Planta (2001) 212: 205–214

Planta

Characterisation of programmed cell death during aerenchyma formation induced by ethylene or hypoxia in roots of maize (*Zea mays* L.)

Arunika H. L. A. N. Gunawardena[1,*], Deborah M. Pearce[1], Michael B. Jackson[2], Chris R. Hawes[1], David E. Evans[1]

[1]Research School of Biological and Molecular Sciences, Oxford Brookes University, Gipsy Lane Campus, Headington, Oxford, OX3 0BP, UK
[2]IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol, BS41 9AF, UK

Received: 12 April 2000 / Accepted: 18 June 2000

Abstract. Aerenchyma is a tissue type characterised by prominent intercellular spaces which enhance flooding tolerance in some plant species by facilitating gas diffusion between roots and the aerial environment. Aerenchyma in maize roots forms by collapse and death of some of the cortical cells in a process that can be promoted by imposing oxygen shortage or by ethylene treatment. Maize roots grown hydroponically in 3% oxygen, 1 μl l^{-1} ethylene or 21% oxygen (control) were analysed by a combination of light and electron microscopy. Use of in-situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) suggested internucleosomal cleavage of DNA. However, chromatin condensation detectable by electron microscopy was preceded by cytoplasmic changes including plasma membrane invagination and the formation of vesicles, in contrast to mammalian apoptosis in which chromatin condensation is the first detectable event. Later, cellular condensation, condensation of chromatin and the presence of intact organelles surrounded by membrane resembling apoptotic bodies were observed. All these events were complete before cell wall degradation was apparent. Therefore, aerenchyma formation initiated by hypoxia or ethylene appears to be a form of programmed cell death that shows characteristics in part resembling both apoptosis and cytoplasmic cell death in animal cells.

Key words: Aerenchyma – Apoptosis – Ethylene – Hypoxia – Programmed cell death – *Zea* (apoptosis)

Introduction

Aerenchyma comprises a series of interconnected intercellular spaces which facilitate gas diffusion between roots growing in waterlogged soil and the aerial environment (Armstrong 1979). In many, but not all species, these spaces are created by a spatially distinct pattern of selective cell death. In maize and rice roots, its formation begins with the death and lysis of cells of the mid cortex in a zone behind the root apex and it spreads radially to form gas spaces separated by radial bridges of living cells (Armstrong and Armstrong 1994) linking the stele and epidermis. The cell walls and contents of cells that form aerenchyma are completely digested and leave no apparent trace (Drew et al. 1979).

Aerenchyma formation occurs as an ordered series of events and thus shows a key characteristic of programmed cell death (PCD) but does not show characteristics of necrotic (or traumatic) lesions. In maize roots it is specifically initiated by ethylene, produced endogenously or applied exogenously (Drew et al. 1979; Jackson et al. 1985).

Previous studies have investigated aerenchyma formation in maize roots, including its initiation by hypoxia (but not anoxia) and the plant hormone ethylene (Drew et al. 1979; Jackson et al. 1985); its inhibition by calcium antagonists (He et al. 1996), the induction of wall degradative enzymes (cellulase, xyloglucan endotransglycosylase; Saab and Sachs 1996) and ultrastructural changes which occur (Campbell and Drew 1983). While aerenchyma formation has been recognised as a form of PCD for some time (Brailsford et al. 1993), the precise nature of the processes involved remains uncertain (Drew 1997; Buckner et al. 1998; Drew at al. 2000). Various forms of PCD have been characterised in animal cells, including apoptosis and cytoplasmic cell death, distinguishable by the order of events in cell degradation. Apoptotic cells show chromatin condensation to the nuclear periphery at an early stage, with blebbing of membranes, cellular condensation, and retention of intact organelles until a late stage of cell death. These intact organelles are observed in membrane-bounded bodies

Present address: Department of Agricultural Biology, University of Peradeniya, Sri Lanka

Abbreviations: PCD = programmed cell death; TEM = transmission electron microscopy; TdT = terminal deoxynucleotidyl transferase; TUNEL = TdT-mediated dUTP nick-end labelling

Correspondence to: D. E. Evans;
E-mail: deevans@brookes.ac.uk; Fax: +44-1865-483242

(called apoptotic bodies; Wyllie et al. (1980, 1984); Bursch et al. 1990). Chromatin condensation is accompanied by oligonucleosomal fragmentation of DNA. Cytoplasmic cell death (CCD), a second type of PCD in animal cells, involves no initial change in nuclei, but large changes in cytoplasm with the formation of large autophagic vacuoles. Later, organelles are absorbed and finally chromatin condensation and oligonucleosomal DNA fragmentation may be detected (Zakeri et al. 1995).

Programmed cell death has been observed in various developmental processes in higher plants (Barlow 1982; Pennell and Lamb 1997), and has led to the conclusion that processes resembling apoptosis also occur. Evidence for internucleosomal DNA fragmentation (a key feature of apoptosis) has been obtained during pathogen-induced PCD (Ryerson and Heath 1996), tracheary element differentiation (Mittler and Lam 1995) and cell shedding at the root cap (Wang et al. 1996a). Wang et al. (1996a) also reported that host-selective phytotoxin-induced PCD exhibits many apoptotic-like characteristics, such as the lobing and fragmenting of nuclei and the formation of apoptotic-like bodies. Chromatin condensation in nuclei has been observed by transmission electron microscopy (TEM) during anther development (Wang et al. 1998, 1999). Other characteristics in animal cells, such as the retention of intact organelles, membrane invagination and the presence of membrane-bound bodies do not appear to have been reported in this context in plants at the electron-microscope level.

A previous ultrastructural study of aerenchyma formation in maize (Campbell and Drew 1983) utilised the developmental progression of cell development within the root to assign an order to some of the cellular events occurring. This study, however, was limited to a consideration of some cytoplasmic events and did not consider changes in the nucleus that are now known to be central to PCD. Recently, Schussler and Longstreth (2000) have also included maize in an ultrastructural study predominantly of aerenchyma in *Sagittaria lancifolia*; however, their investigations of maize were restricted to tissues in which aerenchyma had already formed. In this paper, we present a detailed study of changes in the nucleus and cytoplasm (including the detection of DNA fragmentation and description of membranous structures not previously reported) to characterise the order of both nuclear and cytoplasmic events in aerenchyma formation and cortical cell death in maize roots that are under ethylene control. Our findings are discussed in the light of knowledge about PCD processes in both other plant and non-plant systems.

Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, UK) or BDH/Merck Laboratory Supplies (Poole, Dorset, UK) and were of the highest available grade.

Plant materials and experimental treatments

Maize caryopses (*Zea mays* L. cv LG-20-80; Force Limagrain, Market Rasen, UK) were washed with running tap water for 2–3 h, and soaked overnight in aerated distilled water in a growth cabinet at 18 °C (16-h photoperiod). Imbibed caryopses were sown in dampened vermiculite medium for germination at 18 °C for 3 d. Seedlings with uniform root length (about 20 mm) were selected and marked 10 mm from the root tip with a spot application of charcoal slurry using a fine brush. Then, 7–10 seedlings were transferred to a stainless-steel mesh over 500 μM $Ca(NO_3)_2$ solution bubbled with either a gas stream of 3% oxygen in nitrogen gas (BOC, London, UK), 1 μl l^{-1} ethylene in 18–20% oxygen (BOC), or air (21% oxygen) at a rate of 150 ml min^{-1}. The experiment was repeated at least three times with three replicates per treatment. The oxygen concentration in the gas flow and in solution was measured using a membrane inlet quadrupole mass spectrometer (VG Micromass, Chester, UK).

