

Nitric oxide induces cell death in *Taxus* cells

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Abstract

Sodium nitroprusside (SNP), a nitric oxide donor, or centrifugation at 150 times unit gravity, caused a nitric oxide burst in oocyte-derived *Taxus brevifolia* haploid cultures. This burst, visualized by the specific fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA), preceded a significant increase in nuclear DNA fragmentation and cell death. DNA fragmentation was detected in situ by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) of DNA 3'-OH groups. Nitric oxide formation and cell death were significantly decreased by *N*^G-monomethyl-L-arginine (L-NMMA), a nitric oxide-synthase (NOS; EC 1.14.13.39) inhibitor. Our results show that nitric oxide leads to irreversible DNA fragmentation and cell death under stressful conditions, and that its effect can be prevented by L-NMMA. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Apoptosis; Cell death; Nitric oxide; Oocyte cultures; *Taxus brevifolia*

1. Introduction

Nitric oxide (NO) is a highly diffusible gaseous free radical that has recently been reported to play a key role in plant disease resistance [1–4], ethylene emission [5], plant response to drought stress [6,7], senescence [8] and plant stresses in general [9]. Recent evidence suggests that key components of animal NO signaling are also operating in plants [1,2,4,10–12].

In animals, NO functions as an intra- and inter-cellular messenger, being involved in several patho-physiological processes including pro-

grammed cell death [13–17]. NO is formed from L-arginine by a family of constitutive and inducible nitric-oxide synthase enzymes (NOS; EC 1.14.13.39) [6]. Evidence for the presence of a mammalian-type of NOS has been reported for several plants [2,6,11]. The localization of NOS in plant peroxisomes and chloroplasts was recently reported [18,19]. Also a gene sequence with NOS from *Rattus norvegicus* was recently discovered in *Arabidopsis thaliana* (Wambutt et al., pers. commun.). However, NO is also produced in plants as a by-product of the activity of constitutive nitrate reductase [20,21]. NO can also be generated non-enzymatically from donor molecules, such as sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP), 3-morpholino-sydnomine and *N*-*tert*-butyl- α -phenylnitron [6,22]. In animal systems, NO induces both pro- and anti-apoptotic responses [23–25]. This free radical was recently visualized in animal cells by the fluorescent indicator 4,5-diaminofluorescein diacetate (DAF-2 DA)

Abbreviations: DAF-2 DA, 4, 5-diaminofluorescein diacetate; DAPI, 4, 6-diamino-2-phenylindole; D-NMMA, *N*^G-monomethyl-D-arginine; L-NMMA, *N*^G-monomethyl-L-arginine; NO, nitric oxide; NOS, NO-synthase; SNP, sodium nitroprusside; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; UV, ultraviolet.

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[16]. DAF-2 DA is converted by esterases in the cytoplasm to DAF-2, which reacts specifically with NO to form a highly green-fluorescent triazole form [16]. DAF-2 does not react with stable oxidized forms of N, such as nitrite (NO_2^-), nitrate (NO_3^-), or with other reactive oxygen species, e.g. superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), or peroxyxynitrite anion (ONOO^-) [16,26].

Taxus brevifolia, as other conifers, expresses programmed cell death (apoptosis) at different developmental stages during diploid parthenogenesis and early embryony [27–30]. Exposure of *T. brevifolia* cultures to hypergravity (centrifugation) significantly increased cell death by apoptosis [31]. An increase in apoptosis with increasing centrifugal force was also reported for *Kalanchoë daigremontiana* [32,33]. These findings led us to hypothesize that *Taxus* cell death under stressful conditions (centrifugation; mechanical stress) is preceded by an increase of NO production, directly or indirectly responsible for irreversible nuclear DNA fragmentation. To show the involvement of NO in DNA fragmentation, SNP, a NO donor, was added at different concentrations to cultures to determine if NO artificially induced cell death in *Taxus* cells. As a complement to these experiments, cell death was physically induced in *T. brevifolia* cells by centrifugation with or without N^G -monomethyl-L-arginine (L-NMMA), inhibitor of NO-synthase, or its enantiomer, N^G -monomethyl-D-arginine (D-NMMA; negative control). NO production was correlated to the number of DAF-2 DA-stained cells. Cell death was determined by the number of cells presenting irreversible DNA fragmentation (TUNEL-positive nucleus) and cytological features of apoptosis. Here, we report that NO induces cell death in *T. brevifolia* cells and that DNA fragmentation, and subsequent cell death, can be prevented by a NOS inhibitor.

2. Material and methods

2.1. Plant material

Haploid egg-derived callus cultures from the female gametophyte of a 30-year-old *T. brevifolia* tree, maintained on modified B5 medium, in darkness, at 25°C [34], were used as experimental system.

