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Calvin cycle activity in fruit and the effect of heat stress

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ABSTRACT

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As green fruit mature the permeability of the outer epidermis decreases. Consequently, gas exchange with the outside air becomes more restricted and it is unclear whether or not maturing fruit continue to fix carbon dioxide (CO_2) photosynthetically, possibly utilizing accumulated internal CO_2. To examine this, Calvin cycle activity in fruit was investigated by chlorophyll *a* fluorescence quenching in vivo, the fluorescence emission from the fruit surface being measured with a modulated fluorometer. Fruit of 15 species were examined and all showed evidence of Calvin cycle activity as indicated by relaxation of chlorophyll fluorescence quenching. Allowing for the differences in chlorophyll content, the activity in fruit was comparable with that in leaves. When exposed to CO_2-free air, tomato leaves and discs of avocado peel quickly lost activity, but loss of activity was slow in intact fruit, indicating that most of the CO_2 fixed photosynthetically in fruit was derived from CO_2 accumulated within the fruit. Calvin cycle activity in fruit was especially sensitive to heat, more so than either photosynthetic electron transfer activity or photophosphorylation. Optical monitoring of Calvin cycle activity by fluorescence quenching thus has the potential to detect early symptoms of heat stress in fruit, for instance, as the result of post-harvest heat treatments to disinfest fruit of insects.

Keywords: avocado; Calvin cycle; CO_2 fixation; chlorophyll fluorescence; fruit; heat stress; lemon; tomato.

Abbreviations: PAM = pulse amplitude modulated fluorometer; PFD = photon flux density.

INTRODUCTION

Photosynthetic CO_2 fixation in green fruit differs in several ways from that in leaves. Rates per unit area are lower, generally from 1 to 10% of those in leaves, because the chloroplasts are more sparsely distributed in the photosynthetic tissues of fruit. However, on a per unit chlorophyll basis, calculated photosynthetic rates in apple fruit and leaves were comparable (Šesták and Čatský, 1967). Especially in maturing fruit, gas exchange with the external

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air is severely restricted compared with leaves. Typically, the number of stomata in fruit are fixed at anthesis and as the surface of the developing fruit expands, stomatal frequencies decrease, for instance, in apple from more than 10 stomata mm^{-2} in the young developing fruit to less than one stoma mm^{-2} approaching maturity (Blanke and Lenz, 1985). This compares with a stomatal density of 320 to 390 stomata mm^{-2} on the abaxial surface of the apple leaf (Blanke and Lenz, 1989).

Along with the reduction in stomatal frequency, there is a decrease in the number of functional stomata. Guard cells are active in the immature fruit of apple (Lenz and Blanke, 1983), but in many fruit, the walls subsequently thicken and become covered with wax. Substomatal cells with suberized walls divide and grow, transforming the stomata into lenticels (Clements, 1936) which resist gas exchange. Decreasing permeability of the outer epidermis of the fruit, arising from the combined effects of decreasing stomatal frequency and function, leads to a buildup of CO_2 within fruit cavities, in apple to as much as 5% (v/v) (Reid et al., 1973), a 150-fold increase in concentration compared with the external ambient CO_2.

Carbon, convertible to CO_2, is also stored in many fruits as malate. Fruits contain phosphoenolpyruvate carboxylase with properties characteristic of the enzyme found in C_3 photosynthetic cells and non-chlorophyllous cells that fixes CO_2 into oxalacetate, which is then reduced to malate (Blanke and Lenz, 1989). Most of the malate is in vacuoles which increase in volume as fruit cells grow (Bain and Mercer, 1964). CO_2 is released from malate by decarboxylation catalyzed by malic enzyme, which is highest in activity at ripening (Dilley, 1962), particularly in the peel (Hulme et al., 1963), and by mitochondrial respiration. Consequently, while access by fruit chloroplasts to external CO_2 is increasingly restricted as the fruit matures, a relatively large concentration of CO_2 that is potentially available for photosynthetic CO_2 fixation develops within the fruit.