Roots were marked 10 mm from the root tip every 24 h to establish the age of tissue and measurements taken to estimate the rate of elongation. Also every 24 h, two roots were selected at random from each treatment, and transverse sections made by hand at various distances from the root tip and observed under a light microscope to determine where aerenchyma was forming. Three days after the treatments root sections at 0.5 d (induction of aerenchyma), 1.5 d (developing aerenchyma) and 2.5 d (developed aerenchyma) were fixed for light and electron microscopy.

Preparation of specimens for light microscopy

Segments of root tissue (5 mm) were fixed in 5 ml formalin, 5 ml acetic acid, 90 ml 50% ethanol for 3 h and then dehydrated through a graded ethanol series (10, 20, 30, 50, 75, 100%, 20 min each step) and incubated overnight in 100% ethanol. The dehydrated tissue was then taken through a graded Microclear series (xylene substitute; TAAB) (25, 50, 75, 100% Microclear for 1 h at each step). The tissue was embedded in a paraffin wax series (25, 50, 75, 100%) in Microclear for 3 h at 59 °C. The specimens were finally placed in 100% Paraplast in slightly warmed containers in preparation for sectioning. Tissue sections (5 μm) were obtained using a Spencer 820 rotary microtome and collected onto APES (3-aminopropyltriethoxysilane)-coated slides.

Assay by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL)

Tissue sections prepared for light microscopy were de-waxed in Microclear for 3 min and hydrated through a graded ethanol series. The TUNEL assay was then carried out according to the manufacturer's (Boehringer, Lewes, UK) instructions, and nuclei were stained by incubating in 3% (w/v) propidium iodide (PI) for 2 min. To avoid interference due to the binding of PI to ribonucleic acids, samples were incubated in 0.5 mg ml^{-1} RNase 1 (DNase-free; Sigma, UK) for 10 min at 37 °C beforehand. Samples were observed with a Zeiss LSM 410 inverted confocal laser scanning microscope (CLSM) fitted with the following filter configuration: excitation/488 nm, emission/515 nm for FITC (fluorescein isothiocyanate) and excitation/543 nm, emission/570 nm for PI. The negative control was carried out without TdT enzyme and a positive control was carried out with DNase 1. The number of TUNEL-positive nuclei and PI-stained nuclei were quantified in epidermis, cortex and stele in the different ages: pre-aerenchyma (0.5 d old), developing aerenchyma (1.5 d old) and developed aerenchyma (2.5 d old) of the same root for statistical analysis.

Statistical analysis

Because of wide variations in the lengths of cells across the maize root (Wenzell and McCully 1991), the probability of sectioning through cells with a retained nucleus is not the same in all tissues or even in the cortex. Thus the absolute percentage of staining by

TUNEL is not a sound basis for judging the effects of treatments on DNA cleavage. Instead a statistical approach is required based on the proportion of nuclei visualised by PI that are also stained with the TUNEL reagent. The number of nuclei stained by TUNEL in epidermis, cortex, and stele were counted for three to five sections from three roots at three different ages (0.5 d, 1.5 d, 2.5 d) of root tissue. The percentage of TUNEL-positive nuclei was calculated with respect to the number of nuclei stained with PI in the same section. Analysis of variance (ANOVA) was carried out on Arcsin-transformed data using Statistica (Statsoft, Bedford UK). The Tukey test was used to determine the significance between treatments using the same package.

Isolation of DNA and analysis

After 3 d of treatment, genomic DNA was isolated from 0.5- to 1.5-d-old tissue where aerenchyma was forming. About 0.5–1.0 g of root portions for each treatment was frozen in liquid nitrogen immediately after being collected and ground with a mortar and pestle to a fine powder. Isolation of DNA was carried out using a DNeasy Plant Mini Kit (QIAGEN Crawley, UK) according to the manufacturer's instructions. To observe DNA fragmentation, samples (1.7 μg per lane) were run on a 0.5% agarose gel with 0.5 μg ml^{-1} (final concentration) ethidium bromide at constant 50 V using a *λAva*II molecular weight standard.

Transmission electron microscopy

Root sections (2 mm) were fixed in 1% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 6.9) for 2 h at room temperature (Karnovsky 1961). The segments were washed in the buffer, and post-fixed in 1% aqueous osmium tetroxide for 1 h, before being stained in 0.5% uranyl acetate at 4 °C overnight. Tissue sections were dehydrated in a graded ethanol series (30 min each) and then embedded through ethanol:Spurr resin mixtures (3:1, 1:1, 1:3) for 1 h each. They were finally embedded in 100% Spurr resin (Spurr 1969) and polymerised at 60 °C for 9 h in flat silicone rubber moulds. Ultra-thin sections (90 nm) were cut on a Reichert Ultracut E microtome (Leica, Milton Keynes, UK), collected onto Formvar-coated grids (200-mesh copper hexagonal mesh: Agar Scientific, Stansted, UK), stained in lead citrate (Reynolds 1963) and observed with a JEOL 1200 EXII transmission electron microscope.

Results

Roots grown in 3% oxygen and 1 μl l^{-1} ethylene elongated less than those grown in 21% oxygen (Fig. 1). Aerenchyma was not observed in 0.5-d-old tissue grown in 21% oxygen (10 mm from the root tip; Fig. 2A). However, developing aerenchyma was evident in the central cortex of roots grown in 3% oxygen (Fig. 2B) and throughout the cortex in roots grown in

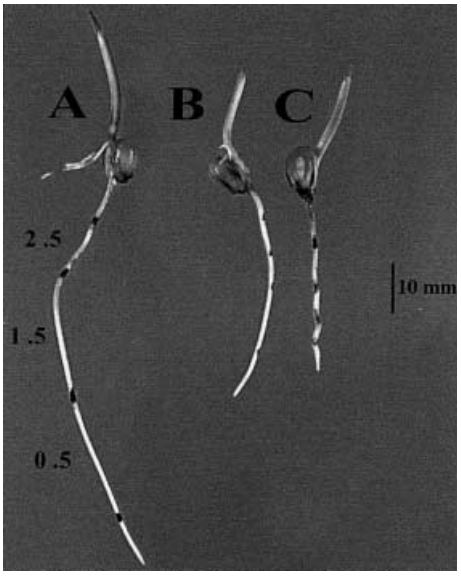

Fig. 1. Root growth. Three maize seedlings grown in 21% oxygen (*A*), 3% oxygen (*B*), or 1 μl l^{-1}ethylene (*C*). Charcoal marks applied daily are visible along the root axes. The numbers along the roots indicate the age of the root tissues (0.5 d, 1.5 d, 2.5 d)