2.2. Nitric oxide and cell death assays

To study the effect of SNP on NO levels and cell death, freshly subcultured friable callus were incubated for 3 h in 10^{-6} , 10^{-4} , and 10^{-2} M SNP (filter-sterilized) in modified B5 liquid medium supplemented with 1 mM L-arginine (ca. 1 ml packed cell volume per 3 ml of medium), a modification of a protocol developed for animal cells [17]. Cells incubated in modified B5 medium without SNP and with and without L-arginine were used as controls. As additional controls, cells were incubated in medium supplemented with 10 mg/ml nitrite (NO_2^-) or nitrate (NO_3^-). Treatments were kept in a shaker at 60 rpm, at $23 \pm 2^\circ\text{C}$. After 3 h, a group of samples (cells and cell aggregates) were washed in sterile, fresh medium, and immediately stained for NO visualization with 10 μM DAF-2 DA (detection limit 5 nM of NO; Calbiochem-Novabiochem Corp., La Jolla, CA, USA) and incubated for 1 h, at 25°C [16,17]. Negative controls consisting of cells boiled in water for 1 min (dead cells) stained with DAF-2 DA and live and dead cells mounted in water were also performed. Staining with aminofluorescein diacetate (4-AF DA), which does not form a fluorescent compound in presence of NO, was used as negative control of the DAF-2 DA probe. The remaining cells and cell aggregates were kept in culture overnight, under agitation, for cell death assays. Cells were fixed in 4% (v/v) formaldehyde solution and processed for a one-step modified terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method (Boehringer Mannheim, Germany) [29] for in situ detection of nuclear DNA fragmentation. Negative controls were performed in the absence of TUNEL enzyme. In positive controls, cells were incubated with DNase I (1 mg/ml) for 10 min, at 25°C, prior to labeling. DAPI (4,6-diamino-2-phenylindole) was used in all TUNEL assays, as a counterstain for the detection of nuclei with intact DNA. Experiments were repeated at least six times with at least eight samples per treatment.

In a second set of experiments, cell death was physically induced by centrifuging *Taxus* cells for 3 h in liquid culture medium, at 150 times unit gravity, with and without 0.5 mM L-NMMA, a competitive substrate inhibitor of NOS (Calbiochem-Novabiochem Corp.) [17,35]. To determine possible non-specific L-NMMA activity,

incubation in medium with its enantiomer (0.5 mM D-NMMA), which does not have any significant effect on animal NOS [35], was also performed as negative control.

Cells were mounted and observed with an epifluorescence microscope, equipped with DAPI (UV light; excitation at 360 nm; emission, ≥ 420 nm) and FITC filters (blue light; excitation at 450–490 nm; emission, 520 nm). The numbers of DAF-2 DA stained cells (bright green fluorescence) versus non-stained cells (mean \pm S.D.) were determined. Statistical analysis using analysis of variation (ANOVA) at $P < 0.05$ was performed (n not less than 8). Cell death was determined by counting the number of TUNEL-positive nuclei (bright green at 450–490 nm and DAPI-negative) versus the total number of DAPI-positive nuclei (blue at 360 nm). Values in graphs are presented as percentage. Confocal images were acquired using a Leica TCS-NT laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany), with UV and Argon/Krypton lasers (ex. splitter RPS500) and an objective UV 40 \times 1NA oil PI FLUOTA. Images of UV and FITC channels were merged using Molecular Dynamics ImageSpace software (Molecular Dynamics Inc., Sunnyvale, CA, USA).

3. Results

3.1. Visualization of nitric oxide

Boiled cells (dead) with and without subsequent staining with DAF-2 DA presented only a yellow autofluorescence at cell walls, as did living cells mounted in water (negative controls) (Fig. 1A). As predicted, no cell-staining was obtained with 4-AF DA.

In general, the cytoplasm of DAF-2 DA-positive *Taxus* cells stained uniformly for NO. Fluorescence was visibly brighter at the cell wall level (Fig. 1B–E) and in cells with dense cytoplasm (not shown). Detailed observations showed that the staining was localized at the cytosol level; the large vacuole, which occupied most of the cell volume, was not stained. Although NO was detected in controls (cells grown in at unit gravity without any additional treatment) (Fig. 1B and Fig. 2), less than 4% of the stained cells showed a fluorescence in the cytosol, comparable to that observed for

SNP-treated and centrifuged cells (Fig. 1C and D). No significant differences were recorded ($P < 0.05$; $n = 8$) between cells incubated in culture medium and cells incubated in medium supplemented with NO_3^- or NO_2^- (additional controls). Also, no significant differences were recorded between *Taxus* cells incubated in culture medium with and without L-arginine. The results presented in Figs. 2 and 3 are for media containing L-arginine.

As predicted, the number of DAF-2 DA-positive cells was the highest for SNP-treated (98%) and centrifuged samples (99%) (Fig. 2). DAF-2 DA-negative cells in those samples, either had no nuclei or were highly damaged. Addition of SNP or centrifugation significantly increased the numbers of cells stained for DAF-2 DA ($P < 0.05$, $n = 10$) compared with those cultured without SNP (controls) (Fig. 2). Also, fluorescence was brighter for SNP-treated and centrifuged cells (Fig. 1C and D) than for controls (Fig. 1B). No statistically significant differences in the number of cells showing NO were detected between 10^{-4} M SNP-treated and centrifuged samples (Fig. 1C and D). Addition of SNP at higher concentrations increased the number of DAF-2 DA-positive cells to 99.5% (not shown). Centrifugation of cells in the presence of L-NMMA, a NOS inhibitor, significantly reduced ($P < 0.05$, $n = 10$) the number of cells stained for NO, compared to those centrifuged without L-NMMA (Fig. 2). Fluorescence brightness was also reduced (Fig. 1E). No significant differences ($P < 0.05$, $n = 10$) were recorded between cells centrifuged with and without D-NMMA (Fig. 2).

