Nitric oxide and hydrogen peroxide involvement during programmed cell death of *Sechium edule* Sw. nucellus

Lara Lombardi[a], Nello Ceccarelli[b], Piero Picciarelli[b], Carlo Sorce[a] and Roberto Lorenzi[a].

a) Department of Biology University of Pisa, Via Ghini 5, 56126 Pisa (Italy)

b) Department of Crop Plant Biology University of Pisa, Via Mariscoglio 34, 56124 (Italy)

Author for correspondence: Lara Lombardi

Via Ghini 5, 56126 Pisa (Italy)

Tel: +39 0502211337

Fax: +39 0502211309

E-mail: llombardi@biologia.unipi.it

Number of figures: 7

Date of submission:

Running title: NO and H_2O_2 involvement in nucellus programmed cell death.

Abstract

The nucellus is a maternal tissue that feeds the developing embryo and the secondary endosperm. During seed development the cells of the nucellus suffer a degenerative process early after

fertilization as the cellular endosperm expands and accumulates reserves. Nucellar cell degeneration has been characterized as a form of developmentally programmed cell death (PCD).

In this work we show that nucellus PCD is accompanied by a considerable production of both nitric oxide and hydrogen peroxide (NO and H_2O_2). Interestingly, each of the two molecules is able to induce the production of the other and to cause cell death when applied to a living nucellus. We show that the induced cell death has features of a PCD characterized by apoptotic hallmarks. Evidences show that cell death is accompanied by profound changes in the morphology of the nuclei and by a huge degradation of nuclear DNA. Moreover we report that NO and H_2O_2 cause an induction of caspase-like proteases previously characterized in physiological nucellar PCD.

Key words*: caspase, cell death, DNA fragmentation, hydrogen peroxide, nitric oxide, nucellus, Sechium edule.*

Abbreviations:

c-PTIO: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline -1-oxyl-3 oxide

CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

DAPI: 4, 6-diamidino-2-25 phenylindole

DMSO: dimethyl sulfoxide

DTT: dithiothreitol

FM 4-64: N-(3- triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl)-hexatrienyl) pyridinium dibromide

HEPES. 4-2-idroxyethil-1-piperazinil-ethanesulfonic acid

L-NAME: N-nitro-L-arginine methyl ester

TUNEL: terminal dUTP nick-end labeling

Introduction

The degeneration of the maternal tissue nucellus in flowering plants is a default process in ovule ontogeny; it takes place right after fertilization to supply the young embryo and the expanding endosperm with nutrients. The major seed storage organ, the endosperm, expands and accumulates reserves at the expense of the nucellus, which is no longer present in mature seeds (Chen and Foolad, 1997; Xu and Chye, 1999; Dominguez *et al*., 2001; Hiratsuka *et al*., 2002).

Nucellar cell degeneration has been described and characterized in several plant species including cotton (Jensen, 1975), barley (Norstog, 1974; Chen and Foolad, 1997), *Oenothera biennis* (deHalac, 1980) and *Ricinus communis* (Greenwood *et al*., 2005). The ultrastructural and cytological alterations reported lead up to the hypothesis that nucellar degeneration occurs by means of a developmentally regulated programmed cell death (PCD).

The large *Sechium edule* seed represents an interesting model system to study nucellar degeneration. Autolysis of the nucellus gradually leaves a cavity inside the ovule that is filled by both the growing endosperm and embryo. Noteworthy, during seed development the extent of nucellar degeneration precisely coincides with the progression of endosperm growth and the time-course of this process can be easily appreciated even with naked eye. Cell death in the nucellus has been recognized as a form of PCD being characterized by several PCD hallmarks as chromatin condensation, profound alterations in nuclear shape, nuclear DNA fragmentation and caspase-like proteases activation (Lombardi *et al*., 2007). The fine temporal and spatial regulation of this phenomenon suggests the presence of an integrated network of signals, coming from the nucellus itself or from the surrounding tissues, able to induce and regulate the execution of the whole process. At the moment, nothing is known about the molecules possibly involved in the induction of nucellar PCD.

Reactive oxygen species (ROS) are now well recognized triggers of cell death in many plant systems (Overmyer *et al*., 2003; Van Breusegem and Dat, 2006). ROS may act as signalling molecules that diffuse from sites of synthesis to target cells or, alternatively, cells committed to die may kill themselves producing high levels of ROS. In fact, ROS homeostasis provides a baseline

level on which spikes of ROS can act as triggers for different physiological processes and the delicate balance between production and scavenging determines whether the cell lives or dies. ROS signals interact directly or indirectly with several other small molecules such as nitric oxide, salicylic acid, jasmonate and ethylene, which are crucial partners in determining cell fate and cell response to various stresses (Overmyer *et al*., 2003; Zaninotto *et al*., 2006).

Nitric oxide (NO) and hydrogen peroxide (H_2O_2), having simple structure, high diffusivity and small dimension, have all the properties for being key signalling molecules in many plant processes (Neill *et al*., 2002; Delledonne, 2005; Besson-Bard *et al*., 2008). It has been increasingly evident that NO plays important roles during seed germination and dormancy, xylogenesis, flowering, stomatal closure, pathogen defence and PCD (Beligni and Lamattina, 2000; Bethke *et al*., 2006; Leitner *et al*., 2009). NO, interplaying with ROS and other small molecules, is a fundamental part of many signal transduction pathways (Qiao and Fan, 2008) and it participates in the regulation of multiple responses toward a variety of abiotic and biotic stresses such as drought, heat and pathogen infection (Garcia-Mata and Lamattina, 2002; Uchida *et al*., 2002; Arasimowicz and Floryszak-Wieczorek, 2007; Mur *et al*., 2006). There is evidence that NO can regulate enzyme activity by S-nitrosylation (Lindermayr *et al*., 2005) and can modulate gene expression (Grun *et al*., 2006; Palmieri *et al.*, 2008).

H_2O_2 participates in many defence and resistance mechanisms, including phytoalexin production and reinforcement of the cell wall (Dempsey and Klessig, 1994). Recently it has been demonstrated that H_2O_2 not only is a defensive molecule but also functions as a key regulator of many plant processes (Mittler *et al*., 2004; Bright *et al*., 2006; Quan *et al*., 2008) and has been recognized as a regulator of the expression of some genes (Neill *et al*., 2002). H_2O_2 acts as a signalling molecule during PCD (Gechev and Hille, 2005): perturbation of H_2O_2 homeostasis through alteration of catalase activity increases oxidative stress and induce cell death (Dat *et al*., 2003; Palma and Kermode, 2003).