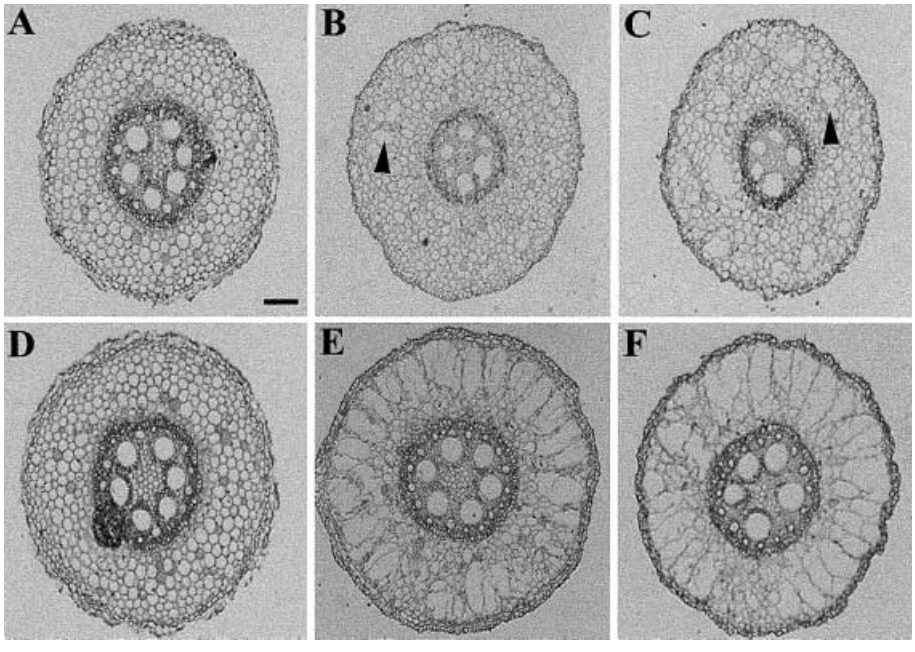

Fig. 2A–F. (**A**) Development of aerenchyma. Haematoxylin- and eosin-stained maize root sections showing development of aerenchyma (*arrowheads*) in 0.5-d-old tissue in 3% oxygen (**B**) or 1 μl l^{-1} ethylene (**C**) but not in 21% oxygen (**A**). Fully developed aerenchyma (2.5-d-old tissue) is shown in 3% oxygen (**E**), or 1 μl l^{-1} ethylene (**F**) but not in 21% oxygen (**D**). Bar = 100 μm

1 μl l^{-1} ethylene (Fig. 2C). Aerenchyma was also not present in roots grown in 21% oxygen (Fig. 2D) in 2.5-d-old tissue but was well developed by this time in 3% oxygen (in which it extended from two to three cells inside the endodermis to two to three cells inside the epidermis; Fig. 2E) or in ethylene (in which widespread disruption of the cortex was evident; Fig. 2F). It was shown that 0.5- to 2.5-d-old tissue encompassed all the stages of cell death leading to the formation of aerenchyma and this tissue was used in developmental order in subsequent experiments.

Root tissues at different stages of aerenchyma formation: pre-aerenchyma (0.5 d), developing aerenchyma (1.5 d) and developed aerenchyma (2.5 d) were stained using the TUNEL assay. Figure 3 shows the results of TUNEL labelling of tissue forming aerenchyma in 0.5-d-old tissue from roots which had received 3 d of treatment (Fig. 3A and D: 21% oxygen; B and E: 3% oxygen; C and F: 1 μl l^{-1} ethylene). Control roots (21% oxygen) showed a few TUNEL-positive nuclei in the epidermis but not the cortex or stele (Fig. 3A). By contrast in 3% oxygen (Fig. 3B) or 1 μl l^{-1} ethylene (Fig. 3C), the nuclei of cells of the cortex were TUNEL positive as well as a few nuclei in the epidermis and stele. The TUNEL-positive nuclei were distributed throughout the cortex. All nuclei in the treatments were also stained with propidium iodide (PI; Fig. 3D–F) and use of co-localisation software facilitated accurate estimation of the percentage of nuclei that were TUNEL positive for each treatment (Table 1). As a negative control, the TUNEL assay was performed without TdT enzyme and no DNA labelling was observed (Fig. 3G) although nuclei readily stained with PI (Fig. 3I). A positive control, carried out with DNase 1, which induces DNA breaks, resulted in all PI-stained nuclei being TUNEL positive (Fig. 3H,J). Similar results were obtained with 1.5- and 2.5-d-old tissue (data not shown).

A statistical analysis of the percentage of TUNEL-positive nuclei in the epidermis, cortex and stele was carried out for three ages of tissue (0.5, 1.5 and 2.5 d). There were no significant differences between the percentage of TUNEL-positive nuclei in the cortex at each age of tissue (though the total number of nuclei and of TUNEL-positive nuclei declined with increasing age as cells died to form aerenchyma). There were significant differences ($P < 0.01$) between the percentage of TUNEL-positive nuclei in 21% oxygen vs. 3% oxygen and 21% oxygen vs. 1 μl l^{-1} ethylene in the cortex. No significant differences ($P > 0.01$) were observed between 3% oxygen and 1 μl l^{-1} ethylene in the cortex; however, significantly ($P < 0.05$) more nuclei were TUNEL positive in the epidermis of tissue treated with 3% oxygen than in 21% oxygen or ethylene although absolute numbers were small (Table 1).

After 3 d of treatment, DNA was isolated from 0.5- to 1.5-d-old root tissue and separated by agarose gel electrophoresis. Degradation of DNA was not observed in roots treated with 21% or 3% oxygen (Fig. 4, lanes 1, 2). Ethylene treatment induced faint laddering which was evident when the agarose gel for ethylene-treated tissue was viewed at high contrast (Fig. 4, compare lanes 3, 6), but not in control or hypoxic tissue, even when viewed in the same way (Fig. 4 lanes 4, 5).

Accurate preservation of ultrastructure in tissue undergoing cell death without introducing fixation or other artefacts is difficult and requires careful consideration of controls. The root material was fixed rapidly (after removal from the plant) in 1% glutaraldehyde and 2% paraformaldehyde and embedded in Spurr's resin. Careful consideration was given to assessing the quality of fixation; in particular, the presence of cortical cells showing normal ultrastructure without plasmolysis or membrane damage was observed, both in control roots (Fig. 5A: 21% oxygen) and frequently close to areas of cell death in treated roots (Fig. 5A–C; 21% oxygen, 4% oxygen, 1 μl l^{-1} ethylene, respectively). Experiments (including fixation and embedding) were replicated three times on different occasions and several roots examined in each case to ensure reproducibility.

Sections were taken from the same root at different ages of tissue (0.5 to 2.5 d) treated with 21% oxygen (control), 3% oxygen or 1 μl l^{-1} ethylene. These were observed sequentially by TEM after lead citrate staining. Stages of aerenchyma formation were identified in order of distance from the root tip and assigned tissue age based on growth analysis. Tissues of like age (rather than distance from the root tip) were therefore compared in each experiment. The sequence presented here is representative of observations made on three to four roots in each treatment from three experiments with hypoxia and two for ethylene treatment. Results obtained from roots treated with 3% oxygen or 1 μl l^{-1} ethylene appeared identical.

The first sign of cell death was observed in the mid cortex of 0.5-d-old tissue treated with 3% oxygen or 1 μl l^{-1} ethylene. In these cells, plasma membrane invagination (Fig. 5D,E, 3% oxygen; Fig. 5F, 1 μl l^{-1} ethylene) was apparent, with abundant vesicles present near the plasma membrane (Fig. 5G, 3% oxygen; H, 1 μl l^{-1} ethylene). The plasma membrane had retracted from the cell wall and the cytoplasm appeared more electron-opaque (Fig. 5C) than in normal cells and vacuoles were filled with granular material (Fig. 5I). The tonoplast was heavily stained (Fig. 5J, 3% oxygen; K, 1 μl l^{-1} ethylene). Cortical cells of control roots (21% oxygen) showed normal ultrastructure with intact cell walls and cytoplasm (Fig. 5L).