3.2. Induction of cell death

TUNEL was negative for negative controls. Only DAPI-positive nuclei (Fig. 1F) and a yellow autofluorescence on some cell walls and in vacuoles (as in Fig. 1A and G) were observed in those samples. Positive controls, besides autofluorescence, presented TUNEL-positive nuclei (bright green fluorescence under blue light) as a result of nuclear DNA fragmentation by DNase-I activity. These TUNEL-positive nuclei were identical to those detected in SNP or centrifuged cells (Fig. 1G). Addition of SNP significantly increased ($P < 0.05$, $n = 8$) the number and percentage of cells with TUNEL-positive nuclei compared to cells

cultured without SNP (controls) (Fig. 3). SNP caused irreversible nuclear DNA fragmentation and, consequently, cell death. The lowest level of SNP (10^{-6} M) increased cell death from 4 to 17%. Cell death also increased (1.6-fold) with the increase of SNP from 10^{-6} to 10^{-4} and 10^{-2} M, respectively (Fig. 3). For all the assays performed, cell death increased with the increase of NO production. Centrifugation in the presence and ab-

sence of L-NMMA, showed that L-NMMA significantly decreased ($P < 0.05$, $n = 10$) the nuclear DNA fragmentation and cell death (Fig. 3). Contrary to L-NMMA, D-NMMA had no significant effect on DNA fragmentation and cell death (Fig. 3). Confocal studies confirmed that NO staining was absent in vacuoles. In cells with amyloplasts, staining was preferentially observed around those organelles (Fig. 1H). Simultaneous

