Effect of Different Gravity Environments on DNA Fragmentation and Cell Death in *Kalanchoë* Leaves

M. C. PEDROSO*† and D. J. DURZAN‡

*Dept. Biologia Vegetal, Faculdade de Ciências de Lisboa, Ed. C2, Piso 1, Campo Grande, 1749-016 Lisboa, Portugal and ‡Dept Environmental Horticulture, University of California, Davis, CA 95616-8587, USA

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Different gravity environments have been shown to significantly affect leaf-plantlet formation and asexual reproduction in *Kalanchoë daigremontiana* Ham. and Perr. In the present work, we investigated the effect of gravity at tissue and cell levels. Leaves and leaf-plantlets were cultured for different periods of time (min to 15 d) in different levels of gravity stimulation: simulated hypogravity (1 rpm clinostats; 2 × 10⁻³ g), 1 g (control) and hypergravity (centrifugation; 20 and 150 g). Both simulated hypogravity and hypergravity affected cell death (apoptosis) in this species, and variations in the number of cells showing DNA fragmentation directly correlated with nitric oxide (NO) formation. Apoptosis in leaves was more common as gravity increased. Apoptotic cells were localized in the epidermis, mainly guard cells, in leaf parenchyma, and in tracheary elements undergoing terminal differentiation. Exposures to acute hypergravity (up to 60 min) showed that chloroplast DNA fragmentation occurred prior to nuclear DNA fragmentation, marginalization of chromatin, nuclear condensation, and nuclear blebbing. Addition of sodium nitroprusside (NO donor) mimicked centrifugation. NO and DNA fragmentation decreased with NG-monomethyl-L-arginine (NO-synthase inhibitor). The variations in NO levels, nucleoid DNA fragmentation, and cell death show how chloroplasts, cells and leaves may respond (and adapt) to gravity changes.

Key words: Apoptosis, chloroplasts, gravitational biology, *Kalanchoë daigremontiana*, mechanical stress, Mother of Thousands, nitric oxide, programmed cell death, TUNEL.

INTRODUCTION

Traumatic (‘accidental’) cell death, often called necrosis, is a non-physiological process caused by overwhelming levels of stresses (e.g. toxins, heat or cold), which involves disruption of membrane integrity and subsequent cellular swelling and lysis (for a review see Schwartzman and Cidlowski, 1993; Gray and Johal, 1998). Programmed cell death (PCD) is a physiological cell death process, dynamically regulated by both developmental and environmental cues, involving the selective elimination of unwanted cells (for a review see Ellis et al., 1991; Havel and Durzan, 1996b; Jacobsen et al., 1997; Pennel and Lamb, 1997; Gilchrist, 1998; Gray and Johal, 1998; Yeng and Yang, 1998). When PCD involves the marginalization of chromatin in the nucleus, nuclear condensation, shrinkage of the cytoplasm, cleavage of nuclear DNA into oligonucleosomes (Ryerson and Heath, 1996), and the breakage of the cytoplasm or nucleus into small, sealed packets, the process is termed apoptosis, both in animals (Kerr et al., 1972) and plants (Havel and Durzan, 1996a; Wang et al., 1996; Yeng and Yang, 1998). The *in situ* detection of DNA fragmentation leading to cell death can be achieved by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling of DNA 3'-OH groups (TUNEL method) (Gavrieli et al., 1992; Wang et al., 1996).

In plants, the continuous generation of organ systems by the plant’s meristems is countered by the programmed senescence and/or abscission (shedding) of existing organs throughout the life of the plant (Bleecker and Patterson, 1997). Senescence involves cell death on a large scale, nutrient recycling and is often interrelated with reproduction (for reviews see Resende, 1951; Barlow, 1982; Bleecker and Patterson, 1997; Pennel and Lamb, 1997; Gray and Johal, 1998; Noodén, 1988). Recent reports have shown that the process of senescence exhibits features of PCD by apoptosis (in Gray and Johal, 1998; Yeng and Yang, 1998).

*Kalanchoë daigremontiana* Ham and Perr. (Crassulaceae), commonly known as Mother of Thousands, reproduces asexually by forming plantlets in leaf indentations (Warden, 1970a, b). In nature, its entire plant body except leaf-plantlets senesces as a consequence of floral differentiation (Resende, 1951). The leaf-plantlets fall to earth and convert into adult plants. At unit gravity, in nature and *in vitro*, PCD precedes plantlet detachment from the mother-leaf, leading to an abscission scar after plantlet fall (Pedroso, 1998). Recent work showed that changes in gravity significantly affected leaf-plantlet formation, development, and asexual reproduction in this species (Pedroso and Durzan, 1998). The cytological, morphological and physiological changes observed suggested that stressful gravity environments (hypergravity) interfered with cellular processes in ways that might trigger apoptosis.

The response of *K. daigremontiana* in natural conditions (Resende, 1951) and in hypergravity (Pedroso and Durzan,
suggested that, under stressful conditions, leaf senescence could be induced and used as a mechanism for plant survival. As a first step to verify this hypothesis, we investigated the effect of gravity (simulated hypogravity, 1 g, and hypergravity) on cell death in Kalanchoë leaves.