Older (1–1.5 d) tissue from the mid cortex of roots that had been treated with 3% oxygen or 1 μl l^{-1} ethylene showed nuclear condensation with chromatin accumulating at the nuclear periphery. Chromatin condensation was not observed in sections from roots treated with 21% oxygen (Fig. 6A). Membrane shrinkage was evident in cells treated with ethylene in which chromatin condensation had not occurred (Fig. 6B); condensation had commenced in 1-d tissue from roots treated with ethylene (Fig. 6C) or 3% oxygen (data not shown). At this stage, the Golgi apparatus appeared intact but with many secretory vesicles (Fig. 6D). Mitochondria were frequently crescent shaped with some evidence of membrane loss (Fig. 6E, 1 μl l^{-1} ethylene; Fig. 6F, 3% oxygen).

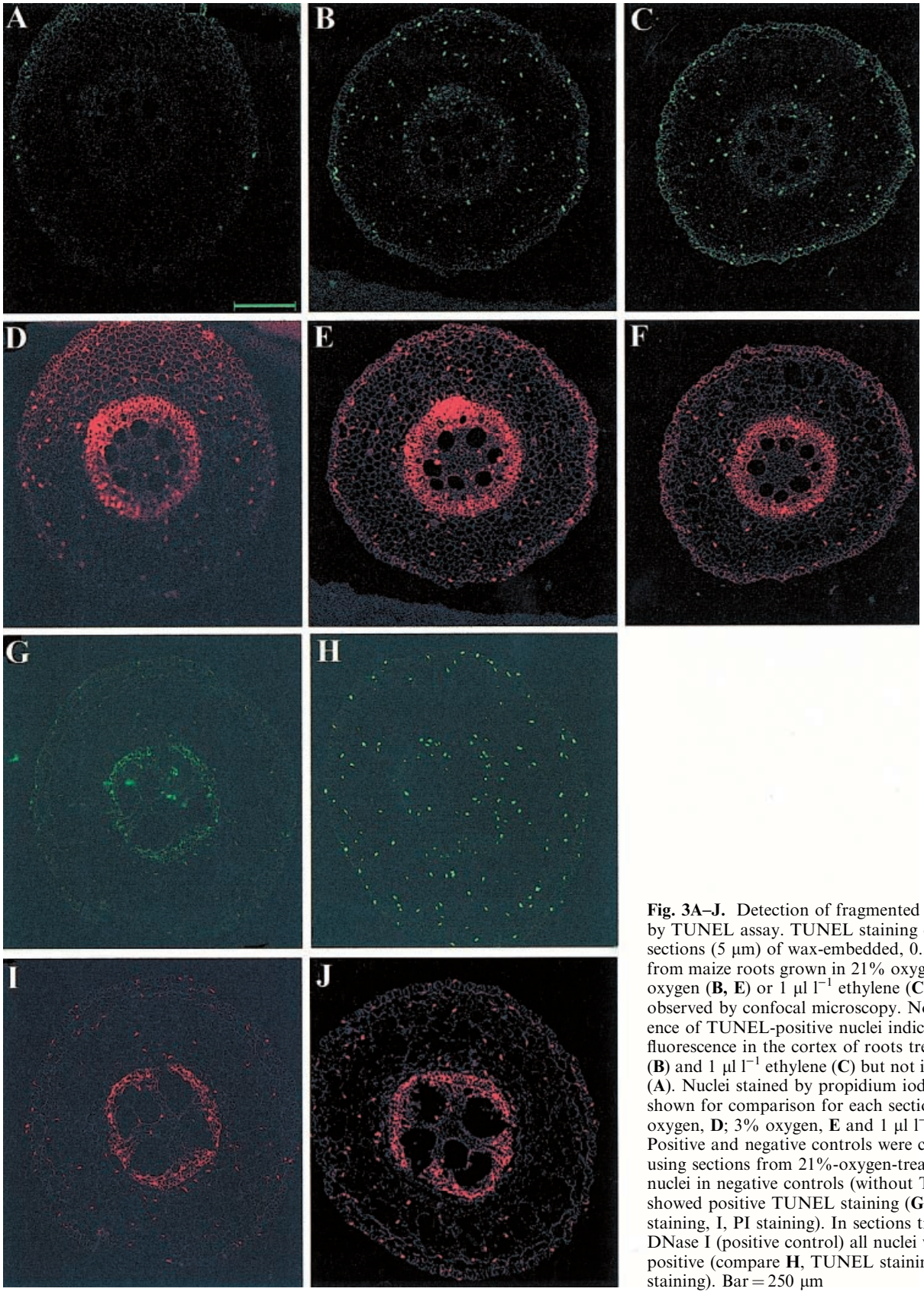

Fig. 3A–J. Detection of fragmented nuclear DNA by TUNEL assay. TUNEL staining of transverse sections (5 μm) of wax-embedded, 0.5-d-old tissue from maize roots grown in 21% oxygen (**A, D**), 3% oxygen (**B, E**) or 1 μl l^{-1} ethylene (**C, F**) was observed by confocal microscopy. Note the presence of TUNEL-positive nuclei indicated by green fluorescence in the cortex of roots treated with 3% (**B**) and 1 μl l^{-1} ethylene (**C**) but not in 21% oxygen (**A**). Nuclei stained by propidium iodide (PI) are shown for comparison for each section (21% oxygen, **D**; 3% oxygen, **E** and 1 μl l^{-1} ethylene, **F**). Positive and negative controls were carried out using sections from 21%-oxygen-treated tissue No nuclei in negative controls (without TdT enzyme) showed positive TUNEL staining (**G**, TUNEL staining, I, PI staining). In sections treated with DNase I (positive control) all nuclei were TUNEL-positive (compare **H**, TUNEL staining, with **J**, PI staining). Bar = 250 μm

In sections from 1.5- to 2-d tissue from roots treated with 1 μl l^{-1} ethylene (Fig. 7A) or 3% oxygen (Fig. 7B,C) mitochondria (Fig. 7A,B), Golgi apparatus (Fig. 7C) and other parts of the cytoplasm or nucleoplasm (Fig. 7A–C) were observed surrounded by membrane. Control (21% oxygen) cells retained a normal

Table 1. Comparison of proportion of TUNEL-positive nuclei in epidermis, cortex and stele for maize roots grown in 3% and 21% oxygen or 1 μl l^{-1} ethylene. Tissue sections were stained with TUNEL reagent and propidium iodide (total nuclei) and observed by confocal microscope. The TUNEL-positive nuclei and total number of nuclei were counted for at least three sections from three roots at three different ages (0.5 d, 1.5 d, 2.5 d) and the percentage of TUNEL-positive nuclei is presented for each treatment. Arcsin-transformed data were analysed by analysis of variance using Statistica and the Tukey test applied to determine significance between treatments (Statsoft, UK)

Treatment	TUNEL-stained nuclei (%)		
	Epidermis	Cortex	Stele
21% O_2	33 ± 6.2	13 ± 2.3	5 ± 2.3
3% O_2	71 ± 3.4	84.5 ± 5.1	5 ± 2.2
Ethylene	59 ± 5.0	83 ± 3.1	17 ± 2.8

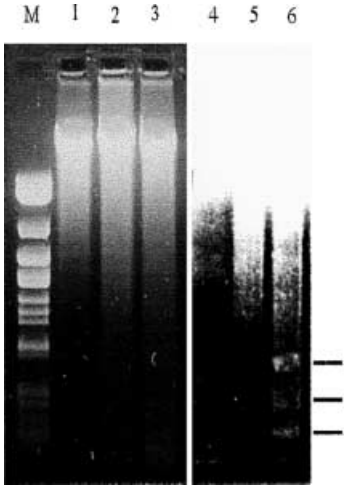

Fig. 4. Fragmentation of nuclear DNA in maize roots during aerenchyma formation. Genomic DNA isolated from maize roots grown in 21% oxygen (*lanes 1*, *4*), 3% oxygen (*lanes 2*, *5*) and 1 μl l^{-1} ethylene (*lanes 3*, *6*) was loaded onto a 0.5% (w/v) agarose gel, stained with ethidium bromide and separated electrophoretically. *M*, molecular weight markers (100 bp – 12 kb λDNA digested with λ*Ava*II). *Lanes 1–3* were scanned digitally and are reproduced at high contrast (*lanes 4–6*). Note the presence of laddering in tissue treated with 1 μl l^{-1} ethylene (*lane* 6) which is not evident in tissue treated with either 21% or 3% oxygen (*lanes 4*, *5*)

ultrastructure (Figs. 7D,E). The cell wall remained intact. After this stage (>2.5 d), cell wall degradation and the formation of air spaces occurred.

