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Programmed cell death features in apple suspension cells under low oxygen culture*

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Abstract: Suspension-cultured apple fruit cells (*Malus pumila* Mill. cv. Braeburn) were exposed to a low oxygen atmosphere to test whether programmed cell death (PCD) has a role in cell dysfunction and death under hypoxic conditions. Protoplasts were prepared at various times after low oxygen conditions were established, and viability tested by triple staining with fluorescein diacetate (FDA), propidium iodide (PI) and Hoechst33342 (HO342). DNA breakdown and phosphatidylserine exposure on the plasma membrane were observed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), and annexin V binding. About 30% of protoplasts from cells after 48 h under low oxygen showed an increased accumulation of HO342, indicating increased membrane permeability. Positive TUNEL and annexin V results were also only obtained with protoplasts from cells under low oxygen. The results suggest that apple cell death under low oxygen is at least partially PCD mediated, and may explain tissue breakdown under controlled atmosphere (low oxygen) conditions in apple fruit.

Key words: Apple, Programmed cell death, Low oxygen
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INTRODUCTION

A number of abiotic stresses have been shown to induce programmed cell death (PCD) responses in plant cells. High temperatures (e.g. 55 ºC) induce nuclear condensation and DNA fragmentation in cultured carrot, cucumber and *Arabidopsis* cells (McCabe *et al*., 1997; McCabe and Leaver, 2000), and also mitochondrial cytochrome *c* release in cucumber cotyledons (Balk *et al*., 1999). The PCD pathway also appears to be activated by ozone. Ozone induces an oxidative burst with accumulation of hydrogen peroxide and superoxide anion (Langebartels *et al*., 2002), and these very active species act as signals to amplify the PCD pathway, as seen for example in DNA degradation visualized in tobacco leaf cells by the TUNEL assay (Pasqualini *et al*., 2003). There is also some evidence for low temperature induction of PCD (Koukalová *et al*., 1997), although close examination of cell sub-populations responding to low temperatures has revealed the likelihood of a necrotic pathway rather than one associated with the conventional PCD processes (Wang *et al*., 2001).

Another stress that might induce PCD is low oxygen conditions or hypoxia. A number of animal

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cell types undergo apoptotic cell death under hypoxic conditions (Jacobson and Raff, 1995; Araya *et al*., 1998). In the plant environment, low oxygen is encountered mainly under waterlogging conditions, and one response in the root is the development of aerenchyma, which allows greater diffusion of oxygen from the atmosphere and the upper parts of the plant. Aerenchyma is developed through the death of root cortical cells, as a response to ethylene induced by low oxygen. It appears that some form of PCD, characterized by DNA fragmentation and chromatin condensation occurs under these conditions, followed by cell wall degradation and the formation of the aerenchymatous airspaces (Drew *et al*., 2000; Gunawardena *et al*., 2001).

The other example of hypoxia, of considerable interest to fruit and postharvest research, is that of imposed controlled atmosphere (CA) storage conditions for long-term fruit storage. The postharvest life of many fruit species, such as apple, pear and kiwifruit, can be prolonged under low oxygen and/or high carbon dioxide concentrations, in combination with low temperature. Such low oxygen storage can, however, result in damage to the tissues, as seen in the 'Braeburn' apple cultivar, where a flesh browning disorder (Lau, 1998) has been characterised; its symptoms include browning, watersoaking and in severe cases, formation of cavities, presumably a result of flesh cell autolysis. The former symptoms are indicative of membrane dysfunction and possible cell death.

PCD involves a series of well-programmed events such as chromatin condensation, cell shrinkage, nucleosomal DNA fragmentation, and ordered cell disassembly (Pennell and Lamb, 1997; Wang *et al*., 2001). Necrosis, the other means of cell death, is distinct from PCD in that the process is a response to severe trauma and is not genetically controlled. The differences between these two death pathways help to establish various methods to discriminate PCD from necrosis (Xu *et al*., 2004). We have used cell viability tests, TUNEL, and detection of annexin V binding as indicators of PCD in suspension-cultured apple fruit cells under low oxygen conditions. Our objective was to see whether low oxygen induces PCD in fruit cells. If this were to be the case, it might give us some understanding of what happens in fruit tissue under these conditions during postharvest storage.