Nitric oxide (NO) is a free radical which plays an important role as an intra- and intercellular messenger in animal systems (for reviews see Lancaster, 1992; Kojima et al., 1998). In plants, NO formation is associated with ethylene biosynthesis (Lesshem and Haramaty, 1996), disease resistance (Delledonne et al., 1998; Durner et al., 1998), drought stress (Lesshem, 1996; Haramaty and Lesshem, 1997), cell death and cell proliferation (Magalhaes et al., 1999; Pedroso et al., 1999, 2000a, b). Evidence for its function as an endogenous maturation and senescence regulating factor was recently provided (Lesshem et al., 1998). Histochemical detection of NO in vivo can now be achieved using the probe 4,5-diaminofluorescein diacetate (DAF-2 DA) (Kojima et al., 1998; Magalhaes et al., 1999; Pedroso et al., 2000a, b). The potential involvement of NO as trigger of cell death in Kalanchoë leaves was also investigated in the present work.

Drastic hypergravity treatments, for short (15 d) and acute (10–60 min) periods of time, were chosen to ascertain the induction of large-scale cell death (for a review see Gray and Johal, 1998), helping the cytological distinction between physiological (PCD) and non-physiological (‘accidental’) cell death. Double staining TUNEL-DAPI, microscopy techniques, a NO probe (DAF-2 DA), a NO donor (sodium nitroprusside, SNP) and a competitive substrate inhibitor (N\(^\text{G}\)-monomethyl-L-arginine, L-NMMA) of the NO-forming enzyme NO-synthase (EC 1.14.13.39; NO-synthase), were used to show that leaf and leaf-plantlet exposure to different gravity environments affects leaf PCD in this species, and that variations in the number of cells correlated with NO formation.

**MATERIALS AND METHODS**

**Plant material and in vitro culture conditions**

*Kalanchoë daigremontiana* Ham. and Perr. shoot cultures were used as a source of leaves and leaf-plantlets (Pedroso, 1998). Stock shoot cultures were maintained by micropropagation on half-strength Murashige and Skoog basal medium (1962), with 5 mg l\(^{-1}\) dithiothreitol, 25 g l\(^{-1}\) D-glucose, without glycine and growth regulators (MS/2 medium); pH was adjusted to 5.5 prior to agar addition (7.5 g l\(^{-1}\)) and medium sterilization (15.5 °C, for 20 min). Subculture was performed every 4 weeks. Unless otherwise stated, cultures were maintained in glass tubes (150 × 22 mm), at 25 °C, under a 16 h photoperiod provided by Phillips F40 AGRO AGROLITE fluorescent lamps [maximum radiation at 350–450 nm (blue) and 650 nm (red)]. Leaves and leaf-plantlets were cultured on solid MS/2 medium, on plastic Petri dishes (90 mm) sealed with transparent film. Leaf-plantlets were cultured in a vertical position with their roots in the medium. Leaves were inoculated horizontally with the upper leaf-page up. Cultures under hypergravity were kept in darkness, whereas those exposed to simulated hypogravity were kept in darkness or under a 16 h photoperiod. Leaf-plantlets and leaves cultured under the Earth’s gravity (1 g), in darkness and under a 16 h photoperiod, were used as controls.

**Hypergravity and simulated hypogravity experiments**

Low speed centrifuges (Braun Knecht-Heimann, Co., San Francisco, International Equipment Company, Boston, MA, USA) with wood carriers were used to simulate hypergravity. The centrifuges were modified to prevent temperature oscillations and vibration. Leaves and leaf-plantlets were exposed to 20 and 150 g for 15 d, or to 150 g for 10, 30 and 60 min. Clnostats equipped with 1 rpm motors and adapted to hold a Modular Incubator Chamber (Billups-Rothenberg, Inc., Burlingame, CA, USA) were used to simulate hypogravity (2 × 10\(^{-4}\) g). Leaves and leaf-plantlets were clinorotated, in darkness and under a 16 h photoperiod, for 15 d. Immediately after each treatment, samples (leaves) were collected and immersed for 24 h, at 4 °C, in 3.7 % formaldehyde fixative. Ninety leaf-plantlets and 30 leaves were cultured in each experiment. Experiments were repeated four times. In both hypergravity and hypogravity experiments, no ethylene production was detected, nor were oxygen, CO\(_2\), temperature and vibration limiting factors (Pedroso, 1998, 1999).