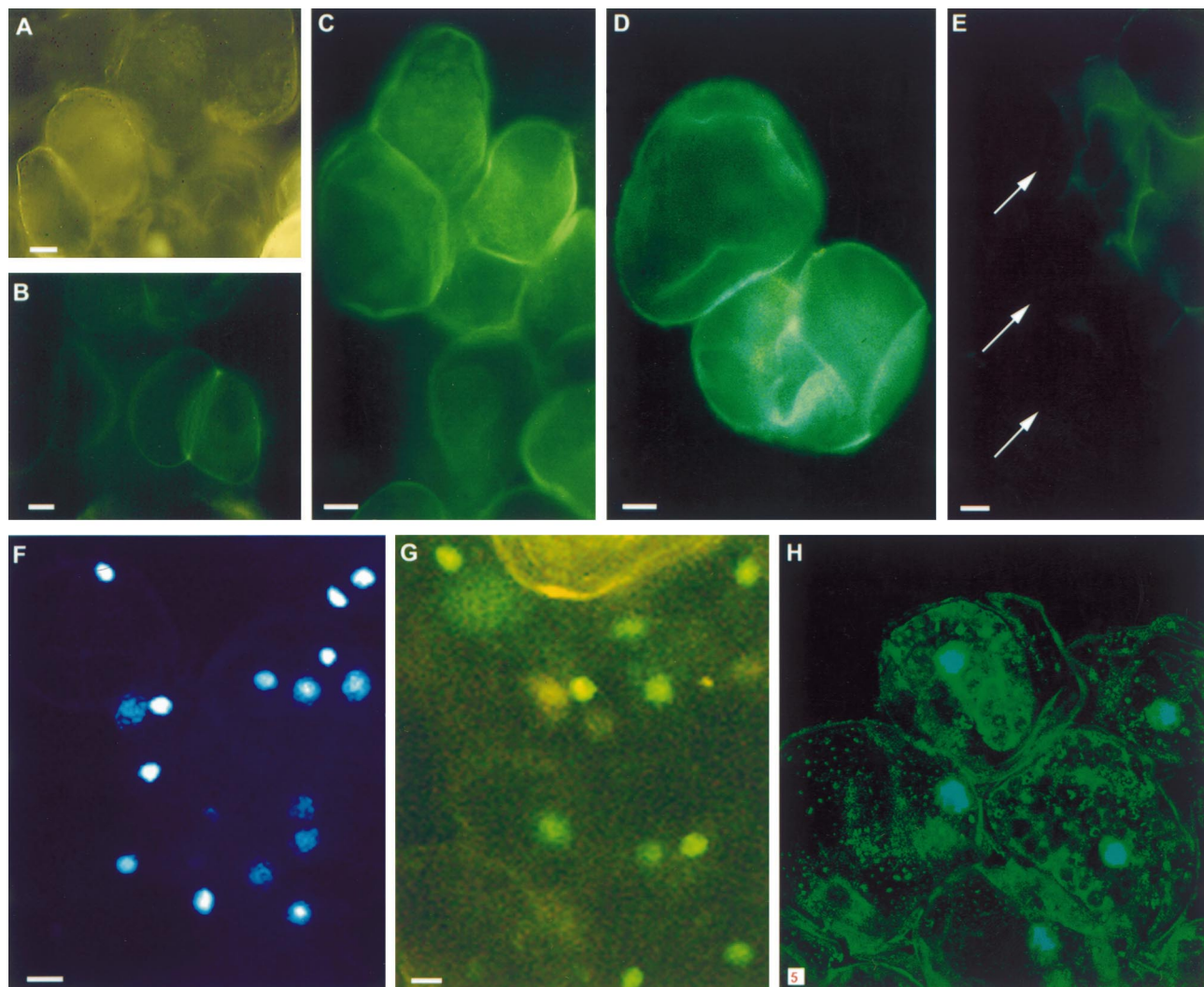


Fig. 1. Visualization of nitric oxide and nuclear DNA fragmentation in *T. brevifolia* cells. (A–G) Fluorescence microscopy images (excitation at 450–490 nm; emission 520 nm); (A) cell wall autofluorescence (yellow) in the absence of any staining (control for TUNEL and DAF-2 DA; cells mounted in water); (B) DAF-2 DA-positive control cells (green) stained for NO (cultured at 1 g without any further treatment). Note the presence of some unstained cells and the intensity of the fluorescence compared with (C) and (D); (C) increase of NO (DAF-2 DA-positive cells; green) after incubation in a medium containing 10^{-4} M SNP (see Fig. 2); (D) increase of NO (DAF-2 DA-positive cells; green) after induction of cell death by centrifugation at 150 g (see Fig. 2); (E) inhibition of NO synthesis after centrifugation in the presence of 5 mM L-NMMA, a NOS inhibitor (arrows) (see Fig. 2; Centrif + In). A few cells (upper right) showed low levels of NO; (F) DAPI-positive nuclei (blue/white) on cells cultured at 1 g; (G) TUNEL-positive nuclei (bright green) indicative of irreversible DNA fragmentation and consequent cell death, after incubation in a medium with SNP or after centrifugation; (H) confocal merged image (UV-FITC channels) of centrifuged *Taxus* cells showing NO (green cytoplasm) and TUNEL-positive nuclei, indicative of DNA fragmentation. Note the drastic plasmolysis a feature of cell death by apoptosis in *Taxus* cells. (A–C, E) Bar, 16 μ m; (D) bar, 13 μ m; (F–G) bar, 10 μ m; (H) bar, 5 μ m.

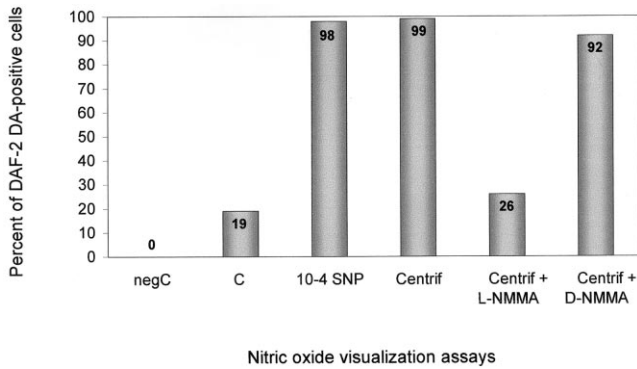