MATERIAL AND METHODS

Plant material

Apple (*Malus pumila* Mill. cv. Braeburn) flesh cells were maintained in suspension culture in the same medium and under the same conditions as described by Wang *et al*.(2001). The cells were subcultured every 5 days, and cells at 3 days after subculture, in the early log phase stage, were used for experiments.

Low oxygen treatment

Cells (20 ml) were sealed in 50 ml Erlenmeyer flasks, in duplicate. Low oxygen conditions were accomplished by flushing the air in the flasks with nitrogen for 5 min. Oxygen concentrations inside the flasks were determined to be near 0.1%. Cells under normal atmosphere served as controls. Cells were cultured under the conditions described above.

Gas measurements

A 1 ml gas sample was taken from the headspace of each flask to measure gas concentrations. Oxygen and carbon dioxide were analysed with an ICA40 system, where infra red is used for the carbon dioxide analysis and the oxygen is measured with paramagnetic technology. Acetaldehyde, ethanol and ethylene were analysed by gas chromatography with a flame ionization detector and alumina column (Philips PU4500, Unicam, Cambridge, UK).

Protoplast Preparation

Protoplasts were prepared according to Wang *et al*.(2001) except that the enzymes were dissolved in CPW 13M (CPW medium with 13% mannitol) (Frearson *et al*., 1973). Protoplasts were washed and held in CPW 13M and subjected to further analyses in 4 hours.

Viability measurements

Cell viability can be determined using non-fluorescent fluorescein diacetate (FDA), which can be absorbed by functional cells and hydrolysed by

esterases to a fluorescent form, and propidium iodide (PI) which can only enter into the nucleus of dead cells and intercalate with nuclear DNA, resulting in red fluorescence under ultraviolet light. Hoechst33342 (HO342) is another DNA fluorochrome which can enter into both live and dead cells. However, apoptotic cells accumulate much more HO342 in a short time compared to non-apoptotic live cells because of increased membrane permeability (Schmid *et al*., 1994). Therefore, the cells stained by both FDA and HO342 can be regarded as cells becoming dysfunctional, by FDA only as functional live cells, and by both PI and HO342 as dead ones.

To 0.5 ml protoplast solution, 1 μl 0.1 mg/ml FDA, 2.5 μl 2 mg/ml PI, and 3.75 μl 1 mg/ml HO342 were added. The solution was gently mixed by inversion and held at room temperature for 10 min, before microscopic imaging under UV, using a Leitz Fluovert FS microscope equipped with a high pressure 50 W mercury arc lamp. Viability was calculated on a basis of more than 200 individual protoplasts for each treatment as the percentage of live protoplasts.

TUNEL Analysis

DNA breakdown during PCD can be specifically detected by TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling reaction (Gavrieli *et al*., 1992). Protoplasts were subjected to TUNEL analysis using the "*In Situ* Cell Death Detection Kit, Fluorescein" (Roche) according to the manufacturer's recommendations, except that the PBS was replaced by PBSPP (PBS for plant protoplasts) (De Jong *et al*., 2000). Briefly, protoplasts were fixed with 2% paraformaldehyde in PBSPP, permeablized with 0.1% Triton X-100 in 0.1% sodium citrate, and then reacted with the TUNEL reaction mixture under catalysis with TdT at 37 ºC for 2 h, and finally photos were taken using the UV microscope.

Annexin V staining and flow cytometry analysis

Cells undergoing PCD expose phosphatidylserine (PS) on the outer surface of the plasma membrane. Normally, PS only locates on the inner plasma membrane, and exposure can be recognized by annexin V binding (O'Brien *et al*., 1997).

To 0.5 ml protoplast solution, 0.5 μl annexin V-FITC stock solution (Immunotech) was added. The solution was mixed by inversion and held at room temperature for 30 min before analysed by flow cytometry according to O'Brien *et al*.(1998), using a Coulter EPICS Elite ESP flow cytometer (Beckman Coulter).

RESULTS

Cell viability under low oxygen

When apple cells were held under low oxygen conditions, there was a rapid accumulation of carbon dioxide, acetaldehyde, and ethanol over the first 8 h. Ethylene was not detected until 24 h, and only at low levels. The concentrations of ethylene, acetaldehyde and ethanol were 0.10 μl/L, 7.85 μl/L, and 131.97 μl/L, respectively at 48 h after the start of the experiment.