**In situ detection of DNA fragmentation**

Nucleoid and nuclei DNA fragmentation in leaf cells was detected in situ using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) method (Pedroso, 1998, 1999). Briefly, fixed leaves were hand-sectioned with a razor blade and the sections washed in PBS (10 mM Na\(_2\)HPO\(_4\), 150 mM sodium chloride, pH 7.2), five times over 1 h, at room temperature (RT). The sections were then sequentially immersed in an enzymatic solution [4 % (v/v) pectinase (Sigma Chemical CO, St Louis, MO, USA) and 2 % (v/v) Cellulase (Fluka Biochemika, CH)] in PBS (30 min, at RT); PBS (5 min at RT); methanol in 1 % acetic acid (20 min); PBS (5 min at RT), and incubated with 10 μg ml\(^{-1}\) proteinase K (Boehringer Mannheim, Germany) in PBS, pH 7.5, for 15 min at 37 °C. Following the proteinase treatment, the sections were rinsed in PBS for 2 min and immersed in TDT buffer (30 mM Trisma base, 140 mM sodium cacodylate, 1 mM cobalt chloride, pH 7.2), for 2 min, at 37 °C. The sections were then incubated for 60 min at 37 °C in the TUNEL reaction mixture (Boehringer Mannheim, Germany) consisting of 45 μl of TUNEL label (fluorescein-dUTP and dNTP) and 5 μl of TUNEL enzyme (terminal deoxynucleotidyl transferase). After incubation, the sections were washed in TB buffer (300 mM NaCl, 30 mM sodium citrate, pH 8.5) for 15 min, at 37 °C, to terminate the reaction, and finally rinsed in PBS, at RT. Controls were performed for each step of the protocol to determine potential sources of artifacts, namely by the use of enzymes and of methanol in acetic acid. Results showed that incubation in the enzymatic mixture is not required, it has to be optimized for each plant...
material, and should be avoided if cells or thin sections are used. Also, higher proteinase K concentrations can lead to false TUNEL-positive nuclei. For negative controls, only TUNEL label was added. Incubation of fixed sections with DNase-I (1 mg ml⁻¹, 10 min, at RT) prior to TUNEL labelling was performed for positive controls. Counterstaining for 15 min, at RT, with 1 μg ml⁻¹ 4-6-diamino-2-phenylindole dihydrochloride (DAPI), which specifically binds to adenine-thymine-rich deoxyribonucleic acids, was performed to distinguish non-apoptotic nuclei from apoptotic ones. Sections were mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and observed by fluorescence and/or confocal laser scanning microscopy (CLSM). TUNEL-positive nuclei or nucleoids fluoresce green (excitation 450–500 nm, detection 515–565 nm), while DAPI-positive nuclei fluoresce blue (emission 365 nm, excitation 510–530 nm). Four leaves per treatment were randomly collected from each of the four experiments performed (see above) and used in TUNEL assays. DAPI and TUNEL-positive cells were counted in ten individual fields from at least six leaf sections per leaf. Experiments reported herein were repeated at least four times, with all results in agreement between experiments. Results are presented as mean values ± s.d. Simple analysis of variance (ANOVA) was performed for \( P < 0.05 \) (results not shown).

**Visualization of nitric oxide**

Fresh leaves, collected 0 and 24 h after each treatment, were sectioned and incubated for 1 h, at 25°C, in darkness, with 10 μM 4,5-diaminofluorescein diacetate (DAF-2 DA; CALBIOCHEM, La Jolla, CA, USA) prepared fresh in distilled water or in PBS. This probe is highly specific for NO (Kojima et al., 1998) and has been successfully tested in plant cells (Pedroso et al., 2000a, b). After incubation, the samples were washed twice in water, mounted in water or Vectashield, and observed with a fluorescence or confocal microscope. DAF-2 fluoresces green (excitation 495 nm; emission 515 nm; long-pass filter of 515 nm; Kojima et al., 1998). Unstained leaf sections and sections stained after boiling (dead) were used as controls. For quantitation, at least six clonal leaves were collected per treatment, and stained cells were counted within three to five sections per leaf. Values are the mean of six independent experiments.

**Induction and inhibition assays**

Chemical agents (sodium nitroprusside and NG-monomethyl-L-arginine) and hypergravity were used to investigate the involvement of NO on DNA fragmentation and cell death in Kalanchoë leaves. Leaves were incubated for 3 h, at 1 g, in filter sterilized MS/2 medium with 10⁻⁴ M sodium nitroprusside (SNP), a NO donor; leaves incubated in medium without SNP were used as controls. Incubation was performed in a shaker at 60 rpm, at 23 ± 2°C.

In independent assays, leaves were exposed to hypergravity (150 g) for 3 h in filter sterilized MS/2 medium with and without (control) 0.5 mM NG-monomethyl-L-arginine, L-NMMA, a competitive NO-synthase inhibitor. Exposure in medium with 0.5 mM D-NMMA, which has no significant effect on NO-synthase, was performed as negative control. Leaves kept for 3 h at 1 g under agitation were used as an additional control. After 3 h, leaves were washed in sterile fresh medium without SNP or NMMA and immediately assayed for NO visualization or kept in culture overnight, for the detection of apoptosis (see above). Double staining for simultaneous detection of NO and DNA fragmentation was also tested; live cells and leaf sections were stained with DAF-2 DA, fixed, and then processed for TUNEL assay and DAPI counterstaining. This staining was possible because nuclei do not stain with DAF-2 DA; even so controls should be performed for every step of the process. Assays in the presence of other NO donors, NO-synthase inhibitors, NO scavengers, nitrite or nitrate suplementations were not included in the present study as the results are described elsewhere (Pedroso et al., 2000a, b).