Fig. 2. Effect of SNP and centrifugation on NO production expressed as percentage of DAF-2 DA-positive cells of *T. brevifolia*. NO was visualized by fluorescence microscopy after 1 h in a medium with 10 μ M DAF-2 DA. negC, negative controls, dead cells incubated in $1 \times g$ for 3 h and stained with DAF-2 DA; live cells incubated in the same conditions and mounted in water; C, cells incubated in $1 \times g$ for 3 h in a medium without SNP. No significant differences were recorded between this control and the additional controls tested (cells incubated in medium supplemented with NO_3^- or NO_2^- , or without L-arginine supplementation); 10^{-4} M SNP, cells incubated in $1 \times g$ for 3 h in medium containing 10^{-4} M SNP; centrif, cells centrifuged at $150 \times g$ for 3 h, to physically induce cell death; Centrif + L-NMMA, cells centrifuged at $150 \times g$ for 3 h in the presence of 0.5 mM L-NMMA. Centrif + D-NMMA, cells centrifuged at $150 \times g$ for 3 h in the presence of 0.5 mM D-NMMA.

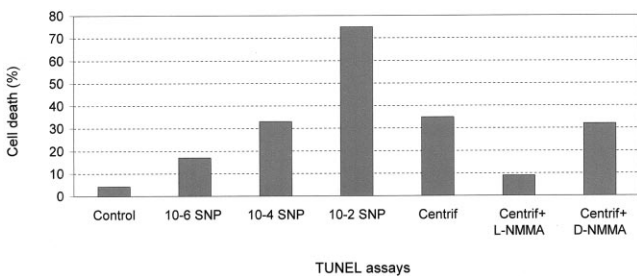


Fig. 3. Percent of cell death in haploid egg-derived cultures of *T. brevifolia* as a function of nitric oxide levels (also see Fig. 2). Control, cells incubated in $1 \times g$ for 3 h without SNP; 10^{-6} , 10^{-4} or 10^{-2} M, cells incubated in $1 \times g$ for 3 h in medium with 10^{-6} , 10^{-4} or 10^{-2} M of SNP; Centrif, cells centrifuged at $150 \times g$ for 3 h; Centrif + L-NMMA, cells centrifuged at $150 \times g$ for 3 h in presence of 0.5 mM L-NMMA; Centrif + D-NMMA, cells centrifuged at $150 \times g$ for 3 h in presence 0.5 mM D-NMMA. TUNEL was negative in negative controls (no TUNEL enzyme) and positive, for samples treated with DNase-I (positive controls). S.D. was lower than 8%.

detection of NO and DNA fragmentation showed that cells with TUNEL-positive nuclei were also positive for NO (Fig. 1H).