NO and H_2O_2 are simultaneously present during various physiological processes and sometimes NO and H_2O_2 production are interdependent (Bright *et al*., 2006; Zhao *et al*., 2007). During ABA-dependent stomatal closure both NO and H_2O_2 plays interconnected roles as signalling molecules (Dong *et al*., 2005; He *et al*., 2005) and, even if the modality of their interaction is still unclear, their functions may converge on MAPK signalling pathways (Desikan *et al*., 2004). Cross-talk between the two molecules is essential for the execution of PCD in some systems. For example, during the defence against pathogens a fine balance between NO and H_2O_2 modulates the process and determines when cell death is activated (Delledonne *et al*., 2001; Hanckock *et al*., 2002). Nitric oxide can induce cell death in carrot cells but its efficacy increases in the presence of H_2O_2 (Zottini et al., 2002).

The present study aims to investigate the involvement of NO and H_2O_2 during PCD of *S. edule* nucellus and their role as signalling molecules. By using pharmacological and biochemical approaches, we studied NO and H_2O_2 production in dying cells. To clarify the ability of these two molecules to induce PCD in living nucellar tissue we analysed intact nucellus treated with NO and H_2O_2 and we could report the appearance of some apoptotic hallmarks. Because of the previous observations we were also interested in elucidating the inter-relationship between NO and H_2O_2 and we were able to demonstrate that the two molecules are able to induce each other.

Materials and methods

Plant material and treatments

Plants of *Sechium edule* Sw. (Cucurbitaceae) were grown in the field and fruits harvested starting from the end of September. Seeds were cut longitudinally and the two halves separated; nucellar tissue was gently removed from the seed and, if not immediately used, stored at –80 °C. All material was kept in an ice bath during the whole procedure.

Intact nucella were incubated for 2 h in the NO donor sodium nitroprusside (SNP, Sigma) 1 mM or for 2 h in 3 mM H_2O_2, both dissolved in 20 mM Na-phosphate buffer pH 7.4. To study the effect of

inhibitors and scavengers, the NO scavenger c-PTIO (Sigma) was applied at the concentration of 200 μM and the nitric oxide synthase (NOS) inhibitor L-NAME (Sigma) was used at the concentration of 1 mM.

Viability staining

Viability of cells in intact nucellus excised from the seed or after treatments was determined by the double staining with fluorescein diacetate (FDA, Sigma) and FM 4-64 (Sigma) as previously described (Lombardi et al., 2007). FDA enters the cells where esterase cleaves off the acetate residues leaving fluorescein, which then accumulates and fluoresces leading to a yellow-green staining of the cytoplasm. FM 4-64 only penetrates dead or damaged cells leading to a red staining of the cellular contents (Fath *et al*., 2001).

Nucella were observed under a Leica DMLB microscope and images captured by a Leica DC 300F CCD camera. An argon and a krypton laser were used for visualization of the FDA (λex 495 nm, λem 518) and FM 4-64 (λex 515, λem 625) signals, respectively.

NO determination and visualization

NO accumulation in intact nucellus was visualized using the specific fluorescent dye 4,5 diaminofluorescein diacetate (DAF-2DA; Sigma). The fluorescent probe DAF-2DA is highly specific for NO and does not react with other ROS (Kojima et al., 1998). It permeates through the cell membrane and can be de-acetylated by either extracellular or intercellular esterases. It then reacts with NO to make a fluorescent compound. Nucella were removed from the seeds and incubated in the dark for 1 h, at 25°C with 10 μM DAF-2DA dissolved in 20 mM Na-phosphate buffer pH 6,8. After staining, samples were washed twice in the fresh buffer to remove excess fluorophore, and mounted in buffer on microscopic slides. Green fluorescence was then observed under a Leica DMLB microscope (λex 495 nm, λem 518) and images captured by a Leica DC 300F CCD camera.

NO content in nucellus was determined by a fluorometric assay based on DAF-2DA. Treated and untreated nucella were homogenized in mortar and pestle 0.5 g of tissue with 1 vol of 20 mM Na-phosphate buffer pH 6,8. After centrifugation, DAF-2DA at the final concentration of 20 μM was added to the cell free extract. After 30 min incubation in the dark, fluorescence was measured in a VersaFluor Fluorimeter (Biorad) (λex 495 nm, λem 518).

H_2O_2 determination and visualization

Hydrogen peroxide concentration in nucellus was determined by the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen), following the manufacturer's instructions. Briefly, 200 mg of tissue was homogenized in 1 vol of 50 mM Na-phosphate buffer pH 7.0. After a centrifugation at 17000 RPM for 15 min, the supernatant was mixed (1:1 v/v) with the reaction buffer and incubated 30 min in the dark. Absorbance was measured at 560 nm and H_2O_2 concentration was calculated from a standard curve made by hydrogen peroxide solutions ranging from 1 to 10 μM and expressed as nmol/g of fresh weight.

H_2O_2 was visualized in intact nucellus by the specific probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA), which is rapidly oxidised to highly fluorescent dichlorofluorescein (DCF) in the presence of H_2O_2. Nucellus excised from the seed was incubated in 20 mM Na-phosphate buffer pH 6,8 containing DCFH-DA 10 μM, at room temperature for1 h. After staining, samples were washed twice in the fresh buffer to remove excess fluorophore, and mounted in buffer on microscopic slides. Green fluorescence was then observed under a Leica DMLB microscope (λex 495 nm, λem 518) and images captured by a Leica DC 300F CCD camera.

Catalase activity

Catalase activity was measured by monitoring the disappearance of H_2O_2 at 240 nm according to the method of Beaumont *et al*., (1990). The enzymatic activity is reported as nmol H_2O_2 decomposed per min per mg of fresh weight (ε=43,6 $M^{-1}cm^{-1}$).

In situ detection of DNA fragmentation (TUNEL assay)

Nucellar tissue, treated or untreated, was fixed over night at 4°C in 4% (w/v) paraformaldehyde in phosphate buffer saline pH 7.4. After dehydration through ethanol series samples were embedded in Paraplast Plus (Sigma). Sections of 12 μm were cut and stretched on poly-lysine coated slides. The sections were then dewaxed in xylene and re-hydrated before examination.