Discussion

The term 'programmed cell death' (PCD) describes the ordered and genetically programmed death and lysis of cells. It is distinguished from necrosis by the orderly nature of events that occur. However, the presence of several forms of PCD makes identifying key characteristics difficult. As aerenchyma formation occurs in cortical tissue undergoing oxygen deficiency, it might be assumed that necrosis was taking place, as necrotic cell death is frequently initiated by anoxia through oxygen starvation and cytoplasmic acidosis (Vartapetian and Jackson 1997). However, cortical cell death associated with spatially specific aerenchyma formation occurs by identical processes in maize roots treated with ethylene or grown in hypoxic conditions. In the latter, endogenous ethylene production is promoted (Jackson et al. 1985). This indicates that the metabolic consequences of hypoxia are not major factors in cortical cell death and suggests the initiation of a cell death pathway (Brailsford et al. 1993; He et al. 1996; Pennell and Lamb 1997; Kawai et al. 1998). Data obtained in this study confirmed this. The time course of aerenchyma formation was similar in roots treated with 3% oxygen and with 1 μl l^{-1} ethylene, the first signs of aerenchyma formation being observed in 0.5-d-old tissue in roots treated for 3 d (Figs. 1, 2) in both cases. Cell changes occurring in ethylene or hypoxic roots were indistinguishable (see below).

Internucleosomal DNA fragmentation frequently occurs in PCD of animal cells. It may precede other changes (in apoptosis) or follow them (e.g. in cytoplasmic cell death). The TUNEL procedure may be used to detect such DNA fragmentation. In this study, it was used to establish the timing and location of DNA degradation. Although some TUNEL-positive nuclei were first detected in 0.5-d tissue in the cortex, epidermis and stele, with only cortical cells showing a significantly ($P < 0.01$) higher proportion of TUNEL-stained nuclei in 3% oxygen or 1 μl l^{-1}ethylene-treated roots compared to normoxic controls. It was noticeable that significantly ($P < 0.05$) more TUNEL-positive nuclei were present in epidermal cells in tissues treated with 3% oxygen compared with controls (21% oxygen) but this was not the case for ethylene-treated tissue. This small, but statistically significant promotion by 3% oxygen in tissues external to the stele and not developing aerenchyma (including the outer cortex) may result from other causes connected with hypoxic metabolism rather than hormonal activation of PCD.

Great caution is necessary in the interpretation of the results of TUNEL staining as TUNEL positives can result from the detection of DNA damage in necrotic cells as well as in PCD. In addition to negative and positive controls (see *Results*), detection of DNA ladders on agarose gels has been used to indicate oligonucleosomal cleavage (e.g. Ryerson and Heath 1996; Yen and Yang 1998; Wang et al. 1999), though such 'ladders' are not always detectable, particularly in heterogeneous tissue in which asynchronous initiation of cell death may have occurred (Wang et al. 1996b). When DNA was extracted from ethylene-treated root tissue and separated by agarose gel, faint DNA laddering was detectable; although this was not observed in DNA extracts from tissue treated with 3% oxygen or controls (Fig. 4). The small amounts of DNA available or the presence of necrotic cells and asynchronous initiation of cell death are likely to have led to loss of clear laddering. It is possible that ethylene treatment results in greater synchrony of cell death than hypoxia, and generates fewer necrotic nuclei (e.g. in the epidermis, see above), thereby giving the faintly detectable ladder.