4. Discussion

4.1. Nitric oxide visualization

Our results showed that the protocol developed for the visualization of NO in animal systems can also be applied to plant cells [17]. As expected, low levels of NO were visually detected in some cells cultured at unit gravity (controls; see Fig. 2). Basal levels of NO in non-treated control cells have also been reported in animal systems [17]. Addition of NO_3^- or NO_2^- did not significantly affect the number of NO-stained cells. This result is important as it confirms that DAF-2 DA does not react with nitrate and/or nitrite. It also shows that, at least in short (3 h) incubation treatments, nitrate and/or nitrite were not used for NO production [21].

NO was detected in the cytosol of *Taxus* cells. This observation is in accordance with the subcellular localization of NOS previously reported for maize cells [12] and epidermal cells of *Kalanchoë daigremontiana* [19]. Recent reports on *Pisum sativum* [18] and *K. daigremontiana* [19] indicate that NOS is present in peroxisomes and chloroplasts of leaf cells.

As NO production through NOS depends on the presence of L-arginine, protocols developed for animal systems include L-arginine in the incubation media [17]. Our results showed that in *Taxus*, medium supplementation with L-arginine is not required. This can be explained by the fact that *Taxus* cells are rich in L-arginine when cultured on medium with ammonia as the main nitrogen source [36,37]. In *Taxus*, contrary to animal cells, an exogenous supply of substrate was not required to ascertain NOS activity. However, we can not yet rule out differences due to the effect of nitrate and/or nitrite supplementation on other experimental conditions.

Incubation in SNP or centrifugation at 150 times unit gravity (severe mechanical stress) led to a drastic increase in NO. The treatment of soybean cell suspensions with 0.5 mM SNP generated a steady state NO concentration of 2×10^{-6} M [1]. In our work, the predicted increases in NO generation by increasing concentrations of 10^{-6} , 10^{-4} and 10^{-2} M SNP were clearly visualized. *S*-nitroso-*N*-acetyl-penicillamine (SNAP), another NO donor which has been recently tested in these cells (Pedroso and Durzan, unpublished results) also led to a drastic increase in the percentage of

DAF-2 DA-stained cells. This result confirmed that the effect reported here for SNP was not due to its action on mitochondrial cytochrome *c* oxidase [4,38].

Centrifugation (150 times unit gravity, for 3 h) significantly increased the number of *Taxus* cells stained for NO. Increases in NO levels as a response to stressful conditions have been reported for other plants [1,2,4,6,9]. Pea and carnation plants increased NO emissions to the atmosphere in response to drought stress [6]. A burst in NO has also been reported to occur during hypersensitive response [1,2,4,10].

L-NMMA, a NOS inhibitor, significantly decreased NO production in centrifuged cells. Centrifugation in the presence of D-NMMA, which has no significant effect on animal NOS [35] (negative control), enabled the demonstration that L-NMMA effectively prevented NO production in 66% of the centrifuged cells. However, NO production in 150 times unit gravity, in the presence of the inhibitor, was higher than that detected for controls in unit gravity. One explanation may be that NOS activity was not completely inhibited by the amount of L-NMMA used. Alternatively, the time lapse between the addition of the NOS inhibitor (ca. 10 min) and the actual enzyme inhibition (not determined), might have accounted for that difference. It is also important to point out that, (1) NO production in plants may also occur as a by-product of constitutive NAD(P)H-nitrate reductase during nitrogen assimilation [20,21]; (2) considering that *Taxus* cells are rich in L-arginine and metabolically related monosubstituted guanidino compounds [36,37], we can not yet rule out differences due to the effects of endogenously produced guanidino products of arginine metabolism, as agmatine and γ -guanidinobutyric acid, among others. However, recent results [19] reaffirm that higher concentrations of NOS inhibitors (L-NMMA and others) are necessary to achieve complete inhibition of NO production.

4.2. Detection of cell death

In *T. brevifolia*, centrifugation was shown to physically induce programmed cell death [31]. In the present work, the addition of SNP or centrifugation, both led to an increase in the number of cells stained for NO (NO burst), which preceded a significant increase in cell death. No TUNEL or

cytological differences were detected between SNP- and centrifugation-induced cell death. Centrifugation of *Taxus* cells in the presence of a NOS inhibitor, L-NMMA, significantly decreased the NO production and cell death. This suggests that NO produced by a NOS-like activity may be involved in programmed cell death in this species. Our results are in accordance with the earlier reports in mouse and rat cells, where the increase in NO production, produced enzymatically or generated by SNP, led to DNA damage [14,39–41] and to programmed cell death [23,41].