Cell growth and division was completely inhibited under low oxygen, whereas control cells continued to grow and divide, with the cell density doubling by around 60 h (data not shown).

Although accumulation of acetaldehyde and ethanol under low oxygen began as early as 8 h, no significant differences in cell viability, as determined by FDA and PI staining, were found between control and the low oxygen-treated cells until after the 48 h sample point (Fig.1). From this time, there

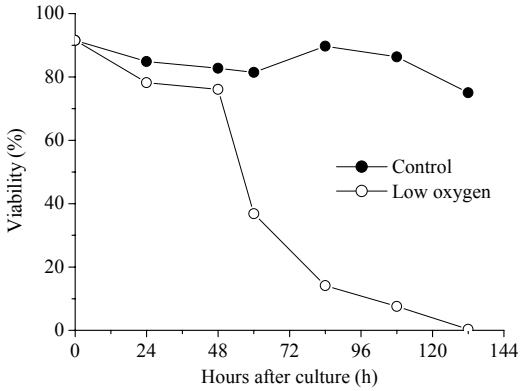

Fig.1 Effects of low oxygen culture on viability of apple cells. Protoplasts were prepared from cells at different times, and viability was calculated on a base of more than 200 individual protoplasts for each sample as the percentage of live protoplasts among total protoplasts

was a rapid decline in viability under low oxygen until all the cells were dead by 132 h. Viability of the control cells slightly increased at 84 h, probably as a result of new cell division.

When cells were stained with FDA and PI, viable cells showed typical green fluorescence, and under low oxygen at 48 h there was an increase in PI binding to nuclear DNA, as shown by the cells with red fluorescence (Figs.2a and 2c). With triple staining, about 30% of the protoplasts from cells under low oxygen were found to have strong blue fluorescence (Fig.2d, indicated by arrows); these can be separated from the normal living cells which have a weak blue or greenish fluorescence and dead cells with purplish fluorescence. This strong blue fluorescence results from the incorporation of HO342 into DNA in the cell nuclei. The strong purplish fluorescence, however, was from leakage of PI fluorescence into the measurement of HO342 fluorescence. Dead cells are permeable to both PI and HO342, but are preferentially stained by PI rather than HO342, and as a result, the purplish fluorescence is observed (Fig.2d). Weak blue or greenish fluorescence was from FDA fluorescence leakage.

In summary, although viability of cells after 48 h under low oxygen was not significantly lower than that of control cells (Fig.1), low oxygen was having an effect by this time, since increased accumulation of HO342 was found for protoplasts from cells under low oxygen (Fig.2d).

DNA fragmentation

TUNEL analysis was applied to detect possible DNA breakdown under low oxygen, and after the TUNEL reaction, protoplasts were incubated with HO342 to stain all nuclei. Nuclei with strong green fluorescence (Fig.3c, indicated by arrow) indicated TUNEL positive protoplasts. As shown in Fig.3, no protoplasts from control cells at 48 h were found to be TUNEL positive (Fig.3a), while about 30% of protoplasts from the low oxygen treatment were TUNEL positive (Fig.3c). The percentage is quite close to that of protoplasts with increased accumulation of HO342 (Fig.2d).

Annexin V binding

The ability of protoplasts to bind annexin V was measured with cells under normal air or low oxygen at 24 h and 48 h after treatment. Fluorescence of protoplasts measured by flow cytometry is expressed as a histogram, where the quantitative response is covered by a set of 1024 channels, from a minimum of fluorescence intensity at 1 to a maximum at 1024 (in Fig.4, this is on a logarithmic scale). The number of protoplasts recorded determine the peak height and area. Both at 24 and 48 h, cells exposed to low oxygen showed increased annexin V binding (Fig.4), indicated by the shift in the peak to the right. This was greater for the cells at 24 h (the peak channel number moving from 2 to 23; Figs.4a and 4b), but still significant at 48 h (Figs.4c and 4d).