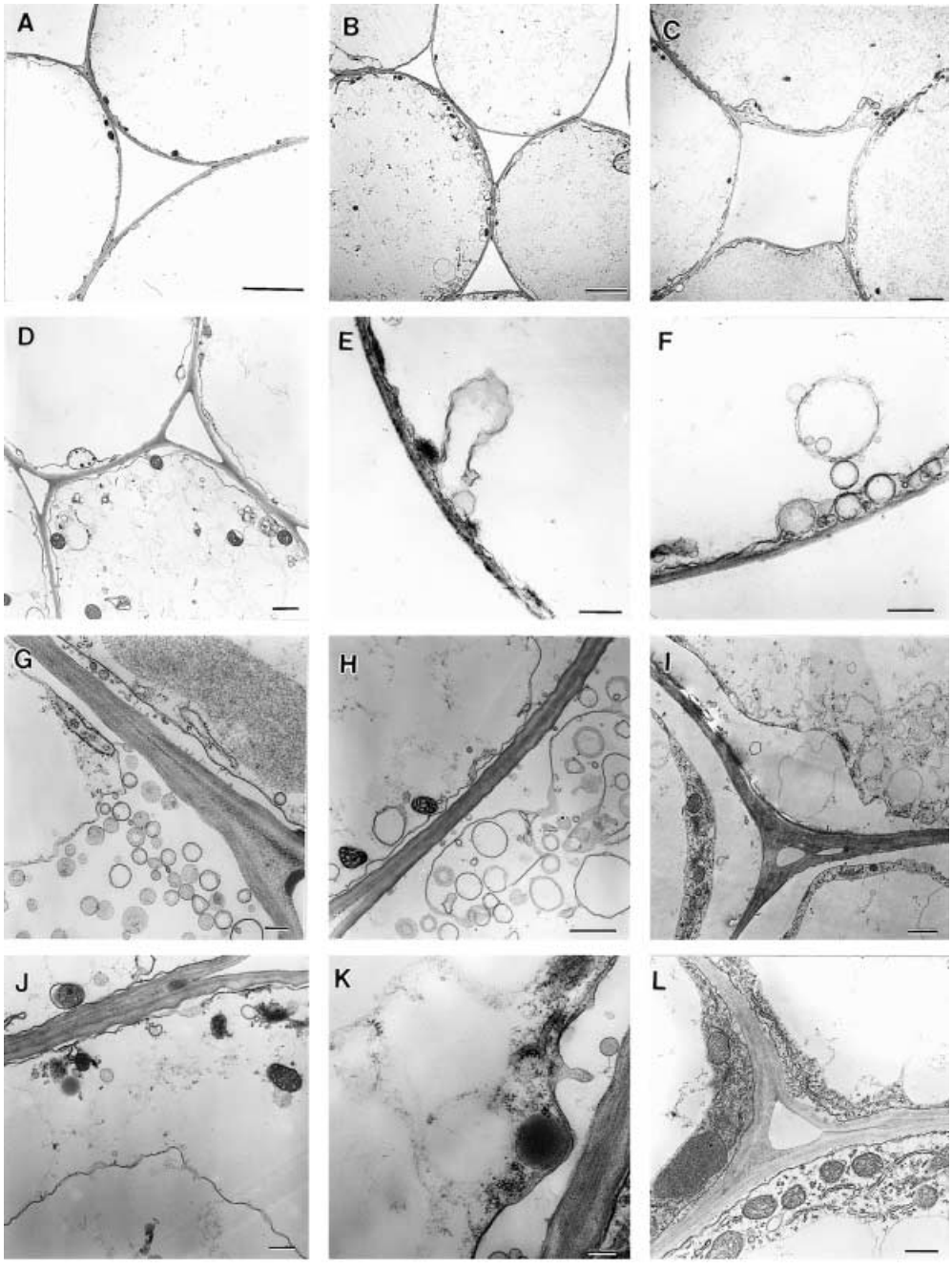

Fig. 5A–L. Ultrastructure of tissue from 0.5- to 1.0-d-old maize root tissue. Tissue from roots treated with 21% oxygen, 3% oxygen or 1 μl l^{-1} ethylene was fixed, embedded in Spurr's resin and sectioned before staining with lead citrate. Low-magnification images of tissue indicate the preservation of normal ultrastructure in 21% oxygen (**A**) and in some cortical cells adjacent to others beginning the cell death process in 3% oxygen (**B**) or 1 μl l^{-1} ethylene (**C**) respectively. Higher-magnification images record the earliest detectable events of cell death in both 3% oxygen (**D, E, G, J**) and 1 μl l^{-1} ethylene (**F, H, I, K**). Plasma membrane invagination and presence of vesicles between the plasma membrane and cell wall (**D, E, G, H**) were noted for both treatments, as was granular staining in the vacuole and a heavily stained tonoplast (**I, J, K**). Control (21% oxygen) sections showed normal ultrastructure (**L**). Bars = 5 μm (**A–C**), 1 μm (**D–F, H, I**), 200 nm (**G, K**), 500 nm (**J, L**)

Ultrastructural analysis provides an alternative approach to the validation and interpretation of TUNEL data, as it permits events in individual cells and tissues to be studied; indeed ultrastructural studies provide the main basis for the identification of apoptosis (as opposed to other forms of PCD) in animal cell systems (Zakeri et al. 1995). Such studies permit events in the nucleus to be described in relation to events in the cytoplasm. This has not previously been carried out for aerenchyma formation. Electron microscopy revealed chromatin condensation and its redistribution to the periphery of the nucleus in cells of the mid cortex at an early stage (1.0 d) of both ethylene and 3% oxygen treatment, though not in 21% oxygen controls, commensurate with the results of TUNEL staining. Chromatin condensation to the nuclear periphery is a characteristic of apoptosis in animal cells.

The first detectable events in the cells of the cortex, both with ethylene and 3% oxygen treatment, were not, however, in the nucleus but in the cytoplasm. In the earliest stage of aerenchyma formation (0.5 d; Fig. 5), plasma membrane invagination, a more electron-opaque cytoplasm and shrinkage of the plasma membrane from the cell wall were observed in cells not showing DNA condensation. Soon after, the presence of granular staining of the vacuolar contents could be seen, together with organelles surrounded by membrane in the vacuole. By (0.5–1 d), numerous vesicles beneath the plasma membrane were apparent; however, their origins were not clear. Campbell and Drew (1983) also reported such

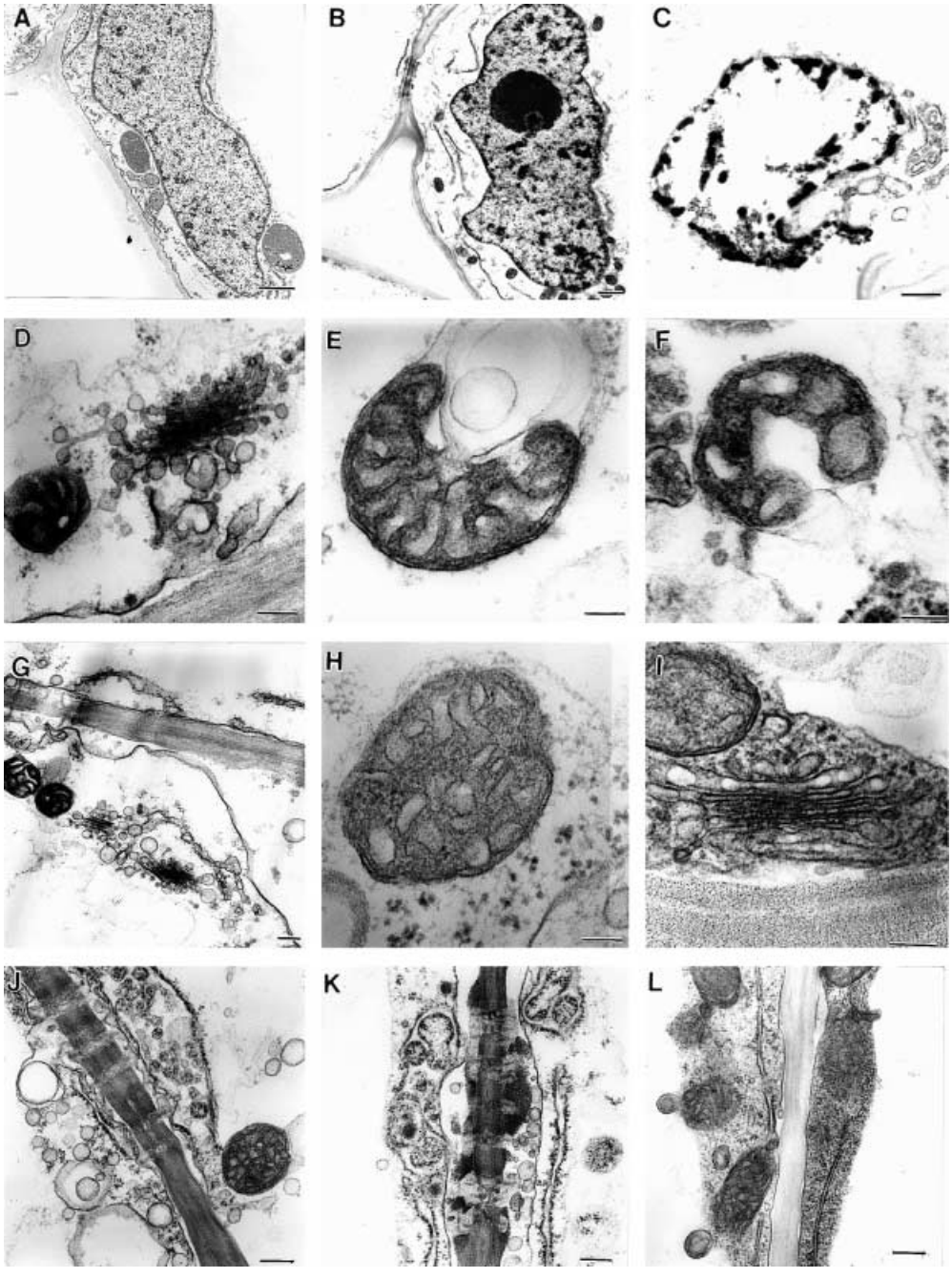

Fig. 6A–L. Ultrastructure of 1.0- to 2.0-d-old maize root tissue. Tissue (1.0–2.0 d) from roots treated with 21% oxygen, 3% oxygen or 1 μl l^{-1} ethylene was fixed, embedded and sectioned as described for Fig. 5. Ultrastructural changes were similar in sections of tissue treated with 3% oxygen (**F, K**) or ethylene (**B–E, G, J**), while controls (21% oxygen) retained a normal ultrastructure (**A, H, L**). The presence of chromatin condensation was not evident in controls (**A**); membrane shrinkage without chromatin condensation was observed (**B**) prior to chromatin condensation (**F**). The ultrastructure of the Golgi apparatus (**D, G**) and mitochondria (**E–G**) from the treatments is compared with that from controls (21% oxygen; **H, I, L**). Plasmodesmata were evident in treated tissue (**J–K**). Bar = 1 μm (**A, B**), 100 nm (**E, F, H, I**), 200 nm (**D, G, K, J, K, L**), 500 nm (**C**)