TUNEL assay was performed using an "In Situ Cell Death Detection Kit" (Promega) according to the manufacturer's instructions. To facilitate the entry of TdT enzyme into the tissue sections, the slides were treated with proteinase K (20 mg ml^{-1}) for 20 min. The labelling reaction was performed at 37 °C in a humidified chamber in the dark for 1 h. A negative control was included in each experiment by omitting TdT from the reaction mixture. As a positive control, permeabilized sections were incubated with DNAse I (10U ml^{-1}) for 10 min before TUNEL assay. The yellow-green fluorescence of incorporated fluorescein-12-dUTP (λex 495 nm, λem 518) was observed using a Leica DMLB microscope and a Leica DC 300F CCD camera. Counter-stain was done with DAPI (1 μg/mL) (λex 358 nm, λem 461).

Experiments were repeated three times, each time labelling five slides for sample.

Light microscopy analysis

Collected nucella were fixed overnight at 4 °C in paraformaldehyde 4% in PBS pH 7.4. After dehydration through ethanol series (30%, 50%, 70%, 96% and 100%), samples were embedded in Paraplast Plus (Sigma). Sections 12 μm-thick were cut and stretched on poly-lysine coated slides. The tissue sections were dewaxed in xylene and rehydrated. After rehydration, the samples were stained with Hematoxylin or Fast Red (Trevigen), observed under a Leica DMLB microscope and images taken by a Leica DC 300F CCD camera. Nuclear diameter was measured with a dedicated program for images analysis, Zeiss Axio Vision 4.6.

In vitro caspase-like protease activity assay

Caspase-like activity was measured in the D and P regions and in nucellus after treatment with H_2O_2 3 mM for 2 h, SNP 1 mM for 2 h with or without c-PTIO 200 µM. Protein extracts were prepared by homogenization of nucellar tissue in 0.8 volumes of ice-cold extraction buffer (50 mM HEPES-KOH pH 7.0, 10% [w/v] sucrose, 0,1% [w/v] CHAPS, 5 mM DTT, 1 mM EDTA). After centrifugation for 10 min at 17000 RPM at 4°C, the supernatant was collected and immediately used or stored at –80°C. Proteolytic activity was measured in 500 µL reaction mixtures containing 50 µg protein and 75 µM of substrates specific for individual mammalian caspase. The following colorimetric substrates were used: Ac-YVAD-pNA, Ac-DEVD-pNA and Ac-VEID-pNA (all from Sigma) dissolved in DMSO. Reactions were incubated for 5 h at 32°C then absorbance at 405 nm was taken against a blank containing buffer and substrate alone. All assays were performed in triplicate.

Results

NO and H_2O_2 are produced by the nucellus during its physiological degeneration

In the degenerating nucellus, dying cells are mostly localized in the border region of the tissue proximal to the expanding edge of the developing endosperm, while the remaining portion of the tissue is alive (Fig 1). The width of the dying portion does not vary from the very early to the late stage of seed development. We refer to this marginal and narrow band of dying cells as "proximal region" (P region) while the rest of the living nucellus is called "distal region" (D region) (proximal or distal with respect to the endosperm). Cells located in the P region go through a process of programmed cell death for which we already identified many of the widely accepted apoptotic hallmarks (Lombardi *et al*., 2007).

Dying cells in the P region produce a noteworthy amount of nitric oxide that can be visualized and quantified by using the NO-specific fluorescent probe DAF-2DA. This probe has been widely used to identify NO and quantitatively measure its production in plant cells (RIF). During PCD cells of

the P region produce 2.2 times more NO than does the D region (Fig 2a). Fluorescence microscopy confirms that NO production is limited to the P region as in the D region fluorescence is very low or undetectable (Fig 2b).

Dying cells in the proximal region are also characterized by a consistent production of H_2O_2, which is also visualized *in situ* by the specific probe DCFH-DA (Fig. 2d). H_2O_2 concentration in cell free extract of the P region is 5,67 nmol/g of fresh weight, about 2.3 times higher than the one measured in the D region (Fig 2c). Interestingly, catalase activity in the P region is much lower than the basal activity reported in vital D region (Fig. 2e) suggesting a possible correlation between H_2O_2 increase and the reduction of the scavenging capability of dying cells in the P region.

H_2O_2 induces NO production and vice-versa

The observation that the increase of both NO and H_2O_2 is spatially and temporally related during physiological PCD that characterizes nucellus degeneration, prompted us to investigate if NO and H_2O_2 production could be interconnected in same way. In order to verify if the presence of one molecule could induce the production of the other, the living D region was treated either with H_2O_2 or with the nitric oxide donor sodium nitroprusside (SNP). On the basis of preliminary experiments and of indications arisen from the literature, we chose SNP 1 mM and H_2O_2 3 mM for all the experiments (Bethke and Jones, 2001; Houot et al., 2001; Murgia et al., 2004; Zhao *et al*., 2007; Ederli et al., 2009).

The exposure of living nucellus to exogenous H_2O_2 at the concentration of 3 mM for 2 hours elicited NO production. This induction by H_2O_2 is clearly visible in the D region after staining the whole nucellus with DAF-2DA (Fig. 3b); the fluorimetric *in vitro* assay indicates a 3 fold increase, a NO content quite higher than the one reported in the dying P region (Fig 2a). To test if a NOS-like activity could be considered the source of the nitric oxide produced as a consequence of H_2O_2 exposure, we added to the H_2O_2 treated nucella the mammalian nitric oxide synthase (NOS) inhibitor L-NAME that has been proven to be effective also in plant systems (Foissner et al., 2000;

Mur et al., 2006). Under these conditions, NO failed to accumulate, indicating that a nucellar NOS-like activity can be considered the prominent source of nitric oxide (Fig 3a,c).

We next investigated whether the addition of NO in nucellar cells could stimulate production of H_2O_2. Noteworthy, the effect seems to be reciprocal because a 2 hours treatment of the D region with SNP 1 mM induced a diffuse production of H_2O_2 in the D region (Fig 3e), where, in physiological conditions, it is almost undetectable (see Fig 2d). For an estimation of H_2O_2 concentration, we performed an *in vitro* assay, which showed that the increase in endogenous H_2O_2 is about 90% more than the control, untreated nucellus (Fig 3d). We made appropriate controls to ensure that the nucellar responses to SNP treatment were specifically due to NO and to exclude the possibility that the observed effects could instead be due to cyanide. H_2O_2 failed to accumulate in the treated nucellus when the NO scavenger c-PTIO was added together with SNP, and this was observed both *in vivo* and *in vitro* (Fig 3d,f). This led us to the conclusion that H_2O_2 production in the treated nucellus was actually enhanced by the action of NO.