DISCUSSION

Our results suggest that the cell death observed under low oxygen conditions in cultured apple fruit has characteristics of PCD. The only other record of plant PCD under low oxygen is with aerenchyma formation in maize roots (Gunawardena *et al*., 2001). This appears to be a process of early cytoplasmic changes (membrane breakdown and vesicle formation), followed by the more characteristic chromatin condensation and DNA breakdown as detected by both TUNEL and DNA laddering. The early cytoplasmic changes are in contrast with the apoptotic process in animal cells where chromatin condensation is the first manifestation of PCD. In PCD induced in tobacco cells by H_2O_2 and other cytotoxic agents, chromatin condensation is the first observed event (O'Brien *et al*., 1998). However, animal and plant PCD mechanisms are increasingly being shown to be different under different inducing conditions (Jones, 2000).

The cell viability stains show that low oxygen is causing membrane dysfunction and death, particularly with the nuclear staining of PI. The results with HO342, which in mammalian cells is associated with PCD (Schmid *et al*., 1994), shows a comparatively early increase in permeability of the cell membranes to this dye. The suggestion that PCD might be involved was confirmed by the TUNEL and annexin V binding assays. It is notable

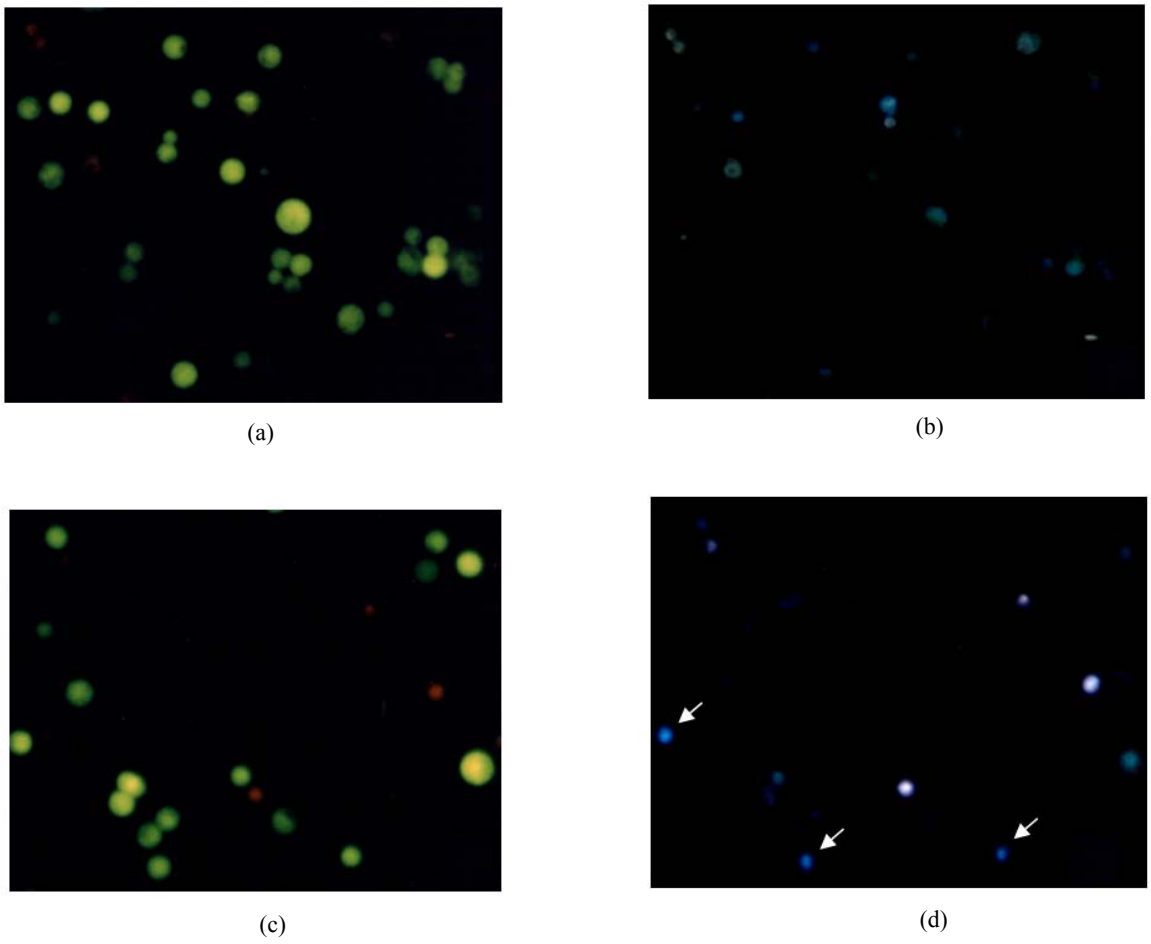

Fig.2 FDA, PI and HO342 staining of apple protoplasts. The protoplasts were prepared from cells after 48 h exposure to normal air (a, b) or low oxygen (c, d). Cells from both treatments were stained with FDA and PI (a, c) or FDA, PI and HO342 (b, d). Arrows (d) indicate protoplasts with increased accumulation of HO342

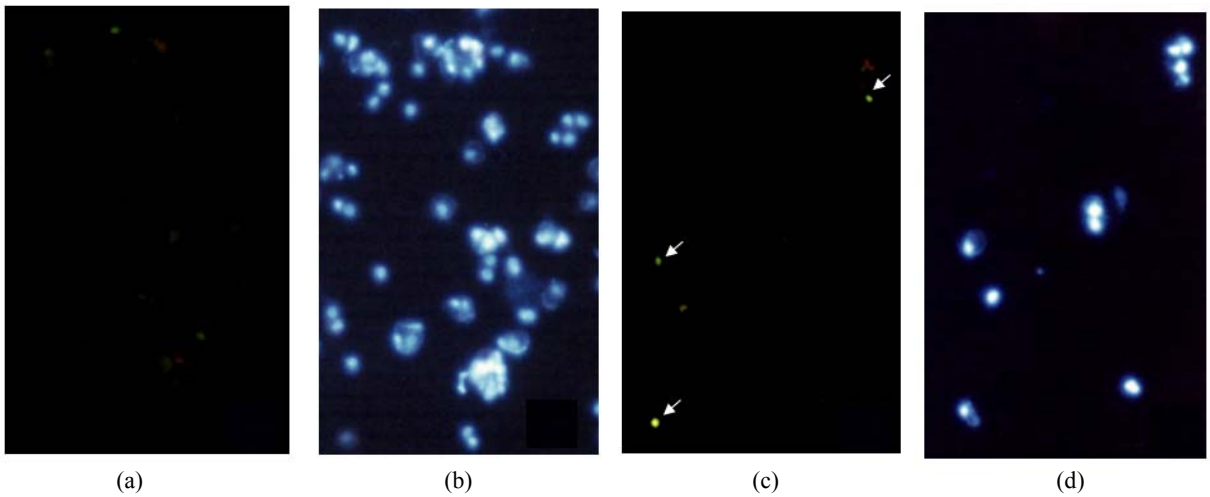

Fig.3 TUNEL assay of apple protoplasts. The protoplasts were prepared from cells after 48 h exposure to normal air (a, b) or low oxygen (c, d) and stained with TUNEL reagents and HO342. Arrows (c) indicate TUNEL positive nuclei