vesicles that they believed expanded and fused with the vacuole. Chromatin condensation was first observed at the end of this stage (1.0 d; Fig. 6), in cells in which cytoplasmic changes were already evident. This is in contrast to the findings of Schussler and Longstreth (2000) who identified nuclear condensation and fragmentation as the earliest events in *Sagittaria lancifolia*. In pathogen-induced PCD in tobacco (Mittler et al. 1997) and in some instances of PCD in animals (Jacobson et al. 1994; Earnshaw 1995), cytoplasmic changes have been observed prior to chromatin condensation or as simultaneous events (e.g. Wyllie 1981). It therefore appears that nuclear condensation detectable by electron microscopy follows (rather than precedes) at least some of the cytoplasmic changes noted. However, it is possible that cleavage of DNA occurs at a very early stage of aerenchyma formation, preceding changes detectable ultrastructurally, as TUNEL-positive cells were present in 0.5-d-old tissue (Fig. 3).

Further changes in the cytoplasm followed detection of condensed chromatin. Cytoplasmic changes at this stage included: retention of organelles (though mitochondria in 3%-oxygen-treated tissue appeared structurally compact (though with some membrane damage), resembling those observed in animal cells undergoing apoptosis (Wyllie 1981). By 1.5 d, plasmodesmata between cortical cells showing cell death became very prominent in both 3% oxygen and in ethylene-treated roots, having been indistinct in control sections (Fig. 6 and data not shown). A breakdown of desmosomal

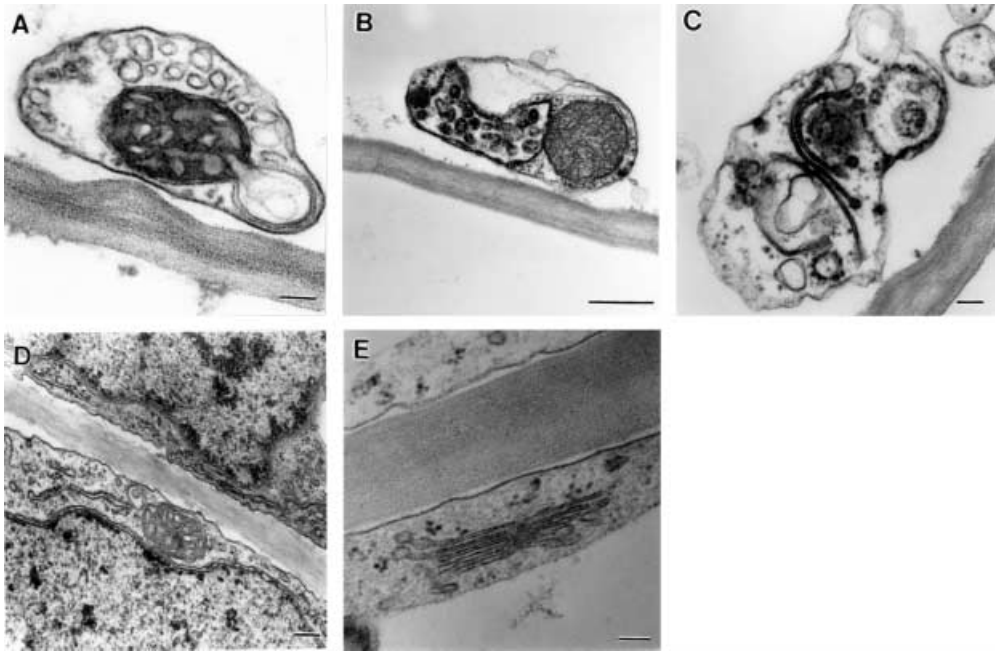

Fig. 7A–E. Ultrastructure of 1.5- to 2.0-d old maize root tissue. Tissue (1.5–2.0 d) from roots treated with 21% oxygen, 3% oxygen or 1 μl l^{-1} ethylene was fixed, embedded and sectioned as described for Fig. 5. Membrane-bounded bodies containing cellular organelles and other material were present in treated tissue (1 μl l^{-1} ethylene, **A**; 3% oxygen, **B, C**) but not controls (21% oxygen, **D, E**). Note the presence of intact mitochondria (**A, B**), Golgi apparatus (**C**) and other inclusions (**A–C**) and the retention of an apparently intact cell wall (**A–C**). Bars = 100 nm (**A, C, E**), 200 nm (**D**), 500 nm (**B**)

contacts between adjacent cells immediately after nuclear condensation is commonly seen in apoptotic animal cells.

Later, in 1.5- to 2-d tissue, membrane-bound bodies containing intact organelles were evident. These bodies enclosed intact mitochondria (Fig. 6) and Golgi bodies and other inclusions (Fig. 6). In animal cells, similar structures, termed apoptotic bodies, occur which may contain only cytoplasmic material (including organelles) or portions of fragmented nuclei and cytoplasmic elements (Russell et al. 1972) including intact mitochondria (Wyllie 1981). We observed different sizes of membrane-bound bodies, containing a variety of material (Fig. 7). In the only previous description of such structures in plants, Wang et al. (1996a) reported apoptotic-like bodies during sloughing of root cap cells, some of which were TUNEL positive. They have not previously been described at the electron-microscope level in plants and were not described by Schussler and Longstreth (2000) in their study of *Sagittaria lancifolia* and other species, or by Campbell and Drew (1983) in their study of maize. Their formation was induced by both 3% oxygen and by ethylene treatment, suggesting that this is an integral part of PCD in aerenchyma formation. However, given the retention of the cell wall at this stage, their function is unlikely to be analagous to apoptotic bodies in animals. They may, however, be involved in the hydrolysis of organelles and other material prior to its absorption by surrounding tissue.

In summary, cell death in aerenchyma formation in maize roots, inducible by low oxygen and ethylene, occurs in a series of events which may conveniently be divided into four stages. Stage 1 (in the mid cortex of 0.5-d tissue) involves cytoplasmic events, including plasma membrane invagination, formation of small vesicles, heavy staining of tonoplast and electron-opaque cytoplasm and vacuole. Stage 2 (1- to 1.5-d tissue) involves nuclear events, including chromatin condensation evident at the electron-microscope level and retention of organelles in the cytoplasm. In stage 3 (usually 1.5- to 2-d tissue) intact organelles remain and membrane-bounded structures. The cell wall remains intact. Stage 4 (usually >2.0-d tissue) involves cell wall degradation and formation of gas space. While TUNEL staining and agarose gels suggest that DNA cleavage may commence in stage 1, ultrastructural changes in the nucleus are not evident until later.