**Fluorescence and confocal microscopy**

Quantitation of NO formation, DNA fragmentation and cell death was performed on a Nikon (Tokyo, Japan) inverted microscope equipped with UV (excitation 360 nm; emission 420 nm) and FITC (excitation 450–490 nm; emission 520 nm) filters and a Nikon camera with 400 ASA Kodak Ektachrome film. Confocal images were obtained using Leitz Fluor 40 × and 100 × 1.3-NA oil PL FLUOTAR objective lenses at a Leica TCS-NT confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with Argon/Krypton and UV lasers (excitation splitter DD488/586; RSP500), and Leica TCS-NT software (TCS-NT version 1.5.451). Serial confocal optical sections were taken at a step size of 1 μm. Molecular Dynamics ImageSpace software (Molecular Dynamics, Inc., Sunnyvale, CA, USA) was used to create 3-D projections of serial confocal optical sections, merged or separated two- or three-channel 3-D images, and sequences of 3-D images at different angles to simulate object rotation. Images were contrast enhanced using image processing software (Photoshop®; Adobe Systems, Inc., Mountain View, CA, USA) and printed on a Kodak ds 8650P Color Printer (Eastman Kodak Company, Rochester, NY, USA).

**RESULTS**

**Detection of DNA fragmentation and cell death in different gravity environments**

The appearance and intensity of the fluorescence after fluorescein-dUTP labelling of free 3'-OH termini (TUNEL; green; FITC channel) was indicative of the extent of DNA fragmentation present in leaf cells following a gravitational force stimuli. To determine accurately the percentage of cell death, all cells were counterstained with DAPI after TUNEL assay and observed in the UV and FITC channels or, in addition, the TRITC channel. No separate UV channel images are presented in this work. Cells with DAPI-positive nuclei (blue; UV channel) showing no green
fluorescence in the FITC channel (results indicative of the absence of DNA breaks), were considered as ‘non-apoptotic’. Cells with simultaneously bright green TUNEL-positive and DAPI-negative nuclei (in the FITC and UV channels, respectively), features indicative of irreversible DNA damage, and presenting cytological features of PCD, were designated as ‘apoptotic’ (Fig. 1A–H). These cells were quantified as death cells. Positive and negative controls of the TUNEL assay further confirmed the absence of artifacts; TUNEL was positive for both nuclei and chloroplast nucleoids in leaf sections treated with DNase-I (positive controls); some nuclei were also DAPI-positive. In negative controls (leaf sections incubated without TUNEL enzyme), only DAPI-positive nuclei and some DAPI-positive nucleoids were observed. Figure 1E shows a merged confocal image of a
non-apoptotic, double stained mesophyll cell. Most chloroplasts, showing starch grains and bright red autofluorescence (chlorophyll), were distributed close to the cell wall, far from the nucleus (Fig. 1E).

Cell death in leaves is differentially affected by different gravity environments

Culture in $2 \times 10^{-4}$ g (clinorotation; simulated hypogravity), 1 g (Earth’s gravity; controls) and 20 and 150 g (hypergravity), in darkness or light were not significant (data not shown). Leaf culture in darkness, in subepidermal parenchyma cells (Fig. 1H) were the most frequently concentrated at the periphery of the nucleus, with or without starch grains, TUNEL-negative (see Materials and Methods) after 15 d exposure to different levels of gravity stimulation. The differences observed between treatments were identical for clinorotated, control and centrifuged cells. Early stages of apoptosis were characterized by bright green TUNEL-positive nuclei with the same shape as DAPI-positive ones (Fig. 1D and F). Disorganization of the nuclear envelope and marginalization of chromatin were also observed in subsequent stages of the process (Fig. 1H and I), and confirmed by transmission electron microscopy (data not shown).

Except for later stages of cell death, the pattern of nuclear degeneration was identical for clinorotated, control and centrifuged cells. Early stages of apoptosis were characterized by bright green TUNEL-positive nuclei with the same shape as DAPI-positive ones (Fig. 1D and F). Disorganization of the nuclear envelope and marginalization of chromatin were also observed in subsequent stages of the process (Fig. 1H and I), and confirmed by transmission electron microscopy (data not shown).

In contrast to apoptotic cells in 1 g (Fig. 1I), the apoptotic cells exposed to hypergravity presented drastic nuclei condensation and blebbing, resulting in nuclei frequently surrounded by bright green fluorescent bodies (Fig. 1G and J). 3-D projections of serial confocal optical sections from images identical to those on Fig. 1G and J showed several stages of bleb formation on the nuclear surface (image not shown). TUNEL-positive globular bodies were observed close to the apoptotic nucleus, both in hypergravity- and hypogravity-exposed leaf cells (Fig. 1J and K).

In simulated hypogravity, nucleus fragmentation in two portions was commonly observed for elongated nuclei (Fig. 1K), with or without the presence of nuclear bodies.