Although many fruits contain chlorophyll, little is known about their photosynthetic CO_2 fixing activity, as the measurement of light-dependent gas exchange in fruit is frequently difficult. The ratio of non-autotrophic to autotrophic tissue is usually large and commercial and most laboratory photosynthesis-measuring systems are not designed for bulky materials. Fruits can be very pale green in colour and possess chlorophyll concentrations too low for accurate measurements of light-dependent gas exchange.

Bilger et al. (1986) and Schreiber and Bilger (1986) have developed an optical method for following Calvin cycle activity in leaves based on changes in chlorophyll *a* fluorescence in vivo. This method is not subject to the constraints associated with measurements of gas exchange in fruit. The aim of this study was to make use of this method to determine the extent to which Calvin cycle activity was present in immature and mature fruit.

As the activity in leaves is quite sensitive to inactivation by heat, more so

than either photosynthetic electron transfer or photophosphorylation (Weis, 1981; Bilger et al., 1986), heat inactivation of Calvin cycle activity in fruit was also investigated. Also it may provide a sensitive, early indicator of heat conditioning and heat injury during high temperature treatments of fruit designed to eradicate insects and fungal pathogens (Couey, 1989).

MATERIALS AND METHODS

Fruit. – The following fruits were obtained from experimental orchards of the NSW Department of Agriculture, Narara: blueberry (*Vaccinium corymbosum* Linn.); *Citrus grandis* L. Osbeck; feijoa (*Feijoa sellowiana* O. Berg); fig (*Ficus carica* L.); guava (*Psidium guajava* L.); kiwifruit (*Actinida deliciosa* A. Chev.); lemon (*Citrus limon* L.) Burm. f cultivar 'Chegwyn Lisbon'), West Indian lime (*Citrus aurantifolia* (Christm.) Swing.); lychee (*Litchi chinensis* (Sonn. cultivar 'Kwai May Pink')); mandarin (*Citrus reticulata* Blanco cultivar 'Ellendale'); Valencia orange (*Citrus sinensis* (L.) Osbeck) and persimmon (*Diospyros kaki* L.). Tomato fruit and leaves (*Lycopersicon esculentum* Mill. cultivar '83G38') were harvested from plants grown in a greenhouse, avocado fruits (*Persea americana* Mill. cultivar 'Fuerte') were obtained from a local commercial orchard and pears (*Pyrus communis* L.) were obtained from a local market. The citrus fruit, guava, kiwifruit and sapote were from half to three-quarters mature. The remaining fruit were almost or fully mature.

High and low CO_2 treatments. – Tomato leaves or fruit and avocado fruit or discs (1.5 cm in diameter) of the peel were exposed to a low CO_2 external atmosphere ($-CO_2$) by placing them on a metal screen above fluted filter paper moistened with saturated KOH in a sealed polyethylene bag. The stems of leaves were immersed in water. Exposure to high CO_2 ($+CO_2$) was done similarly, replacing the KOH with 1 M $NaHCO_3$. The fibre optics of the chlorophyll fluorometer were positioned outside the bag and measurements of fluorescence were made through the plastic so that neither the experimental material nor the surrounding atmosphere was disturbed.

Heat treatments. – Tomato and lemon fruits were heat stressed by immersion for 5 min in a water-bath set to various temperatures. After heating, the fruits were cooled in water at 23°C for 5 min, then in air at 23°C for 5 min before measurements were made of chlorophyll fluorescence.

Measurement of chlorophyll fluorescence. – Chlorophyll fluorescence emission from the surface of fruits (the shoulder of tomato fruit and the equator of the other fruits) or the adaxial surface of leaves was measured using a pulse amplitude modulated (PAM) fluorometer (H. Walz, Effeltrich, Germany), comprising models 101 and 103 and four-armed fibreoptics. The end of the combined fibre bundle was positioned 3 mm above the surface of a fruit or leaf that had been kept in darkness for at least 1 h at 23°C. One arm of the fibre optics was coupled to a unit emitting a weak modulated beam of red

