrots was sufficiently high to induce some isocoumarin formation.

Ethylene was a critical factor for the induction of isocoumarin formation. Wounding and cutting can potentiate ethylene response, but generally wounding itself was insufficient to induce isocoumarin formation. The rate of isocoumarin formation in 5-cm carrot segments exposed to ethylene was about 60% greater than that in whole carrots over a 16-day period. Dropping cut pieces further potentiated ethylene-induced isocoumarin formation. The highest rate of isocoumarin formation observed in the present study occurred in 5-cm pieces that had been dropped. The rate of isocoumarin accumulation in cut and dropped pieces was almost double that of the cut pieces during the first 16 days of storage.

Although high temperatures and high ethylene concentrations caused a rapid formation of isocoumarin, low ethylene concentrations (0.1 to 0.25 ppm) at low temperatures (1 to 5C) eventually resulted in the accumulation of high levels of isocoumarin. It is also important to emphasize that the rate of accumulation of isocoumarin will be greater with younger carrots, freshly harvested carrots, and carrots subjected to physical injury (dropping or cutting). This would become important to the quality of carrots destined for minimal processing, i.e., carrot pieces stored for subsequent peeling and polishing such as baby carrots. These cut pieces are very responsive to ethylene and can accumulate high levels of isocoumarin in the peel and the pulp. However, once the pieces are prepared commercially as baby carrots, the tissues appear to have little capacity to form isocoumarin if they are exposed to ethylene. About 70% to 80% of the isocoumarin formed in carrots is found in the peel tissue. Since about 20 mg/100 g isocoumarin are required for sensory detection, it would be expected that carrot segments containing up to 100 mg/100 g isocoumarin in the peel could still be processed into baby carrots with acceptable flavor.

In conclusion, ethylene increased respiration and induced isocoumarin synthesis in carrots. Factors potentiating ethylene action stimulated both metabolic processes. Our results show that factors such as the physiological state, wounding, and oxygen level noticeably influenced the respiration rate and isocoumarin levels of carrots exposed to low levels of ethylene. The presence of ethylene should be especially avoided during the handling of freshly harvested carrots and cut pieces due to their high capacity to form isocoumarin.

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