Differential TUNEL reaction with chloroplast differentiation and gravitational force

Based on colour, starch content and TUNEL reaction, chloroplasts were divided into three types: (1) bright red, with or without starch grains, TUNEL-negative (see Fig. 1, In situ detection of nuclear and chloroplastidial DNA fragmentation and cell death (apoptosis) in Kalancheë daigremontiana leaves. A–C, Fluorescence microscopy images of leaf sections obtained using a FITC filter (excitation 450–490 nm; emission 520 nm) after TUNEL assay to detect DNA fragmentation (green fluorescence). TUNEL-positive nuclei are in green; red is due to chlorophyll autofluorescence. Leaves were collected and processed for TUNEL (see Materials and Methods) after 15 d exposure to different levels of gravity stimulation. The differences observed between treatments were identical for different focal planes. A, Hypogravity, $2 \times 10^{-4}$ g; B, Earth’s gravity, 1 g (control); C, hypergravity, 20 g. Bar = 20 mm. D-O, Confocal laser scanning microscopy images acquired after TUNEL assay (FITC channel) and DAPI counterstaining (UV channel); red, when present, is due to chlorophyll autofluorescence (TRITC channel). D, DAPI-blue and TUNEL-positive nuclei (green) in a cross-section from a leaf exposed to 20 g, for 15 d (merged image; UV-FITC). Bar = 11 mm. E, Non-apoptotic, double stained leaf parenchyma cell from a leaf cultured in 1 g, showing a DAPI-positive nucleus and TUNEL-negative chloroplasts (red autofluorescence only) (3-D projection; UV-FITC-TRITC). Bar = 5 mm. F, Apoptotic guard cells showing nuclear and chloroplastidial DNA fragmentation (green spots) at 20 g (merged image; UV-FITC channels only to eliminate chlorophyll autofluorescence). Bar = 3–5 mm. G, Nuclear DNA fragmentation in an epidermal cell. Note the presence of nuclear bodies (bright spots) migrating towards the cell wall at 20 g (merged image, UV-FITC). Bar = 3–0.5 mm. H, Nuclear and chloroplastidial DNA fragmentation in mesophyll cells after 15 d of exposure to 150 g (3-D projection; UV-FITC-TRITC). Bar = 11 mm. I, Pseudo-colour image of an apoptotic nucleus from a leaf cell cultured in 1 g. Note the marginalization of the chromatin. Hot colours correspond to areas of high DNA fragmentation (FITC channel; no fluorescence on UV and TRITC channels). Bar = 1–2 mm. J, Example of an apoptotic nucleus from a cell of a leaf cultured in hypergravity. Note the presence of nuclear bodies detaching from the condensed nucleus (UV-FITC-TRITC). Bar = 1–7 mm. K, Example of an apoptotic nucleus from a cell of a leaf cultured in simulated hypogravity. Note the nuclear fragmentation and presence of nuclear bodies (UV-FITC-TRITC). Bar = 1–6 mm. L, TUNEL-positive nucleoids (green spots) in chloroplasts of mesophyll cells after 15 d of leaf exposure to hypergravity (3-D projection; UV-FITC-TRITC). Bar = 2–2 mm. M–O, TUNEL-positive cell from a leaf exposed to hypergravity (3-D projection; UV-FITC). Images artificially coloured to enhance contrast. N, M, O, TUNEL-positive chloroplasts concentrate around the nucleus or irradiate toward the cell wall, as shown by the red lines drawn over the image. Bar = 31 mm. O, This image shows the intricate network of connections (yellow lines) observed between labelled and unlabelled chloroplasts.
Fig. 2. Cell death (TUNEL-positive cells) in the epidermis and mesophyll of Kalanchoë leaves after 15 d exposure to different levels of gravity stimulation (2 × 10⁻⁴, 1, 20 and 150 g) and light conditions (darkness and a 16 h photoperiod). Leaves were fixed immediately after each treatment, sectioned, processed for TUNEL and counterstained with DAPI. Leaves cultured at 1 g, in darkness and light, were used as controls. Leaf sections treated with DNase-I after fixation and permeabilization (positive control) displayed 85% TUNEL-positive cells. Leaf sections processed without addition of terminal transferase did not label. DAPI-positive vs. TUNEL-positive cells in the epidermis (□) and mesophyll (■) were counted in ten individual microscope fields from at least six leaf sections from each of the four leaves randomly collected per treatment per experiment. Values are the means of four independent experiments. Error bars are lower than 8%.

Fig. 3. Quantitation of nitric oxide (NO) formation and of nuclear and chloroplast DNA fragmentation following acute hypergravity treatments. A, Leaves, collected at the times indicated after hypergravity exposure (150 g; hyper-G), were sectioned, stained for 1 h with 10 μM DAF-2 DA for nitric oxide visualization, fixed, processed for TUNEL and counterstained with DAPI. Leaf sections, fresh and fixed, not assayed for TUNEL, treated with DNase-I, processed without terminal transferase, and without DAPI staining, were used as controls of this staining assay. B, Hyper-G treated leaves, kept for 24 h in 1 g under a 16 h photoperiod, were processed as described for A. Leaves not exposed to hypergravity (0 + 24 h) were also collected and used as controls. Three to five leaf sections (90 μm), performed in at least six clonal leaves per treatment, were used to quantify the percentage of leaf cells with NO (shaded line) and, within TUNEL-positive cells, those presenting nucleoid (▲) and nuclear (■) DNA fragmentation. Values are the mean of six independent experiments. Error bars are lower than 7%.