We asked if, similarly to what happens in the P region during physiological cell death, this increment in H_2O_2 concentration in SNP treated nucellus could be attributed to a diminished activity of the scavenging enzyme catalase. SNP treatment on the nucellus indeed caused a remarkable decrease in catalase activity. However, since cyanide, released from SNP, is known to strongly inhibit catalase, we used the NO scavenger c-PTIO together with SNP to test the specificity of the NO effect. We observed that c-PTIO addition is ineffective; consequently we can affirm that the observed SNP effect on catalase activity is actually mediated by cyanide and not by nitric oxide (data not shown).

Exogenously applied H_2O_2 and NO induce cell death

Both NO and H_2O_2, besides inducing a mutual increase of concentration, caused an induction of cell death when applied to living and healthy samples of nucellar D region (Fig 4). As a consequence of treatment with 1 mM SNP or 3 mM H_2O_2 the red-orange fluorescence due to FM 4-64 inside

damaged or dead cells, was no more restricted to the P region but was extended to the D region (Fig 4b,c), which is vital in control untreated nucellus (green fluorescence in Fig 4a). The D region showed no sign of cell death when c-PTIO was added to the SNP treatment as a control, indicating that nucellar cell death was actually due to a signal initiated by NO and not a cyanide side effect (Fig 4d).

Some cytological markers were analysed to obtain information on the nature of cell death (PCD or necrosis) induced by the two treatments. As shown in Fig 5b and 5c cell death induced by both SNP and H_2O_2 treatment is characterized by cell shrinkage and membrane collapse. Treated cells completely lacked plastids otherwise visible in untreated control cells (Fig. 5a). Fast-red staining also showed the presence of nuclei suffering various morphological and structural changes, typical markers of an apoptotic-like cell death (Reape and McCabe, 2008). Compared to the nuclei present in the living nucellus (Fig 5d) nuclei in SNP-treated tissue appeared smaller, due to a pronounced chromatin condensation (Fig 5e). Average diameter of condensed, altered nuclei was 6 μm, one-third the diameter of control nuclei (18 μm). Nuclei lost their normal round shape and became in some cases stretched and elongated. Moreover, some nuclei acquired a half-moon shape, resembling what observed during animal apoptosis (Ihara *et al*., 1998).

On the other hand, H_2O_2 treatment, though causing some nuclear alterations, has a less intense effect on morphology (Fig 5f). In fact, not the totality of the nuclei in the H_2O_2 treated tissue suffered chromatin condensation and alterations in their shape, some of them remaining rounded and decondensed. Some nuclei showed condensed chromatin in punctuate structures at the periphery while others were widely condensed but still bigger than the ones observed after SNP treatment (average diameter 12 μm).

We investigated if these nuclear alterations were accompanied by the PCD hallmark of DNA fragmentation, by using the TUNEL assay. As shown in Fig. 6b no TUNEL-positive nuclei were present in the control D region, while nuclear DNA degradation became evident as a consequence of both NO and H_2O_2 treatment, as demonstrated by the presence of green-fluorescent TUNEL-

positive nuclei (Fig 6d, 6f). In detail, fluorescein label reveals DNA fragmentation in condensed morphologically altered nuclei yet evidenced by Fast-red staining. Noteworthy, non-apoptotic decondensed nuclei in H_2O_2 treated nucellus are TUNEL-negative (arrows in Fig 6e).
Appropriate control treatments were conducted for every set of slides. If the DNAse I treatment preceded the TUNEL assay, all nuclei were fluorescent (positive control, not shown). On the other hand, when TdT enzyme was omitted from the labelling mixture no fluorescence was observed (negative control, not shown).

Exogenously added H_2O_2 and NO affect nucellus caspase-like activities

In a previous work (Lombardi *et al*., 2007) we reported an induction of the two caspase-like activities YVADase and DEVDase in cells undergoing PCD in the P region. Noteworthy, treatments with 1mM SNP and 3 mM H_2O_2 were able to induce YVADase and DEVDase activity in the D region even if the increase in proteolytic activity was not as high as the one observed in the P region during PCD (Fig 7). Comparing the two treatments, H_2O_2 seems to be more effective on nucellar caspase-like proteases; in fact YVADase and DEVDase were 33-35% more active than the control when nucellus was exposed to H_2O_2 and only 20-25% more active after SNP treatment. The induction by NO is to be considered specific as YVADase and DEVDase activity is comparable to the control if the NO scavenger c-PTIO is added together with SNP during treatments of the nucellus.

Discussion

After fertilization, *Sechium edule* nucellus becomes a dispensable tissue and dies. Its degeneration is a typical example of physiological PCD, being precisely regulated during development as the ones of other seed tissues like aleurone and endosperm (Bethke *et al*., 1999; Young and Gallie, 1999). PCD in Sechium nucellus is well characterized from the cytological and the biochemical point of view (Lombardi *et al*., 2007), nevertheless the signals responsible for the induction and the

regulation of this degenerative process are still unknown. Molecules as diverse as hormones, ROS and nitric oxide have emerged as regulators of plant PCD in several systems (Bethke and Jones, 2001; Steffens and Sauter, 2005; Van Breusegem and Dat, 2006). Here we report that, *Sechium edule* nucellar cells undergoing PCD in the region proximal to the expanding edge of the endosperm produce NO and H_2O_2. Apart from their prominent role in stimulating cellular defence and in regulating many physiological processes, there is now compelling evidence for their function in regulating programmed cell death, both individually and together.

Generation of endogenous NO or H_2O_2 have been associated to PCD in some plant systems. For example, ozone-induced cell death in *Arabidopsis* leaves follows a rapid accumulation of nitric oxide (Ahlfors *et al*., 2009) and a NO burst has been visualized in *Taxus* and *Kalanchoe* cells undergoing PCD after being exposed to mechanical stress (Pedroso *et al*., 2000). *Arabidopsis* suspension cultures generate NO in response to avirulent bacteria, and this is sufficient to induce cell death (Clarke *et al*., 2000). On the other hand, H_2O_2 is also an important mediator of programmed cell death in guard cells of pea (Samuilov et al., 2008) and in epidermis of rice during adventitious roots formation (Steffens and Sauter, 2009). Moreover, *Arabidopsis* plants deficient in catalase undergo cell death for accumulation of H_2O_2 (Dat *et al*., 2003).