light peaking at around 680 nm to activate chlorophyll fluorescence, and another to a fluorescence detector unit. The third arm was coupled to a fibre illuminator (model KL1500, Schott, Weisbaden, Germany) which provided continuous white light at a photon flux density (PFD) of 60 μmol m^{-2} s^{-1} unless otherwise stated. The fourth arm was coupled to a second Schott illuminator modified for triggering by the PAM 103. This delivered saturating pulses of white light (PFD of 2100 μmol m^{-2} s^{-1}) of 0.7 s duration every 10 s to the surface of the fruit. Voltage output from the PAM 101 unit was recorded on a Goerz potentiometric recorder (model SE420, Kent Instruments, Sydney). Selective amplification of the modulated fluorescence signal in the PAM 101 unit meant that the unmodulated fluorescence and reflected light generated by the actinic illumination and saturation pulse were ignored; thus only the effect of altered photosynthetic metabolism on the original modulated fluorescence was measured. The temperature of the experimental materials during all fluorescence measurements was 23°C. PFD (400–700 nm) was measured with a Quantum meter (model Li-185A, Li-Cor Inc., Lincoln, NE, USA).

RESULTS AND DISCUSSION

Calvin cycle activity in tomato leaves and fruit. – Figure 1 (A) shows changes with time in chlorophyll fluorescence induced in a dark-adapted leaf of tomato. The fluorescence kinetics obtained were typical of those described in the literature for leaves of other plants. Turning on the modulated measuring beam activated fluorescence emission (F_o, Fig. 1) from Photosystem II reaction centres in the leaf chloroplasts. As the intensity of the measuring beam was set too low to generate photosynthetic activity, the primary acceptor for Photosystem II, Q_A, remained in the fully oxidized state, as if the leaf were still in darkness. Chlorophyll fluorescence in vivo can be thought of as being in competition for absorbed photon energy with other energy dissipative systems, the principal ones being photosynthetic electron transfer and the establishment of a transthylakoid proton gradient and the associated synthesis of ATP. Switching on the continuous white light induced photosynthetic activity which in turn generated changes in chlorophyll fluorescence. The fluorescence rose to a peak (F_p, Fig. 1) as Q_A was reduced, that is, as the availability of the acceptor for Photosystem II (oxidized Q_A) decreased, more absorbed photon energy was lost as fluorescence. The early fluorescence changes thus largely monitored the oxidation/reduction state of Q_A. As photooxidative reactions linked to Photosystem I began to exert their effect, Q_A again became more oxidized and the fluorescence decreased, eventually reaching a steady-state level higher than F_o.

Simultaneously imposing a short saturation pulse of light at the start of the continuous illumination, transiently drove Q_A to the fully reduced state. As