Nuclear DNA fragmentation is preceded by nucleoid DNA fragmentation

In order to determine how fast leaves responded to changes of gravity, in situ detection of DNA fragmentation was performed in clonal leaves immediately (0 h) and 24 h after 10, 30 and 60 min at 150 g. Ten minutes’ (T10) exposure to 150 g drastically increased the number of TUNEL-positive cells. This increase was entirely due to nucleoid DNA fragmentation, as the number of cells with nuclei presenting DNA fragmentation was identical to 1 g controls (Fig. 3A). Guard cells and subepidermal parenchyma cells were the most affected. Longer exposures, T30 and T60 min, further increased nucleoid DNA fragmentation (Fig. 3A). DNA fragmentation in brightly DAPI-stained nuclei also increased, compared to T0 and T10 min, mainly in epidermal cells (Fig. 3A).

Detection of DNA fragmentation 24 h after leaf exposure to 150 g showed that the number of TUNEL-positive nuclei and the extent of nuclear DNA fragmentation had further increased compared to the previous day (Fig. 3B). The nuclei were no longer DAPI-positive, the number of cells with TUNEL-positive nucleoids decreased significantly (Fig. 3B), and death cells were detected in all samples. The highest number of labelled chloroplasts and...
nuclei were recorded for T60 and T60 + 24 h, respectively (Fig. 3A and B), in subepidermal parenchyma cells.

Nitric oxide formation precedes and correlates with DNA fragmentation

DAF-2 DA staining (green) of fresh leaves immediately and 24 h after 10, 30 and 60 min in hypergravity (150 g), showed that NO formation significantly increased after exposure, decreasing to levels higher than those detected for controls 24 h later (Fig. 3A and B). NO was detected diffused in the cytosol of epidermal cells, mainly in guard cells (Fig. 4A and B), or it was just detected in chloroplasts (Fig. 4C and D). Sectioned and stained controls (leaves in 1 g for the same period of time) confirmed that the NO burst detected was a consequence of hypergravity exposure and not of sample manipulation.

To investigate whether NO formation was involved in DNA fragmentation, leaves were incubated for 3 h in 1 g, in medium with and without sodium nitroprusside (SNP), a NO donor, and in hypergravity, in medium with and without N\textsuperscript{G}-mono-methyl-L-arginine (L-NMMA), a NO-synthase inhibitor. Incubation in hypergravity in medium with D-NMMA, which has no significant effect on NO-synthase, was performed as negative control. Leaves collected immediately after the treatments were sectioned, stained with DAF-2 DA, processed for TUNEL, and then counterstained with DAPI. Figure 5 shows the results obtained immediately after treatment. SNP and hypergravity both increased the number of cells showing NO and DNA fragmentation, compared to 1 g controls (Fig. 5). Culture in hypergravity with L-NMMA significantly decreased NO formation and DNA fragmentation, in both epidermal and mesophyll cells, compared to centrifuged samples without the inhibitor, and to 1 g controls (Fig. 5). NO formation and DNA fragmentation was identical for samples cultured in hypergravity with and without D-NMMA (data not shown), reconfirming the inhibitory effect of L-NMMA. These results showed that NO formation was directly correlated with DNA fragmentation. Additional assays in which leaves were fixed and processed for TUNEL without previous in vivo incubation.
for 1 h in DAF-2 DA, showed that nucleoid DNA fragmentation increased during the first hour immediately after exposure (data not shown). Figure 5 shows that the inhibition of NO formation by L-NMMA was more effective in chloroplasts (empty bars; mesophyll cells) than in the cytosol (shaded bars; epidermal cells).

3-D projections showed that chloroplasts brightly stained for NO were concentrated close to the cell wall (Fig. 4E), whereas dimly stained chloroplasts converged to the nucleus. The chloroplasts next to the nucleus were not stained (Fig. 4E, dark shadows on the nucleus). At that moment (1 h after the 3 h treatment), DNA fragmentation was already detected in the nucleus (Fig. 4E), although DAPI fluorescence was still bright in the UV channel (image not shown).

NO was detected diffused in the stroma of the DAF-2 DA-positive chloroplasts preferentially around starch grains (Fig. 4F–H). The red autofluorescence of the chlorophyll frequently surrounded, completely or partially, the green fluorescent core (Fig. 4F and G). The appearance of a yellow colour after merging the confocal images from the FITC and TRITC channels confirmed that NO could also be detected close to the thylakoid membranes (Fig. 4H).

DAF-2 DA-positive connections between stained-stained and stained-unstained chloroplasts were also observed (Fig. 4H). However, in the latter, the side of the tubular connection in contact with the unstained chloroplast was not fluorescent, suggesting the possibility of NO diffusion between chloroplasts.

Identical results were obtained for the other hypergravity treatments. As for the TUNEL assay, it was the epidermal (mainly guard cells) and subepidermal parenchyma cells (chloroplasts) that were preferentially stained for NO (Fig. 4A–D).

All apoptotic cells were positive for NO, but not all NO-positive cells were apoptotic. In all assays, leaf-cell response was identical for both leaves and plantlets. In both, leaf-plantlet formation paralleled the cell death increase with the increase of gravitational force (results not shown).