In addition to their individual roles, NO and H_2O_2 may also be simultaneously present during some plant responses. During HR, maybe the best studied example of H_2O_2 and NO mediated PCD, H_2O_2 operates in a dose-dependent manner in synergy with NO and the ratio of NO to H_2O_2 determines when cell death is activated (Delledonne *et al*., 2001; Zago *et al*., 2006). A simultaneous generation of NO and H_2O_2 is necessary also during the induction of PCD in tobacco and in *Arabidopsis* cells because H_2O_2 or NO alone are not sufficient to trigger cell death (de Pinto *et al*., 2002; Murgia *et al*., 2004). Endogenously produced NO and H_2O_2 also mediate the cytotoxic effect of cadmium in *Arabidopsis* suspension cultures (De Michele et al., 2009).

However, in the above reported examples, NO and H_2O_2 production is mainly a consequence of a cell death process initiated by exogenous treatments with pathogens or with other cell death triggers

on living tissues or cell cultures. The relevance of our results lays in the observation of an endogenous physiological generation of the two molecules by the intact nucellus undergoing PCD. In our system, NO and H_2O_2 production correlates with a developmentally regulated form of PCD. It's interesting to note that in the P region catalase enzymatic activity is compromised; it's tempting to speculate about a link between the physiological raise in H_2O_2 level and the decrease in the scavenging capability of the dying nucellar cells.

In addition to our work reported here, there are very few other reports of NO and H2O2 production during physiological PCD. For instance, H_2O_2 is related to natural senescence: its production increase with age, its concentration being higher in old leaves than in young leaves (Bhattacharjee, 2005; Peng *et al*., 2005). Endogenous levels of H_2O_2 increase in daylily petals as they starts to senesce and decreases if senescence is delayed by antioxidants (Panavas and Rubinstein, 1998). Moreover, Bethke and Jones (2001) assigned to H_2O_2 a role as mediator of the developmentally regulated PCD of aleurone layer. On the other side, endogenous production of nitric oxide in vascular tissue regulates programmed cell death and lignification during xylogenesis (Gabaldon *et al*., 2005).

Our study clearly indicates that not only the production of NO and H_2O_2 is spatially and temporally related to nucellus physiological PCD but that the two diffusible molecules are also simultaneously present in the dying cells. This suggests a relationship and cooperation in the execution of nucellus cell death. The fact that NO and H_2O_2 are generated in the same place may facilitate their spatial interaction. The relationship between NO and H_2O_2 is a particularly intriguing question. In some cases they act in parallel pathways while in other they are generated in short succession (Delledonne *et al*., 2001; Clarke *et al*., 2000; dePinto *et al*., 2002; He *et al*., 2005; Bright *et al*., 2006). By using a pharmacological approach, we have demonstrated that NO and H_2O_2 production in the nucellus is indeed interconnected. In our system, exposure of the D region to H_2O_2 results in a noteworthy production of NO dependent on a NOS-like enzymatic activity, as demonstrated by the capability of L-NAME to prevent NO accumulation. This is consistent with other reports. For

example the addition of H_2O_2 causes a rapid production of NO in mung bean leaves (Lum *et al*., 2002) and in tobacco cells (dePinto *et al*., 2006). In guard cells it has been shown that H_2O_2 treatment induces NO synthesis, linking the two molecules in the regulation of stomatal closure (Bright *et al*., 2006).

Notably, the effect seems to be reciprocal in the nucellus, as treatment of the D region with the NO donor SNP causes an increase in H_2O_2 concentration. This result is of particular interest in the light of the still numerous inconsistencies regarding the possible positive effect of NO on H_2O_2 cellular level. Some authors report NO regulation of H_2O_2 synthesis in guard cells (She *et al*., 2004; He *et al*., 2005) while other do not show any link between NO and increase in H_2O_2 (Orozco-Cardenas and Ryan, 2002; Dong *et al*., 2005). Recently an induction of H_2O_2 accumulation following SNP treatment was clearly observed in P*elargonium* (Floryszak-Wieczorek *et al*. 2007) and in *Cupressus* cultured cells (Zhao *et al*., 2007). The use of SNP as a source of nitric oxide has been demonstrated to be very effective in many plant systems, anyway it has potential drawbacks. Apart from NO, SNP delivers also gaseous cyanide that can be responsible of some of the observed biological effects (Bethke et al., 2006). Even if in the same paper of Bethke et al (2006) and in Ederli et al (2006) it is reported that significant cyanide concentration in treated samples was detected only after 5 h of incubation with SNP 5 mM, we used the NO scavenger c-PTIO as a control to separate the biological effect of NO from the one of cyanide in all our experiments with SNP. The effect of c-PTIO is offered as strong evidence in support of the hypothesis that NO actually mediates the increase of H_2O_2 cellular level.

The present data suggest that the consistent co-accumulation of NO and H_2O_2 in the P region may result from mutual effects but it has still to be clarified if the effect is on synthesis or metabolism. One hypothesis is that the high levels of H_2O_2 could be at least partially due to the inhibitory effect of NO on catalase. The activity of the H_2O_2 -scavenging enzyme catalase, which is reduced during physiological PCD of nucellar cells, is not compromised by exogenously added NO as demonstrated by the fact that catalase activity is strongly reduced also in the presence of c-PTIO.

This is in contrast with what observed during pathogen induced PCD, in which catalase inhibition by NO is reported (Mittler *et al*., 1998; Clark *et al*., 2000).

The fact that both NO and H_2O_2 are produced by dying nucellus raises the question whether they play an active role in inducing cell death or if their production is rather a consequence of tissue degeneration. Besides inducing a mutual increase of concentration, both NO and H_2O_2 are able to induce cell death when applied as single treatments to vital and healthy cells of nucellar D region; this suggest their possible role as part of the signalling pathway required for the cell death program. The application of c-PTIO resulted in the prevention of cell death. It is well known that a single application of H_2O_2 is sufficient to trigger a process of cell death in many plant systems (Houot *et al*., 2001; dePinto *et al*., 2006), while, according to some authors, NO is not able to induce cell death without the presence of ROS (Delledonne et al, 2001). In our system exposure to NO causes cell death in a living nucellus. It has been suggested that NO can have a dual role: it can induce cell death but it can also protect cells from oxidative damage by scavenging ROS (dePinto *et al*., 2002; Beligni *et al*., 2002). Which of the two roles is exploited depends on concentration of NO in the tissue, on the physiological state of the cells and finally, on how the signal is perceived or amplified (Clarke *et al*., 2000). Moreover, it has been demonstrated that the ability of NO to induce programmed cell death also depends on the nature of the NO-donor used, SNP being more effective than others (Murgia *et al*., 2004).