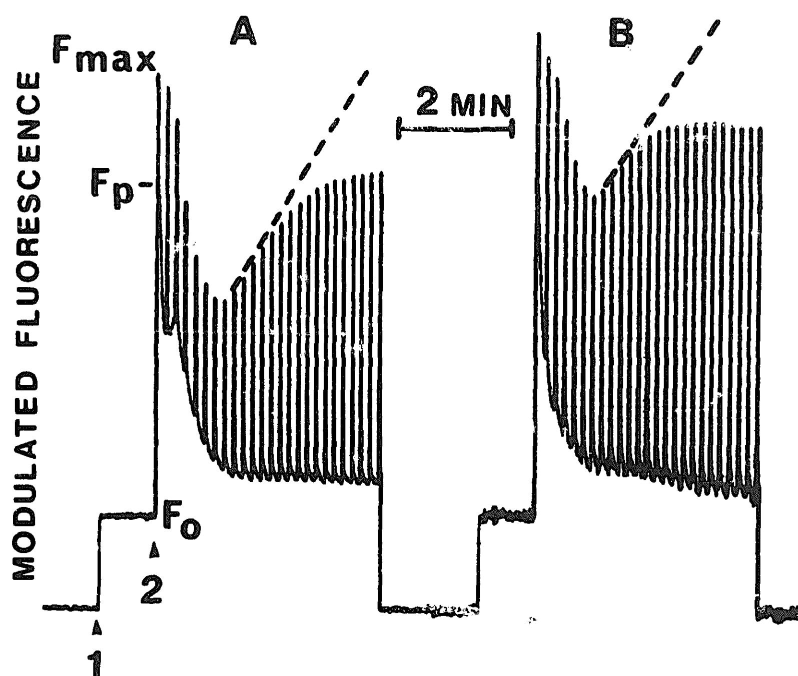

Fig. 1. Recordings of modulated chlorophyll fluorescence in (A) tomato leaf and (B) tomato fruit, measured with a modulation fluorometer. 1, Switching on the modulated measuring beam gave F_o. 2, Simultaneous application of continuous actinic light (PFD of 51 μmol m^{-2} s^{-1}) and the start of a series of saturation pulses, 10 s apart, produced a fluorescence induction curve rising to a peak, F_p, and then falling, while F_{max} was given at 10 s intervals. The dashed line indicates rate of relaxation of quenching of f_{max}.

acceptor availability was now at a minimum, fluorescence emission was at the maximum (F_{max}, Fig. 1).

In subsequent light saturation pulses the same condition pertained with regard to photoreduction, that is, Q_A became completely reduced during each pulse. However, F_{max} did not remain constant but decreased (fluorescence quenching) with successive pulses because of the onset of the other major energy-draining system of the thylakoid membrane resulting in ATP formation. Subsequently, as CO_2 was assimilated into the metabolites of the Calvin cycle and ATP was utilized in the cycle, ATP levels decreased and F_{max} increased again (relaxation of fluorescence quenching), eventually reaching a steady-state yield. The rate of relaxation of quenching of F_{max} is indicated by the dashed lines in Fig. 1 and gives a measure of Calvin cycle activity (Schreiber and Bilger, 1986; Bilger et al., 1986).

Other factors which may affect non-photochemical fluorescence quenching have been discussed by Krause and Weis (1984). An increase in the proportion of excitation energy directed to Photosystem I, thought to be regulated by phosphorylation of the light harvesting chlorophyll *a/b* protein, could increase fluorescence quenching, but the size of the effect was considered to be

a minor one (Krause and Weis, 1984). The remaining factors, high temperature, Mg^{2+} depletion and photoinhibition, are not germane to the present study. While the previous studies were carried out on leaves, there are no observations to suggest that, in these aspects, the chlorophyll-containing cells of fruit differ substantially from the mesophyll cells of leaves.

Figure 1(B) shows the kinetics of chlorophyll fluorescence in a green tomato fruit at the breaker stage. The changes in quenching of F_{max} and its subsequent relaxation followed the same trends observed in the leaf, with relaxation of quenching indicating that photosynthetic CO_2 fixation was taking place. An obvious difference between fruit and leaf was the more rapid decrease in fluorescence after F_p in response to continuous illumination. This has been observed by the author in comparisons between fruit and leaves of other species and may point to a more dominant Photosystem-I linked photooxidation of Q_A relative to its photoreduction in fruit compared with leaves at the light intensities used.

Figure 2 shows how the rate of relaxation of quenching of F_{max}, normalized with respect to F_o, varied with the PFD of the continuous actinic light. The rate increased with increasing PFD to a maximum at 50–70 μmol m^{-2} s^{-1} and then declined. As the highest PFD used was too low to cause photooxidation of the pigments, the likely reason for the decline was that at high PFD, the rate of ATP production exceeded the rate of its utilization by Calvin cycle reactions. In subsequent experiments, a PFD of 60 μmol m^{-2} s^{-1} was used for the continuous actinic illumination.