**DISCUSSION**

The present results provide microscopic and TUNEL staining evidence that cell death is induced in response to gravity changes. Cell death increased with increased levels of gravity stimulation, and was shown to be increased or reduced by chemical agents (SNP or L-NMMA, respectively). Plantlet and leaf culture for 15 d under multiples of Earth’s gravity was expected to induce a stress response in leaf cells, which could eventually lead to cell death. These results are in accordance with previous reports showing that centrifugation and other forms of mechanical stress can lead to cell damage and/or cell death (Pedroso and Durzan, 1998, 1999; Magalhaes et al., 1999). Cell death was increased by exposure to a NO generator (SNP) and reduced by a NO-synthase inhibitor (L-NMMA). Addition of D-NMMA did not affect cell death. This indicates that the cell death process induced was under physiological control and, possibly, mediated by NO.

In both hyper- and hypogravity, cell death was characterized by distinct chromatin marginalization, nuclear condensation and blebbing, resulting in the formation of nuclear-apoptotic bodies. These are typical features of apoptosis in animal (Bowen et al., 1996) and plant cells (Havel and Durzan, 1996a; Wang et al., 1996; Yeng and Yang, 1998). In simulated hypogravity, nuclei often broke into two fragments (see Fig. 1K), a phenomenon not observed in cell death induced by hypergravity (see Fig. 1J). We did not determine the cause of this difference. Nuclear breakage after formation of small nuclear-apoptotic bodies might represent a later stage of nuclear degeneration. Based on the above, these results provide strong evidence that the cell death process induced in Kalanchoë leaves was apoptosis.

The in vivo visualization of NO by DAF-2 DA required an incubation period of 1 h, whereas determinations of DNA fragmentation were made on destructively sampled leaves. Nevertheless, using acute hypergravity treatments (10–60 min) and several experimental controls, it was possible to show that the NO bursts were caused by exposure to hypergravity and not by sample manipulation and/or sectioning, and that they preceded chloroplast DNA fragmentation. Chloroplasts were the first visible organelles to show NO and DNA fragmentation. K. daigremontiana chloroplasts have small, uniformly dispersed nucleoids in the matrix between the thylakoid membranes and/or the grana stalks (Kuroiwa et al., 1981). Treatments with SNP and L-NMMA enabled us to show a direct correlation between NO and DNA fragmentation in chloroplasts and in tissues (epidermis and mesophyll). Identical results were
recently reported for *Arabidopsis thaliana* (Garces et al., 1999) and *Taxus brevifolia* (Pedroso et al., 2000b). No DAF-2-positive cells or DNA fragmentation were observed when centrifugation was performed in the presence of carboxy-PTIO, a NO scavenger (Pedroso and Garces, pers. comm.). The consistent staining of *Kalanchoë* chloroplasts with DAF-2 DA suggests that NO was being produced in these organelles, and that NO was directly or indirectly responsible for the damage of chloroplast DNA. This reinforces previous results on the possible involvement of free radicals in signal pathways leading to PCD (Jacobsen, 1996; Panavas and Rubinstein, 1998), and supports the involvement of NO in DNA damage and plant cell death, as described for animal systems (Wink et al., 1991; Brüne et al., 1998; Tannenbaum, 1998).

Chloroplast DNA fragmentation was detected 10 min after exposure to hypergravity. Nuclear DNA fragmentation was first detected 20 min later (at 30 min of exposure), increasing until 24 h after the treatments, when chloroplast degeneration was severe. In our study, at 30 and 60 min of exposure, the nuclei that labelled for TUNEL were also brightly stained for DAPI. This indicates that most of their DNA was still intact. After 24 h, no intact DNA was detected on TUNEL-positive nuclei. Morphological changes occurring in chloroplasts before nuclear changes were also reported for resistant tobacco plants infected with TMV (Mittler et al., 1996), and during xylogenesis in *Zinnia* cells (Fukuda et al., 1998). Our results are also in accordance with those described for PCD in tracheary elements, a process estimated to be completed in 8 to 12 h (Groover and Jones, 1999), and for toxin-induced cell death in asc/asc tomato protoplasts (6 to 16 h) (Wang et al., 1996).

In mesophyll cells, environmental stresses are readily perceived by chloroplasts and elicit an oxidative response (Noodén, 1988; Allen, 1993; Brüggemann et al., 1994; Green and Fluhr, 1995; Allen and Raven, 1996; Allan and Fluhr, 1997; Buchanan-Wollaston, 1997). NO reacts with superoxide (O$_2^-$) to form peroxynitrite (OONO$^-$) (Pryor and Squadrito, 1995), which damages DNA, RNA, proteins and lipids (Stamler et al., 1992). Thus it is not surprising that chloroplast-containing cells closest to the region of stimuli/stress application (guard cells and subepidermal parenchyma cells) were the most sensitive to external changes (light or gravity). The increase in cell death recorded in darkness was also probably related to senescence in photosynthetic tissues (Panavas and...
Rubinstein, 1998). A direct consequence of the exposure to increasing levels of gravity stimulation was the increase and spread of the population of TUNEL-positive parenchyma cells to deeper cell layers. Because nitrate was used in the culture medium, we cannot exclude the conversion of nitrite to NOx (NO + NO₂) by nitrate reductases, and/or non-enzymatic ones, resulting from chemical reactions between plant metabolites and accumulated NO₂⁻ and/or decomposition of nitrous acid (Dean and Harper, 1986, 1988; Klepper, 1990; Vliet et al., 1997; Durner et al., 1998; Yamasaki et al., 1999). The preferential localization of DAF-2 DA staining around the starch grains (see Fig. 4F) might indicate non-enzymatic NO generation (Nishimura et al., 1986). However, the inhibitory effect of L-NMMA on NO production (see Fig. 5) led us to reject this possibility. Our results suggest the presence of an inducible NO-synthase in Kalanchoë leaf cells. The recent report of a gene in Arabidopsis thaliana with sequence similarity with animal NO-synthase (Mayer et al., 1999), further supports this assumption. The localization of the NO-staining, in the cytosol and/or chloroplasts coincides with sites of known location of soluble L-arginine (Ludwig, 1993), and those previously reported in in situ localization assays (Barroso et al., 1999; Pedroso et al., 2000a, b).