Because it has been reported consistently that there is a threshold for ROS below which PCD is induced and above which necrotic death prevails (Houot *et al*., 2001; dePinto *et al*., 2006), we looked for cytological and biochemical markers of PCD. Both SNP and H_2O_2 treatment cause the appearance of typical apoptotic hallmarks and the nuclear alterations evidenced resembled those observed during the natural PCD of the nucellus (Lombardi *et al*., 2007). Our observations are consistent with other reports showing the induction of an active program of cell death, characterized by well defined cytological markers, in cells exposed to both H_2O_2 and NO (Houot *et al*., 2001; Zottini et al., 2002; dePinto *et al*., 2006; Zhao *et al*., 2007). Interestingly, NO alone is able to cause

a TUNEL-positive programmed cell death also in *Taxus* cells (Pedroso *et al*., 2000) and in *Pelargonium* leaves (Floryszak-Wieczorek *et al*. 2007).

Notably, the caspase-like activities previously identified as related to nucellus PCD, are sensitive to NO and H_2O_2. Both YVADase and DEVDase hydrolytic activities are in fact induced in the D region exposed to NO or H_2O_2 even if the increase in proteolytic activity is not as high as the one observed in the P region during PCD. To our knowledge this is the first report of an induction of caspase-like activities involved in PCD by NO treatment; Garcia-Heredia *et al*. (2008) recently reported an induction of the only DEVDase activity upon treatment with 35 mM H_2O_2.

Our observations demonstrate that cell death induced by both NO and H2O2 in nucellar cells is a programmed event and not merely a necrosis caused by excess ROS.

Taken together, our results show that nitric oxide and H2O2 are involved in developmentally regulated PCD of the nucellus. Nucellar cells undergoing PCD produce a considerable amount of the two molecules and in this work we present strong evidence for a mutual interaction between NO and H2O2. Remarkably, living nucellar cells are sensitive to NO and H2O2. In fact, SNP and H2O2 administration is sufficient to initiate an active program of cell death accompanied by some typical features resembling what happens during physiological PCD. Furthermore, the cyto-morphological alterations induced by exposure to NO and H2O2 are strikingly similar to the ones observed during natural PCD of the nucellus. In conclusion, our observations suggest that the simultaneous presence of NO and H2O2 at the site where cells are dying is not non-specific or merely a consequence of the alteration in cellular metabolism, but rather the two molecules are linked and specifically involved in the execution of the nucellus PCD program.

Acknowledgement – The authors wish to thank Dr. Hilary Rogers for critically reviewing and improving the manuscript.

References

Ahlfors R, Brosché M, Kollist H, Kangasjärvi J. 2009. Nitric oxide modulates ozone-induced cell death, hormone biosynthesis and gene expression in *Arabidopsis thaliana. The Plant Journal*. **58**, 1-12.

Arasimowicz M, Floryszak-Wieczorek J. 2007. Nitric oxide as a bioactive signalling molecule in plant stress responses. *Plant Science* **172**, 876–887.

Beaumont F, Jouve HM, Gagnon J, Gaillard J, Pelmont J. 1990. Purification and properties of a catalase from potato tubers (*Solanum tuberosum*). *Plant Science* **72**, 19-26.

Beligni MV , Lamattina L. 2000. Nitric oxide stimulates seed germination, de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. *Planta* **210**, 215–221.

Beligni MV, Fath A, Bethke PC, Lamattina L, Jones RL. 2002. Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiology* **129**, 1642-1650.

Besson-Bard A, Pugin A, Wendehenne D. 2008. New insights into nitric oxide signalling in plants. *Annual Review of Plant Biology* **59**, 21-39.

Bethke PC, Lonsdale JE, Fath A., Jones RL. 1999. Hormonally regulated cell death in barley aleurone cells. *The Plant Cell* **11**, 1033-1045.

Bethke PC, Jones RL. 2001. Cell death of barley aleurone protoplasts is mediated by reactive oxygen species. *The Plant Journal* **25,** 19-29.

Bethke PC, Libourel IG , Jones RL. 2006. Nitric oxide reduces seed dormancy in *Arabidopsis*. *Journal of Experimental Botany* **57**, 517–526.

Bhattachrjee S. 2005. Reactive oxygen species and oxidative burst: roles in stress, senescence and signal transduction in plant. *Current Science* **89**, 1113–1121.

Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ. 2006. ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. *The Plant Journal* **45**, 113–122.

Chen F, Foolad MR. 1997. Molecular organization of a gene in barley which encodes a protein similar to aspartic protease and its specific expression in nucellar cells during degeneration. *Plant Molecular Biol*ogy **35**, 821–831.

Clark D, Durner J, Navarre DA, Klessig DF. 2000. Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. *Molecular Plant-Microbe Interaction*. **13,** 1380–1384.

Clarke A, Desikan R, Hurst RD, Hancock JT, Neill SJ. 2000. NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *The Plant Journal* **24**, 667–677.

Dat JF, Pellinen R, Beeckman T, Van De Cotte B, Langebartels C, Kangasjärvi J, Inzé D, Van Breusegem F. 2003. Changes in hydrogen peroxide homeostasis trigger an active cell death process in tobacco. *The Plant Journal* **33**, 621-632.

de Halac IN. 1980. Fine structure of nucellar cells during development of the embryo sac in *Oenothera biennis* L. *Annals of Botany* **45**, 515–521.

Delledonne M, Zeier J, Marocco A, Lamb C. 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proceedings of the National Academy of Sciences USA* **98**, 13454–13459.

Delledonne M. 2005. NO news is good news for plants. *Current Opinion in Plant Biology* **8**, 390–396.

De Michele R, Vurro E, Rigo C, Costa A, Elviri L, Di Valentin M, Careri M, Zottini M, Sanita` di Toppi L, Lo Schiavo F. 2009. Nitric oxide is involved in cadmium-induced programmed cell death in *Arabidopsis* suspension cultures. *Plant Physiology* **150**, 217-228.