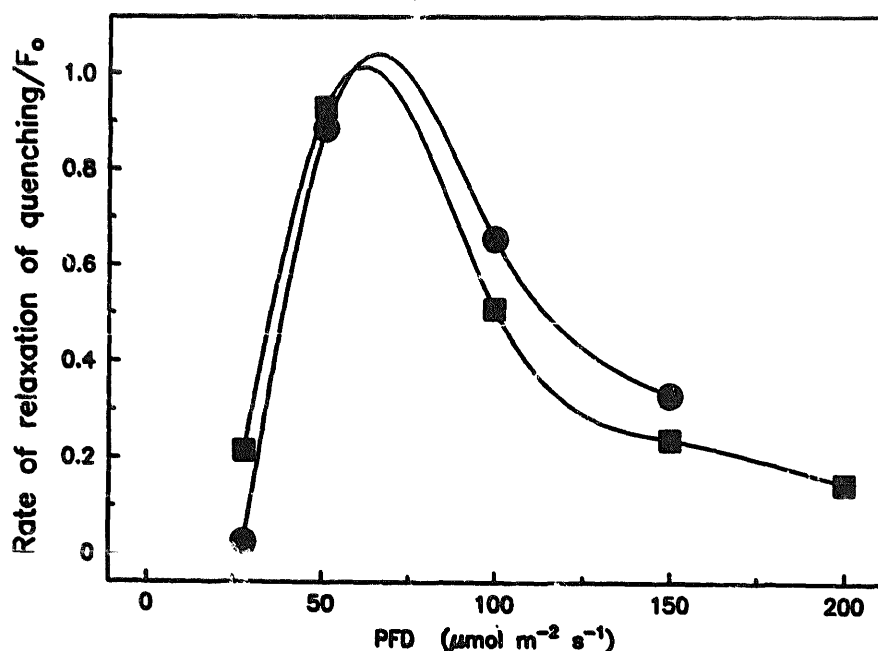

Fig. 2. Rate of relaxation of quenching in tomato fruit and leaf at different PFD. ●, fruit; ■, leaf.

Other fruits. – Non-photochemical quenching measured as described above using saturation pulses is commonly expressed by the non-photochemical quenching coefficient, q_{NP} (Schreiber and Bilger, 1986). This is the ratio of the decrease in F_{max} relative to the original unquenched F_{max} value. Thus

$$q_{NP} = \frac{(F_v)_{max} - (F_v)s}{(F_v)_{max}}$$

where $(F_v)_{max}$ is the difference between the maximal fluorescence intensity and F_0 obtained during the first exposure of dark-adapted tissue to a saturating light pulse; and $(F_v)s$ is the difference between maximal fluorescence intensity and F_0 at any subsequent given time during a saturation pulse. Figure 3 shows changes in q_{NP} for the fruit of four species during fluorescence induction. At first there was a rapid increase in q_{NP}, but then marked relaxation of the quenching took place after about 40 s in lychee fruit, 50 s in lime and blueberry and 80 s in fig. These decreases in q_{NP}, corresponding to the relaxation of quenching of F_{max} shown in Fig. 1, indicated the presence of strong Calvin cycle activity in all four fruit.

Values of relative rates of relaxation of fluorescence quenching for fruit of 14 species are shown in Table 1 together with, for several of the species, values obtained with leaves harvested from the same trees as the fruit. All fruit and leaves sampled showed evidence of Calvin cycle activity as indicated by the relaxation of fluorescence quenching, with values for fruit generally being comparable with those of leaves (mean values per min of 0.46 ± 0.08 for