The detection of NO and DNA breaks in chloroplasts was accompanied by a decrease in chlorophyll autofluorescence, increase in starch content, and followed by severe nuclear DNA damage. Chloroplast degeneration was confirmed by electron microscopy and by a concomitant loss of photosynthetic capacity (unpubl. res.). Membrane lipids of chloroplasts could be one of the primary targets for NO attack, detrimentally affecting the efficiency of photosynthesis (Leshem et al., 1997). The differential TUNEL reaction with chloroplast differentiation and gravitational forces was probably due to the ‘redox state’ of the plastid at the moment of stimuli/stress exposure and its ability to cope with an additional stress (Allen and Raven, 1996).

TUNEL-positive chloroplasts were linked by cytoplasmic connections and concentrated around the nucleus (see Fig. 1M). Linkages irradiated in lines to the cell wall (see Fig. 1N). Exchange of protein molecules through connections between chloroplasts was recently demonstrated (Köhler et al., 1997; Shiina et al., 2000). Confocal images from parenchyma cells double stained with DAF-2 DANTUNEL (see Fig. 4E), suggested that NO diffused along a cytoplasmic network to the nucleus where endonuclease activity was then initiated.

How the chloroplast stress responses leading to cell death were related to the network of connections remains unclear. This intricate network of connections was similar to a cortical endoplasmic reticulum (see Fig. 1M) (Gunning and Steer, 1996). The network could engulf NO-producing chloroplasts to prevent further cellular DNA damage, and promote calcium influx favouring DNA damage and chloroplast elimination. It could also increase molecule transport among degenerating organelles and the neighbouring cells, prior to cell death. Information transfer to the nucleus, by direct chloroplast-nucleus or chloroplast-nucleolus interaction, or by the release of signalling molecules like octadecanoid 12-oxo-phytodienic acid (Stelmach et al., 1998), could trigger silent gene-activation leading to cell adaptation and survival (for reviews see Forsburg and Guarente, 1989; Oelmuller, 1989; Taylor, 1989; Zachleder et al., 1989, 1995; Ehrara et al., 1990; Susek and Chory, 1992; Susek et al., 1993; Gillham, 1994). Cellular degradation products from apoptosis (Havel and Durzan, 1996a, b) would be salvaged to sustain adaptation, viability, and ontogeny of the neighbouring cells, assuring the survival of the species in a harsh(er) environment. Specific checkpoints that regulate plant cell cycle progression in response to oxidative stress (Reichheld et al., 1999), could explain why some cells undergo programmed cell death, while others undergo division leading to leaf-plantlet formation. NO could also play a role in such a situation depending on its concentration and reactivity with superoxide concentration (Shen et al., 1998).

At the organ level (leaf), the increases/decreases in NO formation and cell death were paralleled by increases/decreases in leaf-plantlet formation (data not shown; Pedroso and Durzan, 1998). Thus, the involvement of NO in DNA damage in Kalanchoë cells does not exclude a possible role of NO as a regulator of leaf senescence, as suggested by Leshem et al. (1998). Senescence leads to a decrease in chlorophyll content, chloroplast degeneration (including nucleoid DNA fragmentation) and, ultimately, nuclear condensation and internucleosomal DNA fragmentation (for a review see Hensel et al., 1993; Smart, 1994; Humbeck et al., 1996; Bleecker and Patterson, 1997; Buchanan-Wollaston, 1997; Orzáez and Granell, 1997; Gray and Johal, 1998), exhibiting cytological features of PCD by apoptosis (Yeng and Yang, 1998). Our results suggest that Kalanchoë leaves responded to a gravity change by forming NO, which triggered a senescence-like process that exhibited characteristics of apoptosis. Simulated gravity from 2 × 10⁻⁴ to 150 g, increased NO-related cell death (Fig. 6). In vitro cultured leaves of K. daigremontiana provide a unique experimental system for studying the effect of environmental stresses (gravity or other) (Resende and Pereira-da-Silva, 1965; Pedroso and Durzan, 1999), on a potential homeostatic balance between plantlet formation and apoptosis in this species.

In conclusion, cell death in Kalanchoë leaves increases proportionally with the increase of gravitational force, from 2 × 10⁻⁴ to 150 g. Stressful gravity environments lead to a NO burst in chloroplasts. In some cells this triggers a sequence of events that leads to chloroplast DNA damage, chloroplast degeneration, followed by nuclear degeneration and cell death by apoptosis.

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