Dempsey DA, Klessig DF. 1994. Salicylic acid, active oxygen species and systemic acquired resistance in plants. *Trends in Cell Biology* **4**, 334-338.

de Pinto, MC, Tommasi F, De Gara L. 2002. Changes in the antioxidant systems as part of the signalling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco BY-2 cells. *Plant Physiology* **130**, 698–708.

de Pinto MC, Paradiso A, Leonetti P, De Gara L. 2006. Hydrogen peroxide, nitric oxide and cytosolic ascorbate peroxidase at the crossroad between defence and cell death. *The Plant Journal* **48**, 784-795.

Desikan R, Cheung,MK, Clarke A, Golding S, Sagi M, Fluhr R, Rock C, Hancock J, Neill S. 2004. Hydrogen peroxide is a common signal for darkness- and ABA-induced stomatal closure in *Pisum sativum. Functional Plant Biology* **31**, 913–920.

Dominguez F, Moreno J, Cejudo FJ. 2001. The nucellus degenerates by a process of programmed cell death during the early stages of wheat grain development. *Planta* **213**, 352–360.

Dong L, Zhang X, Jiang J, An GY, Zhang LR, Song CP. 2005. NO may function in the downstream of H_2O_2 in ABA-induced stomatal closure in *Vicia faba* L. *Journal of Plant Physiology and Molecular Biology* **31**, 62–70.

Ederli L, Morettini R, Borgogni A, Wasternack C, Miersch O, Reale L, Ferranti F, Tosti N, Pasqualini S. 2006. Interaction between nitric oxide and ethylene in the induction of alternative oxidase in ozone-treated tobacco plants, *Plant Physiology* **142**, 595–608.

Ederli L, Reale L, Madeo L, Ferranti F, Gehring C, Fornaciari M, Romano B, Pasqualini S. 2009. NO release by nitric oxide donors in vitro and in planta. *Plant Physiology and Biochemistry* **47**, 42-48.

Fath A, Bethke PC, Beligni MV, Spiegel YN, Jones RL. 2001. Signalling in the cereal aleurone: hormones, reactive oxygen and cell death in cereal aleurone. *Plant Molecular Biology* **44**, 255-266.

Floryszak-Wieczorek J, Arasimowicz M, Milczarek G, Jelen H, Jackowiak H. 2007. Only an early nitric oxide burst and the following wave of secondary nitric oxide generation enhanced effective defense responses of *Pelargonium* to a necrotrophic pathogen. *New Phytologist* **175**, 718–730.

Gabaldon C, Gomez-Ros LV, Pedreño MA, Barcelò AR. 2005. Nitric oxide production by differentiating xylem of *Zinnia elegans*. *New Phytologist* **165**, 121–130.

García-Heredia JM, Hervás M, De la Rosa MA, Navarro JA. 2008. Acetylsalicylic acid induces programmed cell death in *Arabidopsis* cell cultures. *Planta* **228**, 89-97.

Garcia-Mata C, Lamattina L. 2002. Nitric oxide and abscisic acid cross talk in guard cells. *Plant Physiology* **128**, 790–792.

Gechev TS, Hille J. 2005. Hydrogen peroxide as a signal controlling plant programmed cell death. *Journal of Cell Biology* **168**,17-20.

Greenwood JS, Helm M, Gietl C. 2005. Ricinosomes and endosperm transfer cell structure in programmed cell death of the nucellus during *Ricinus* seed development. *Proceedings of National Academy of Sciences USA* **102**, 2238–2243.

Grün S, Lindermayr C, Sell S, Durner J. 2006. Nitric oxide and gene regulation in plants. *Journal of Experimental Botany* **57**, 507-516.

Hancock JT, Desican R, Clarke A, Hurst RD, Neill SJ. 2002. Cell signalling following plant/pathogen interactions involves the generation of reactive oxygen and reactive nitrogen species. *Plant Physiology and Biochemistry* **40**, 611–617.

He JM, Xu H, She XP, Song XG, Zhao WM. 2005. The role and the interrelationship of hydrogen peroxide and nitric oxide in the UV-B-induced stomatal closure in broad bean. *Functional Plant Biology* **32**, 237–247.

Hiratsuka R, Yamada Y, Terasaka O. 2002. Programmed cell death of *Pinus* nucellus in response to pollen tube penetration. *Journal of Plant Research* **115**, 141-148.

Houot V, Etienne P, Petitot AS, Barbier S, Blein JP, Suty L. 2001. Hydrogen peroxide induces programmed cell death features in cultured tobacco BY-2 cells in a dose-dependent manner. *Journal of Experimental Botany* **52**, 1721–1730.

Ihara TI, Yamamoto T, Sugamata M, Okumura M, Ueno Y. 1998. The process of ultrastructural changes from nuclei to apoptotic body. *Virchows Archiv* **433,** 443-447.

Jensen WA. 1975. The composition and ultrastructure of the nucellus in cotton. *Journal of Ultrastructural Research* **13**, 112–128.

Kojima H, Nakatsubo N, Kikuchi K, Urano Y, Higutchi T. 1998. Direct evidence of NO production in rat hippocampus and cortex using a new fluorescent indicator: DAF-2DA. *Neuroreport* **9**, 3345-3348.

Leitner M, Vandelle E, Gaupels F, Bellin D, Delledonne M. 2009. NO signals in the haze. Nitric oxide signalling in plant defence. *Current Opinion in Plant Biology* **12**, 451-458.

Lindermayr C, Saalbach G, Durner J. 2005. Proteomic identification of *S*-nitrosylated proteins in *Arabidopsis*. *Plant Physiology* **137**, 921–930.

Lombardi L, Casani S, Ceccarelli N, Galleschi L, Picciarelli P, Lorenzi R. 2007. Programmed cell death of the nucellus during Sechium edule Sw. seed development is associated with activation of caspase-like proteases. *Journal of Experimental Botany* **58**: 2949-2958.

Lum HK, Butt YKC, Lo SCL. 2002. Hydrogen peroxide induces a rapid production of nitric oxide in mung bean (*Phaseolus aureus*). *Nitric Oxide: Biology and Chemistry* **6**, 205–213.

Mittler R, Feng X, Cohen M. 1998. Post-transcriptional suppression of cytosolic ascorbate peroxidase expression during pathogen-induced programmed cell death in tobacco. *The Plant Cell* **10**, 461–473.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. 2004. Reactive oxygen gene network of plants. *Trends in Plant Science* **9**, 490–498.

Mur LAJ, Carver TLW, Prats E. 2008. NO way to live; the various roles of nitric oxide in plant–pathogen interactions. *Journal of Experimental Botany* **57**, 489-505.