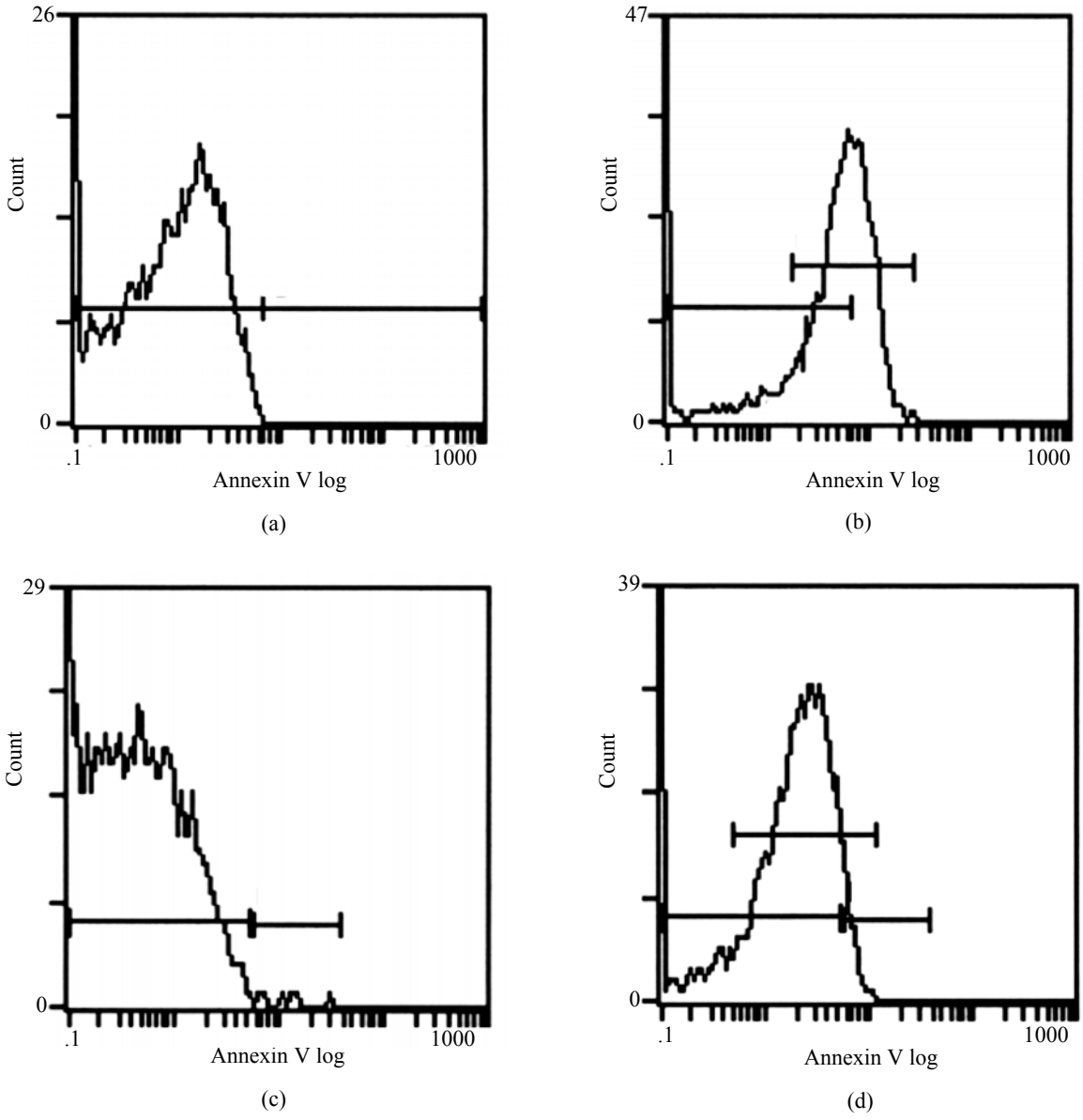

Fig.4 Annexin V assay for binding capacities of apple protoplasts. The protoplasts were prepared from cells after 24 h (a, b) or 48 h (c, d) under normal air (a, c) or low oxygen (b, d)

that positive results from both of these assays were observed at 48 h (and in the case of the annexin V assay, at 24 h), at a time just prior to the rapid decline in cell viability (Fig.1). This suggests that PCD events were under way about 24 h after low oxygen conditions were established.

PCD processes in plants are variable and do not necessarily follow the pathways observed in animal cells. While most examples include endonuclease-induced DNA fragmentation, observed by *in situ* TUNEL or laddering, there are inconsistent results regarding condensation events, and there are currently no identified homologues of caspases in plants (Woltering *et al*., 2002). However, what is relatively consistent is that PCD-associated events are induced by the same agents in plants and animals, including reactive oxygen species, nitric oxide, heat and cytotoxic agents. Low oxygen now appears to be one of these.

Cultured cells have frequently been used to study PCD successfully (McCabe *et al.*, 2000). While care must be taken in extrapolating to whole organs such as fruit, the results on fruit cells do give a guide to events that may occur in intact tissue. The

sequence of events occurring under low oxygen appear to be accumulation of acetaldehyde and ethanol, exposure of phosphatidylserine, increased permeability of membrane and specific breakdown of DNA, and finally cell death. This sequence may help explain the behavior of fruit in low oxygen storage, and the tissue breakdown and cell death commonly observed in some fruit.

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