NO reacts with superoxide to form peroxynitrite (ONOO⁻) [42]. One predicted reaction of ONOO⁻ is the nitration of tyrosine residues in proteins [43,44]. This post-translational modification would affect cell cycling and alter the structure of tyrosine residues in cell walls. DAF-2 green fluorescence at the cell wall might indicate the local presence of NO. In fact, nitro-tyrosine was detected in *Taxus* cells following SNP and centrifugation treatments (Durzan, pers. commun.).

In the present experiments, the effect of NO might have been magnified by the stress-induced formation of paclitaxel and other taxanes [45], and by monosubstituted guanidines that occur naturally in *T. brevifolia* [36,37,46,47]. Al-almi et al. [48] showed that NO played a vital role in mediating paclitaxel-induced programmed cell death by monocytic cells. The exposure of animal cells to paclitaxel increases protein-tyrosine phosphorylation in macrophages [49]. Paclitaxel (Taxol®), an anti-mitotic taxane produced by *T. brevifolia*, when fed to animal cells induces P 53-independent programmed cell death [49,50]. The present results do not rule out the possibility that the NO-mediated cell death in *T. brevifolia* could be operative through a similar signaling pathway [31].

It is important to point out, however, that NO has been reported to have a protective effect against oxidative stress in plants [10,38,51–57], reducing DNA fragmentation and consequently cell death [51,52]. SNP-treated potato plants were resistant to chlorosis, ion leakage, DNA fragmentation and apoptotic-like cell death produced by methylviologen herbicide diquat [56]. Recent evidence suggests that the combination of NO with ROS can either have a toxic or protective effect depending on the circumstances [9,51]. In *K. daigremontiana*, NO (and NO modulators) showed to have a differential effect on leaf-plantlet formation

in different gravity environments [58]. Using L-NMMA, we demonstrated that NO is implicated in the death of *Taxus* cells under stressful conditions. Our results do not exclude the possibility that under different circumstances NO can have a protective role in *Taxus* cells.

In addition to interacting with ROS, NO appears to activate defense responses, at least in part, through a salicylic acid (SA)-dependent signaling pathway [10]. The hypothesis that SA mediates NO-induced *Taxus* cell death during centrifugation also merits investigation.

In summary, DAF-2 DA can be used to visualize NO in plant cells. Centrifugation (mechanical stress) increased NO production and cell death in *Taxus* cultures. Addition of SNP had a similar effect. L-NMMA significantly decreased NO production and cell death under stressful conditions.

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References

- [1] M. Delledonne, Y. Xia, R.A. Dixon, C. Lamb, Nitric oxide functions as a signal in plant disease resistance, *Nature* 394 (1998) 585–588.
- [2] J. Durner, D. Wendehenne, F. Klessig, Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose, *Proc. Natl. Acad. Sci. USA* 95 (1998) 10328–10333.
- [3] D.G. Gilchrist, Programmed cell death in plant disease: the purpose and promise of cellular suicide, *Annu. Rev. Phytopathol.* 36 (1998) 393–414.
- [4] W. Van Camp, M. Van Montagu, D. Inzé, H₂O₂ and NO: redox signals in disease resistance, *Trends Plant Sci.* 3 (1998) 330–334.
- [5] Y.Y. Leshem, E. Haramathy, The characterization and contrasting effects of the nitric oxide free radical in vegetative stress and senescence of *Pisum sativum* Linn. foliage, *J. Plant Physiol.* 148 (1996) 258–263.
- [6] Y.Y. Leshem, Nitric oxide in biological systems, *Plant Growth Regul.* 18 (1996) 155–159.
- [7] E. Haramathy, Y.Y. Leshem, Ethylene regulation by the nitric oxide (NO) free radical: a possible mode of action of endogenous NO, in: Kanellis, et al. (Eds.), *Biology and Biotechnology of the Plant Hormone Ethylene*, Kluwer Academic Publishers, Dordrecht, 1997, pp. 253–258.
- [8] Y.Y. Leshem, R.B.H. Wills, V.-V.V. Ku, Evidence for function of the free radical gas-nitric oxide (NO)- as an endogenous maturation and senescence regulating factor in higher plants, *Plant Physiol. Biochem.* 36 (1998) 825–833.
- [9] J.R. Magalhaes, M.C. Pedroso, D.J. Durzan, Nitric oxide, apoptosis and plant stresses, *Physiol. Plant Mol. Biol.* 5 (1999) 115–125.
- [10] J.D. Durner, D.F. Klessig, Nitric oxide as a signal in plants, *Curr. Opin. Plant Biol.* 2 (1999) 369–374.
- [11] M. Cueto, O. Hernandez-Perera, R. Martin, M.L. Buntura, J. Rodrigo, S. Lamas, M.P. Golvano, Presence of nitric oxide synthase activity in roots and nodules of *Lipinus albus*, *FEBS Lett.* 398 (1996) 159–164.
- [12] E.A. Ribeiro Jr, F.Q. Cunha, W.M.S.C. Tamashiro, I.S. Martins, Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells, *FEBS Lett.* 445 (1999) 283–286.
- [13] J.R. Lancaster Jr, Nitric oxide in cells, *Am. Scientist* 80 (1992) 248–259.
- [14] U.K. MeBmer, M. Ankarcrona, P. Nicotera, B. Brüne, p53 Expression in nitric oxide-induced programmed cell death, *FEBS Lett.* 355 (1994) 23–26.
- [15] K. Xie, S. Huang, Y. Wang, P.J. Beltran, S.H. Juang, Z. Dong, J.C. Reed, T.J. McDonnell, D.J. McConkey, I.J. Fidler, Bcl-2 protects cells from cytokine-induced nitric-oxide-dependent programmed cell death, *Cancer Immunol. Immunother.* 43 (1996) 109–115.
- [16] H. Kojima, K. Sakurai, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Nagano, Development of a fluorescent indicator for nitric oxide based on the fluorescence chromophore, *Chem. Pharm. Bull.* 46 (1998a) 373–375.
- [17] H. Kojima, N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Nagano, Detection and imaging of nitric oxide with novel fluorescent indicators: diamino fluoresceins, *Anal. Chem.* 70 (1998b) 2446–2453.
- [18] J.B. Barroso, F.J. Corpas, A. Car0reras, L.M. Sandalio, R. Valderrama, J.M. Palma, J.A. Lupianez, L.A. del Rio, Localization of nitric-oxide synthase in plant peroxisomes, *J. Biol. Chem.* 274 (1999) 36729–36733.
- [19] M.C. Pedroso, J.R. Magalhaes, D.J. Durzan, A nitric oxide burst precedes apoptosis in angiosperm and gymnosperm callus cells and foliar tissues, *J. Exp. Bot.*, in press.
- [20] J.V. Dean, J.E. Harper, The conversion of nitrite to nitrogen oxide(s) by the constitutive NAD(P)H-nitrate reductase enzyme from soybean, *Plant Physiol.* 88 (1988) 389–395.
- [21] H. Yamasaki, Y. Sakihama, S. Takahashi, An alternative pathway for nitric oxide production in plants: new feature of an old enzyme, *Trends Plant Sci.* 4 (1999) 128–129.
- [22] R. Rucki, Sodium nitroprusside, in: K. Florey (Ed.), *Analytical Profiles of Drug Substances*, vol. 6, Academic Press, New York, 1997, pp. 781–792.
- [23] S. Dimmerler, A.M. Zeiher, Nitric oxide and programmed cell death: another paradigm for the doubled edged role of nitric oxide, *Nitric Oxide* 1 (1997) 275–281.