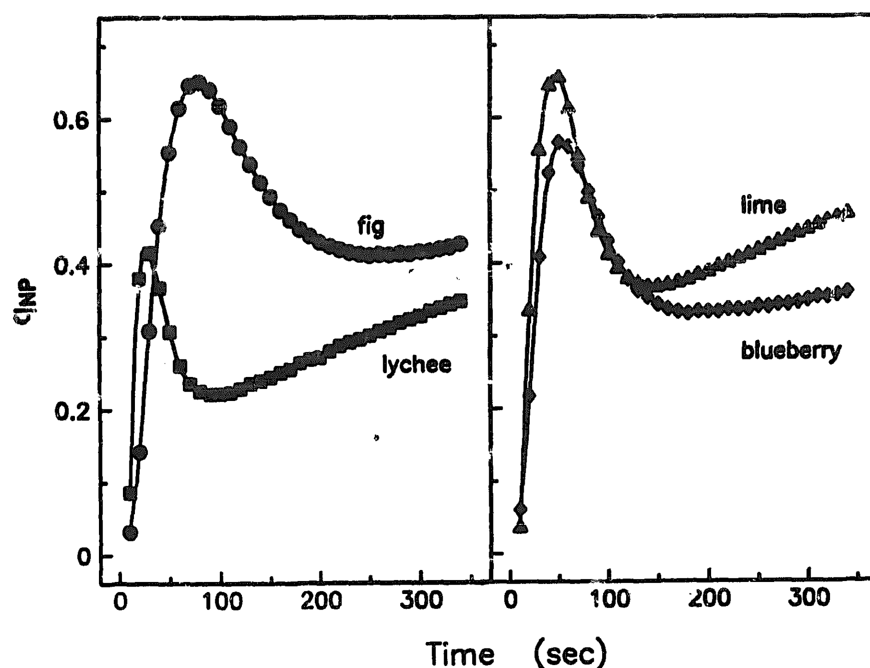

Fig. 3. Changes in the non-photochemical quenching coefficient, q_{NP}, during chlorophyll fluorescence induction in fruit of fig, lychee, lime and blueberry. The initial increase in q_{NP} upon illuminating the fruit surface was followed by a relaxation of quenching as ATP was consumed in the Calvin cycle.

TABLE 1

Relaxation of chlorophyll fluorescence quenching in fruit and leaves

Plant	Organ	Rate of relaxation of quenching/F_o (min^{-1})	$(F_v)_{max}/F_o$
Avocado	Fruit	0.41	4.08
Blueberry	Fruit	0.68	4.54
Citrus grandis	Fruit	0.55	4.77
Feijoa	Fruit	0.16	4.36
Fig	Fruit	0.40	4.61
Fig	Leaf	0.28	4.15
Guava	Fruit	0.29	4.61
Kiwifruit	Fruit	0.05	5.20
Lime	Fruit	1.05	5.32
Lychee	Fruit	0.84	4.27
Lychee	Leaf	0.21	4.29
Mandarin	Fruit	0.23	5.62
Orange	Fruit	0.47	5.07
Orange	Leaf	0.97	5.56
Pear	Fruit	0.32	3.21
Persimmon	Fruit	0.17	5.42
Persimmon	Leaf	0.07	5.53
Tomato	Fruit	0.85	5.15
Tomato	Leaf	0.92	5.19

fruit and 0.49 ± 0.19 for leaves). Table 1 also shows ratios of $(F_v)_{max}$ to F_o obtained during the first saturation pulse. All ratios exceeded four, except for the pear fruits which were over-mature and beginning to soften, indicating that all the other fruits had normal photoreductive systems. A value of four is equivalent to a value of 0.8 when $(F_v)_{max}$ is expressed as a ratio of F_{max} instead of F_o. Björkman and Demmig (1987), who measured $(F_v)_{max}/F_{max}$ in healthy leaves of 37 species of C_3 plants, found a mean value of 0.832 ± 0.004.

The effect of a CO_2-free atmosphere. – Although gas exchange with the outside air may be constrained in maturing fruit, the evidence presented above suggests that photosynthetic CO_2 fixation is nonetheless quite active. To investigate whether much of the CO_2 fixed photosynthetically was derived from CO_2 accumulated internally, Calvin cycle activity in tomato and avocado fruits was observed after placing the fruits in air that was essentially free of CO_2. Placing a tomato leaf in CO_2-free air resulted in a fairly rapid decrease in the rate of relaxation of fluorescence quenching (Table 2). Activity was regained by exposing the leaf to ambient CO_2 in air. The responses of the fruit were much less marked. Rates of relaxation of quenching declined only slowly and were likewise slowly regained after transferring fruit (avocado) to a high CO_2 atmosphere. The prolonged continuation of Calvin cycle activity in the fruit, despite the absence of CO_2 in the external air suggests use of CO_2 accumu-

TABLE 2

Relaxation of chlorophyll fluorescence quenching in different atmospheres. Procedures used to obtain $-CO_2$ and $+CO_2$ atmospheres are described in the text