Much interest is being shown in PCD in plants and inevitably, comparisons with apoptosis are being made. Sensu strictu, ''Apoptosis'' refers to the morphology of the dying or dead cell, which can only be determined by electron microscopy (Zakeri et al. 1995). It is evident that there are marked differences in the later stages of PCD between plants and animals, as the presence of a cell wall prevents macrophage digestion at the closing stages of the process. However, many other parallels exist between the two processes. In classic apoptosis, in animals, the early morphological changes occur in the nucleus, the cell shrinks or fragments but relatively impermeable membranes remain which surround cells and their organelles until late in the process. In maize aerenchyma formation, chromatin condensation to the nuclear periphery suggests an apoptotic process. Cytoplasmic changes in maize, as in animal cells, appear to end with the inclusion of relatively intact organelles and cytoplasm surrounded by membrane structures that may be analogous to apoptotic bodies. However, the data presented here suggest the early events of aerenchyma formation may show some significant differences from apoptosis, as membrane changes are detectable at an early stage, preceding changes to nuclear ultrastructure. Membrane changes at an early stage are characteristic of a second form of PCD in animals, known as cytoplasmic cell death (CCD). In this process, nuclear changes do not occur until vesiculation and the formation of autophagic vacuoles has taken place. However, by contrast, in CCD

organelles are frequently degraded in specific sequence, prior to the condensation of chromatin. Our findings therefore indicate that aerenchyma formation is a unique form of PCD, in part resembling apoptosis and in part resembling CCD.

We acknowledge the support of the Hyam Wingate Foundation and the Royal Society for partial support for the work. A.H.L.A.N.G.was a Presidential Scholar of the Government of Sri Lanka. We thank Prof. R.O. Thattil (University of Peradeniya, Sri Lanka) for advice on data analysis. We are also grateful to Dr. Federica Brandizzi (Oxford Brookes University) for advice and discussion.

References

Armstrong W (1979) Aeration in higher plants. Adv Bot Res 7: 225–332

Armstrong J, Armstrong W (1994) Chlorophyll development in mature lysigenous and schizogenous root aerenchymas provides evidence of continuing cortical cell viability. New Phytol 126: 493–497

Barlow PW (1982) Cell death – an integral part of plant development. In: Jackson MB, Grout B, Mackenzie IA (eds) Growth regulators in plant senescence. British Plant Growth Regulator Group, Wantage, pp 27–45

Brailsford RW, Voesenek LACJ, Blom CWPM, Smith AR, Hall MA, Jackson MB (1993) Enhanced ethylene production by primary roots of *Zea mays* L. in response to sub-ambient partial pressures of oxygen. Plant Cell Environ 16: 1071–1080

Buckner B, Janick-Buckner D, Gray J, Johal GS (1998) Cell-death mechanisms in maize. Trends Plant Sci 3: 218–223

Bursch W, Kleine L, Tenniswood M (1990) The biochemistry of cell-death by apoptosis. Biochem Cell Biol 68: 1071–1074

Campbell R, Drew MC (1983) Electron-microscopy of gas space (aerenchyma) formation in adventitious roots of *Zea mays* L. subjected to oxygen shortage. Planta 157: 350–357

Drew MC (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. Annu Rev Plant Physiol Plant Mol Biol 48: 223–250

Drew MC, Jackson MB, Giffard S (1979) Ethylene promoted adventitious rooting and development of cortical air spaces in *Zea mays* L. Planta 147: 83–88

Drew MC, Chuan-Jui H, Morgan PW (2000) Programmed cell death and aerenchyma formation in roots. Trends Plant Sci 5: 123–127

Earnshaw WC (1995) Apoptosis – lessons from in-vitro systems. Trends Cell Biol 5: 217–220

He CJ, Morgan PW, Drew MC (1996) Transduction of an ethylene signal is required for cell death and lysis in the root cortex of maize during aerenchyma formation induced by hypoxia. Plant Physiol 112: 463–472

Jackson MB, Fenning TM, Drew MC, Saker LR (1985) Stimulation of ethylene production and gas-space (aerenchyma) formation in adventitious roots of *Zea mays* L by small partial pressures of oxygen. Planta 165: 486–492

Jacobson MD, Burne IF, Raff MC (1994) Programmed cell-death and bcl-2 protection in the absence of a nucleus. EMBO J 13: 1899–1910

Karnovsky MJ (1961) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 27: 137A

Kawai M, Samarajeewa PK, Barrero RA, Nishiguchi M, Uchimiya H (1998) Cellular dissection of the degradation pattern of cortical cell death during aerenchyma formation of rice roots. Planta 204: 277–287

Mittler R, Lam E (1995) Identification, characterization, and purification of a tobacco endonuclease activity induced upon hypersensitive response cell-death. Plant Cell 7: 1951–1962

Mittler R, Simon L, Lam E (1997) Pathogen-induced programmed cell death in tobacco. J Cell Sci 110: 1333–1344

Pennell RI, Lamb C (1997) Programmed cell death in plants. Plant Cell 9: 1157–1168

Reynolds ES (1963) The use of lead citrate at a high pH as an electron opaque stain in electron microscopy. J Cell Biol 17: 208–212

Russell SW, Rosenau W, Lee JC (1972) Cytosis induced by human lymphotoxin. Am J Pathol 69: 103–118

Ryerson DE, Heath MC (1996) Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. Plant Cell 8: 393–402

Saab IN, Sachs MM (1996) A flooding-induced xyloglucan endotransglycosylase homolog in maize is responsive to ethylene and associated with aerenchyma. Plant Physiol 112: 385–391

Schussler EE, Longstreth DJ (2000) Changes in cell structure during the formation of root aerenchyma in *Sagittaria lancifolia* (Alismataceae). Am J Bot 87: 12–19

Spurr AR (1969) A low viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 49: 31–43

Vartapetian BB, Jackson MB (1997) Plant adaptations to anaerobic stress. Ann Bot 79s: 3–20

Wang H, Li J, Bostock RM, Gilchrist DG (1996a) Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. Plant Cell 8: 375–391

Wang M, Oppedijk BJ, Lu X, Van Duijn B, Schilperoort RA (1996b) Apoptosis in barley aleurone during germination and its inhibition by abscisic acid. Plant Mol Biol 32: 1125–1134

Wang M, Oppedijk BJ, Caspers MPM, Lamers GEM, Boot MJ, Geerlings DNG, Bakhuizen B, Meijer AH, Van Duijn B (1998) Spatial and temporal regulation of DNA fragmentation in the aleurone of germinating barley. J Exp Bot 49: 1293–1301

Wang M, Hoekstra S, van Bergen S, Lamers GEM, Oppedijk BJ, van der Heijden MW, de Priester W, Schilperoort RA (1999) Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum vulgare* L. Plant Mol Biol 39: 489–501

Wenzell CL, McCully ME (1991) Early senescence of cortical cells in the roots of cereals. How good is the evidence? Am J Bot 78: 1528–1541

Wyllie AH (1981) Cell death: a new classification separating apoptosis from necrosis. In: Bowen ID, Lockshin RA (eds) Cell death in biology and pathology. Chapman and Hall, London, pp 9–29

Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68: 251–306

Wyllie AH, Morris RG, Smith AL, Dunlop D (1984) Chromatin cleavage in apoptosis – association with condensed chromatin morphology and dependence on macromolecular-synthesis. J Pathol 142: 67–77

Yen CH, Yang CH (1998) Evidence for programmed cell death during leaf senescence in plants. Plant Cell Physiol 39: 922–927

Zakeri Z, Bursch W, Tenniswood M, Lockshin RA (1995) Cell-death – programmed, apoptosis, necrosis, or other. Cell Death Differ 2: 87–96