- [24] P. Nicotera, B. Brune, G. Bagetta, Nitric oxide: inducer or suppressor of programmed cell death?, *Trends Pharmacol. Sci.* 18 (1997) 189–190.
- [25] Y.Y. Shen, X.L. Wang, D.E. Wilcken, Nitric oxide induces and inhibits programmed cell death through different pathways, *FEBS Lett.* 433 (1998) 1125–1131.
- [26] N. Nakatsubo, H. Kojima, K. Kikuchi, H. Nagoshi, Y. Hirata, D. Maeda, Y. Imai, T. Irimura, T. Nagano, Direct evidence of nitric oxide presence from bovine aortic endothelial cells using new fluorescence indicators: diaminofluoresceins, *FEBS Lett.* 427 (1998) 263–266.
- [27] P.R. Bell, Commentary. Apomictic features revealed in a conifer, *Int. J. Plant Sci.* 155 (1994) 621–622.
- [28] L. Havel, D.J. Durzan, Apoptosis in plant, *Botanica Acta* 109 (1996) 1–10.
- [29] L. Havel, D.J. Durzan, Apoptosis during diploid parthenogenesis and early somatic embryogenesis of Norway Spruce, *Int. J. Plant Sci.* 157 (1996b) 8–16.
- [30] L. Havel, D.J. Durzan, Apoptosis during early somatic embryogenesis in *Picea* sp, in: S.M. Jain, P.K. Gupta, R.J. Newton (Eds.), *Somatic Embryogenesis in Woody Plants*, vol. IV, Kluwer Academic Publishers, Dordrecht, 1998, pp. 125–147.
- [31] D.J. Durzan, Gravisensing, programmed cell death, and drug recovery in *Taxus* cell suspensions, *Am. Soc. Grav. Space Biol. Bull.* 12 47–55.
- [32] M.C. Pedroso, D. Durzan, Effect of hypergravity and simulated hypogravity on leaf-plantlet development and asexual reproduction in *Kalanchoë daigremontiana*, *Grav. Space Biol. Bull.* 12 (1998) 74–146.
- [33] M.C. Pedroso, D. Durzan, Detection of apoptosis in chloroplasts and nuclei in different gravitational environments, *J. Grav. Biol.* 6 (1999) 19–20.
- [34] D.J. Durzan, F. Ventimiglia, Free taxanes and the release of bound compounds having taxane antibody reactivity by xylanase in female haploid-derived cell suspension cultures of *Taxus brevifolia*, *In Vitro Cell Dev. Biol.* 30P (1994) 219–227.
- [35] M.A. Marletta, Cellular signaling with nitric oxide, in: *Calbiochem Signal Transduction Catalog and Technical Resource*, Calbiochem-Novabiochem, San Diego, CA, USA, 1999, pp. 419–422.
- [36] R.G.S. Bidwell, D.J. Durzan, Some recent aspects of nitrogen metabolism, in: P.J. Davies (Ed.), *Historical and Current Aspects of Plant Physiology*, Cornell University Press, Ithaca, NY, 1975, pp. 152–225.
- [37] D.J. Durzan, F.C. Steward, Nitrogen metabolism, in: F.C. Steward (Ed.), *Plant Physiology. An Advanced Treatise*, vol. VIII, 1983, pp. 55–265.
- [38] A.H. Millar, D.A. Day, Nitric oxide inhibits the cytochrome *c* oxidase but not the alternative oxidase of plant mitochondria, *FEBS Lett.* 398 (1996) 155–158.
- [39] D.A. Wink, S.K. Kasprzak, C.M. Maragos, R.K. Ele-spuru, M. Misra, T.M. Dunams, T.A. Cebula, W.H. Koch, A.W. Andrews, J.S. Allen, L.K. Keefer, DNA deaminating ability and genotoxicity of nitric oxide and its progenitors, *Science* 254 (1991) 1001–1003.
- [40] S.R. Tannenbaum, Effects of NO on DNA damage and DNA synthesis, In: *Nitric Oxide: Basic Research and Clinical Applications*, Extensive abstracts <http://www.pasteur.fr/Conf/nitric-abstracts.html>, 1998.
- [41] U.K. Messmer, B. Brune, Nitric oxide-induced programmed cell death: P 53-dependent and P 53-independent signaling pathways, *Biochem. J.* 1 (1996) 299–305.
- [42] C. Ducrocq, B. Blanchard, B. Pignatelli, H. Oshima, Peroxynitrite: an endogenous oxidizing and nitrating agent, *Cell. Mol. Life Sci.* 55 (1999) 1068–1077.
- [43] J.P. Eiserich, C.E. Cross, A.D. Jones, B. Halliwell, A. Vliet, Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid, *J. Biol. Chem.* 271 (1996) 19199–19208.
- [44] A. Vliet, J.P. Eiserich, B. Halliwell, C.E. Cross, Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite, *J. Biol. Chem.* 272 (1997) 7617–7625.
- [45] G. Appendino, Phytochemistry of the yew tree, *Natl. Prod. Reports* 12 (1995) 349–360.
- [46] D.J. Durzan, F. Ventimiglia, L. Havel, Taxane recovery from cells of *Taxus* in micro- and hypergravity, *Acta Astronautica* 42 (1998) 455–463.
- [47] O.K. Kim, A. Murakami, Y. Nakamura, H. Ohigashi, Screening of edible Japanese plants for nitric oxide generation inhibitory activities in RAW 264.7 cells, *Cancer Lett.* 125 (1998) 199–207.
- [48] O. Al-almi, J. Sammons, J.H. Martin, H.T. Hassan, Divergent effect of taxol on proliferation, programmed cell death and nitric oxide presence in MHH 225 CD 34 positive and U937 CD34 negative human leukaemia cells, *Leukaemia Res.* 22 (1998) 939–945.
- [49] C.L. Manthey, M.E. Brandes, P.Y. Perera, S.N. Vogel, Taxol increases steady state levels of lipopolysaccharide-inducible genes and protein-tyrosine phosphorylation in murine macrophages, *J. Immunol.* 149 (1992) 2459–2465.
- [50] S.S. Lanni, S.W. Lowe, E.J. Lictria, J.O. Liu, T. Jacks, P-53-independent programmed cell death induced by paclitaxel through an indirect mechanism, *Proc. Natl. Acad. Sci. USA* 94 (1997) 9679–9683.
- [51] M.V. Beligni, L. Lamattina, Is nitric oxide toxic or protective?, *Trends Plant Sci.* 4 (1999) 299–300.
- [52] M.V. Beligni, L. Lamattina, Nitric oxide counteracts cytotoxic processes mediated by reactive oxygen species in plant tissues, *Planta* 208 (1999) 337–344.
- [53] D.A. Navarre, D. Wendehenne, J. Durner, R. Noad, D.F. Klessig, Nitric oxide modulates the activity of tobacco aconitase, *Plant Physiol.* 122 (2000) 573–582.
- [54] A. Caro, S. Puntarulo, Nitric oxide decreases superoxide anion generation by microsomes from soybean embryonic axes, *Physiol. Plant* 104 (1998) 357–364.
- [55] J. Dangl, Plants just say NO to pathogens, *Nature* 394 (1998) 525–527.
- [56] M.V. Beligni, L. Lamattina, Nitric oxide protects against cellular damage produced by methylviologen herbicides in potato plants, *Nitric oxide: Biol. Chem.* 3 (1999) 199–208.
- [57] B. Halliwell, K. Zao, M. Whiteman, Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of the recent controversies, *Free Radic. Res.* 31 (1999) 651–669.
- [58] M.C. Pedroso, D.J. Durzan, Leaf-plantlet formation in different gravity environments is affected by nitric oxide, *Grav. Space Biol. Bull.* 13 (1999) 31–66.