Experimental material	Atmosphere	Time (min)	Rate of relaxation of qunching/F_o (min^{-1})
Tomato leaf	Air	-	1.25
	$-CO_2$	5	0.59
		25	0.06
	Air	10	0.75
		25	1.00
Tomato fruit	Air	-	1.03
	$-CO_2$	30	0.85
		90	0.70
		150	0.48
Avocado fruit	Air	-	0.40
	$-CO_2$	30	0.43
		90	0.40
		140	0.21
	$+CO_2$	10	0.20
		60	0.36
		140	0.36
Avocado peel	Air	-	0.41
	$-CO_2$	5	0.22
		12	0
	$+CO_2$	12	0.33

lated internally for photosynthesis. As the closed system employed would result in a CO_2 gradient being formed between the KOH and internal CO_2, the slow decline in activity may have resulted from a decrease in internal CO_2 concentration. Further evidence for the utilization of CO_2 from within the fruit by chloroplasts in the avocado peel was obtained using discs of avocado peel. Immediately after cutting the peel from the fruit, rates of relaxation of quenching were high, but quickly declined to zero when discs were placed in a CO_2-free atmosphere (Table 2). The loss of activity was reversible and could be regained by transferring discs to a high CO_2 atmosphere. In avocado peel this cycle of activity loss in the absence of external CO_2 and the resumption of activity in high CO_2 could be repeated several times (data not shown). It would seem that like chloroplasts in leaves, those in avocado peel respond quickly to CO_2 depletion, but that in the intact fruit, these chloroplasts continue to fix CO_2 photosynthetically by using CO_2 present within the fruit.

Heat stress. – The photosynthetic system in leaves is especially vulnerable to heat stress, becoming inactivated at temperatures several degrees below those

damaging respiration and several other cellular processes (Alexandrov, 1964). Of the partial reactions of photosynthesis, Calvin cycle activity is more sensitive to inactivation by heat than either photosynthetic electron transfer or photophosphorylation (Weis, 1981; Bilger et al., 1986). The heat-sensitive step in the cycle appears to be the fixation of CO_2 by Rubisco (Weis, 1981), other enzymes of the cycle being relatively heat stable (Santarius, 1975). Figure 4 shows the effect of heating lemon fruit at 48°C for 5 min on the chlorophyll fluorescence induction curve. The initial rise to F_{max}, decrease in fluorescence after F_p and quenching of F_{max} were only partially affected (Fig. 4(B)), indicating that photoreduction of Q_A by Photosystem II, photooxidation linked to Photosystem I, and ATP formation, respectively, were marginally affected by the heat treatment. However, the subsequent relaxation of quenching of F_{max} evident in the fruit before heating (Fig. 1A) was not present following heating. This effect of heat was partially reversible (Fig. 4(C)). Table 3 shows rates of relaxation of quenching in tomato and lemon fruit heated to various temperatures. After some treatments, the fruit were allowed to recover at 23°C for 3 days. Photoreduction, indicated by $(F_v)_{max}/F_o$, was still measurable in tomato and lemon at temperatures which abolished relaxation of quenching of F_{max}. In both these fruits, Calvin cycle activity appears to be more sensitive than the other major photosynthetic processes. Still higher temperatures generally are needed to inactivate respiration in plant tissues (Alexandrov, 1964).

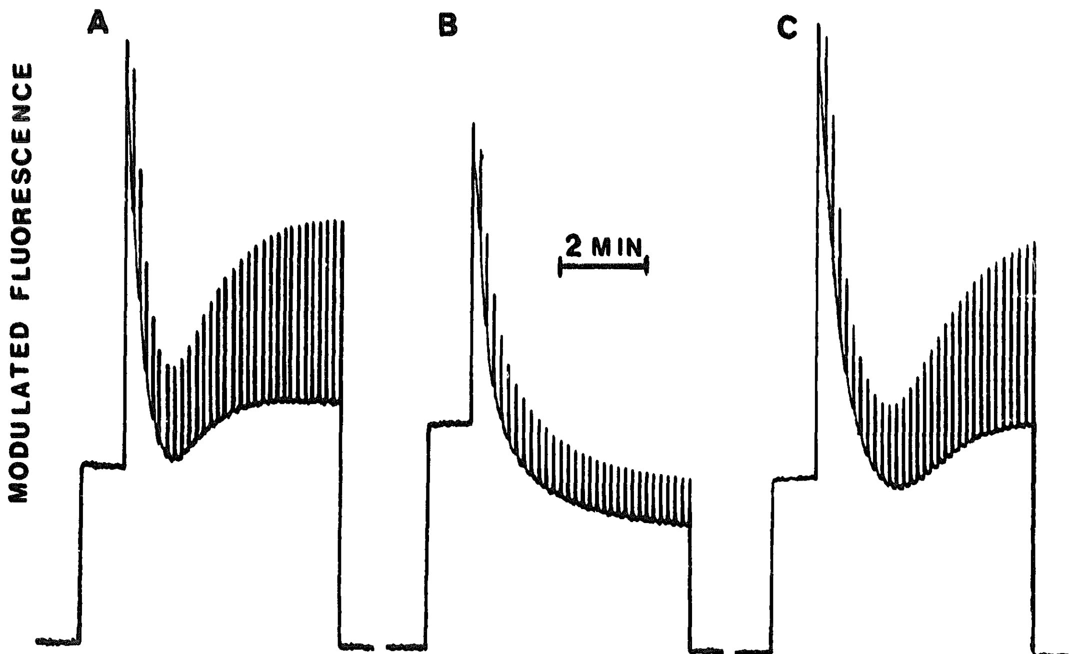

Fig. 4. The effect of heat stress on relaxation of F_{max} quenching in lemon fruit. (A) Before heating; (B) after heating at 48°C for 5 min and cooling to 23°C; (C) heat-treated fruit after a further 4 days at 23°C.

TABLE 3

Heat inactivation of chlorophyll fluorescence emission in tomato and lemon fruit. All heating times were for 5 min

Treatment	Rate of relaxation of quenching/F_o (min^{-1})	$(F_v)_{max}/F_o$
Tomato fruit		
None	0.80	5.48
45°C	0.42	4.26
50°C	0	0.98
50°C, +1 day at 23°C	0.07	2.09
50°C, +3 days at 23°C	0.17	3.17
Lemon fruit		
None	0.51	4.32
44°C	0.38	3.55
48°C	0.05	3.40
51°C	0	0.87
54°C	0	0.42
48°C, +3 days at 23°C	0.37	3.56
51°C, +3 days at 23°C	0.07	1.89
54°C, +3 days at 23°C	0.02	0.29

TABLE 4

Recovery from heat stress in lemon fruit. Fruit were heated at 48 or 49°C for 5 min. The recovery rate of relaxation of quenching at various times after heating is shown. Values are expressed as a percentage of the rate obtained before heating

After heating at 48°C		After heating at 49°C	
Time	Rate of relaxation of quenching/F_o (% of control)	Time	Rate of relaxation of quenching/F_o (% of control)
10 min	9	10 min	0
30 min	49	30 min	11
45 min	55	60 min	15
90 min	62	130 min	24
2 h	66	3 h	28
4 days	85	4 days	83
6 days	87	6 days	87

Temperatures of 48–49°C for 5 min were close to the limit of exposure to high temperature that still allowed reasonable recovery of Calvin cycle activity to occur in lemon (Tables 3 and 4). Recovery was initially fast after heating at 48°C but much slower after heating at 49°C (Table 4).

Currently, there is pressure from consumers for greater use of non-chemical postharvest treatments of fresh fruit. Controlled heat treatment of fruit shows promise for disinfesting fruit of insects, delaying ripening and slowing development of fungal infections. Non-destructive monitoring of Calvin cycle activity by fluorescence quenching should provide a sensitive means of detecting early symptoms of heat stress in chlorophyllous fruits as well as in other horticultural produce such as green vegetables and cut foliage. It should also be useful as an easy-to-measure indicator of the effectiveness of preconditioning treatments designed to maximize the tolerance of fruit to heat stresses.

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