Murgia I, de Pinto MC, Delledonne M, Soave C, De Gara L. 2004. Comparative effects of various nitric oxide donors in plant cells. *Journal of Plant Physiology* **61**, 777–783.

Neill SJ, Desikan R, Clarke A. 2002. Hydrogen peroxide and nitric oxide as signaling molecules in plants. *Journal of Experimental Botany* **53**, 1237–1242.

Norstog K. 1974. Nucellus during early embryogeny in barley: finestructure. *Botanical Gazette* **135**, 97–103.

Orozco-Cardenas ML, Ryan CA. 2002. Nitric oxide negatively regulates wound signalling in tomato plants. *Plant Physiology* **130**, 487-493.

Overmyer K, Brosché M, Kangajarvi J. 2003. Reactive oxygen species and hormonal control of cell death. *Trends in Plant Science* **8**, 335–342.

Palma K, Kermode AR. 2003. Metabolism of hydrogen peroxide during reserve mobilization and programmed cell death of barley (*Hordeum vulgare* L.) aleurone layer cells. *Free Radical Biology and Medicine* **35**,1261-1270.

Palmieri MC, Sell S, Huang X, Scherf M, Werner T, Durner J, Lindermayr C. 2008. Nitric oxide-responsive genes and promoter in *Arabidopsis*: a bioinformatic approach. *Journal of Experimental Botany* **59**, 177-186.

Panavas T, Rubinstein B. (1998). Oxidative events during programmed cell death od daylily (*Hemerocallis* hybrid) petals. *Plant Science* **133**, 125-138.

Pedroso MC, Magalhaes JR, Durzan DJ. 2000. A nitric oxide burst precedes apoptosis in angiosperm and gymnosperm callus cells and foliar tissues. *Journal of Experimenatl Botany* **51**, 1027–1036.

Peng LT, Jiang YM, Yang SZ, Pan SY. 2005. Accelerated senescence of fresh-cut Chinese water chestnut tissues in relation to hydrogen peroxide accumulation. *Journal of plant physiology and molecular biology* **31**, 527-532.

Qiao W, Fan LM. 2008. Nitric oxide signaling in plant responses to abiotic stresses. *Journal of Integrative Plant Biology* **50**, 1238-1246.

Quan LJ, Zhang B, Shi WW, Li HY. 2008. Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. *Journal of Integrative Plant Biology* **50**, 2-18.

Reape TJ, McCabe PF. 2008. Apoptotic-like programmed cell death in plants. *New Phytologist* **180**, 13-26.

Samuilov VD, Kiselevsky DB, Shestak AA, Nesov AV, Vasil'ev LA. 2008. Reactive oxygen species in programmed cell death of pea guard cells. *Biochemistry (Mosc)* **73**, 1076-1084.

She XP, Song XG, He JM. 2004. Role and relationship of nitric oxide and hydrogen peroxide in light/dark regulated stomatal movement in *Vicia faba*. *Acta Botanica Sinica* **46,** 1292-1300.

Steffens B, Sauter M. 2005. Epidermal cell death in rice is regulated by ethylene, gibberellin and abscisic acid. *Plant Physiology* **139**, 713-721.

Steffens B, Sauter M. 2009. Epidermal cell death in rice is confined to cells with a distinct molecular identity and is mediated by ethylene and H2O2 through an autoamplified signal pathway. *The Plant Cell* **21**, 184-196.

Uchida A, Jagendorf AT, Hibino T, Takabe T. 2002. Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice. *Plant Science* **163**, 515–523.

Van Breusegem F, Dat J F. 2006. Reactive oxygen species in plant cell death. *Plant Physiology* **141**, 384-390.

Xu FX, Chye ML. 1999. Expression of cysteine proteinase during developmental events associated with programmed cell death in brinjal. *The Plant Journal* **17**, 321-327.

Young TE, Gallie DR. 1999. Analysis of programmed cell death in wheat endosperm reveals differences in endosperm development between cereals. *Plant Molecular Biology* **39**, 915-926.

Zago E, Morsa S, Dat JF, Alard P, Ferrarini A, Inzé D, Delledonne M, Van Breusegem F. 2006. Nitric oxide- and hydrogen peroxide-responsive gene regulation during cell death induction in tobacco. *Plant Physiology* **141**, 404-411.

Zaninotto F, La Camera S, Polverari A, Delledonne M. 2006.Cross talk between reactive nitrogen and oxygen species during the hypersensitive disease resistance response. *Plant Physiology* **141**, 379-383.

Zhao J, Fujita K, Sakai K. 2007. Reactive oxygen species, nitric oxide, and their interactions play different roles in *Cupressus lusitanica* cell death and phytoalexin biosynthesis. *New Phytologist* **175**, 215-229.

Zottini M, Formentin E, Scattolin M, Carimi F, Lo Schiavo F, Terzi M. 2002. Nitric oxide affects mithocondrial functionality in vivo. *FEBS Letters* **515**, 75-78.

Figure legends

Fig. 1. Longitudinal section of S. edule seed showing the inner tissues. The endosperm is removed and pulled aside, leaving a 'heart-shaped' mark where the nucellus is no longer present. The nucellus is degenerating making the integuments visible. End: endosperm; D: distal nucellus; P: proximal nucellus; Int: integuments. Bar = 1 cm.

Fig. 2. Nitric oxide and hydrogen peroxide are produced by the proximal (P) region of the nucellus during physiological cell death. (A) Nitric oxide content in P and D regions of the nucellus has been estimated by an in vitro fluorometric assay based on the reaction with DAF-2DA. (B) The same probe has been used to visualize NO in the whole nucellus, making it possible to localize NO in the P region. (C) Nucellar hydrogen peroxide concentration has been calculated by the Amplex red Assay and visualized in the nucellus by the specific fluorescent probe DCFH-DA (D). (E) Parallel to an increase in H2O2 level, catalase activity is strongly reduced in the P region during cell death. Bars = 500 μm. FU = fluorescence units. Error bars represent ± SE , n = 5.

Fig. 3. H2O2 induces NO production and vice-versa. (A) Fluorometric assay showing nitric oxide content in the D region treated for 2 h with 3 mM H2O2 with or without the presence of the

mammalian NOS inhibitor L-NAME. (B) Nitric oxide is visualized by the specific fluorescent probe DAF-2DA in the whole nucellus after treatment for 2 h with 3 mM H_2O_2. The addition of the NOS inhibitor L-NAME prevented NO formation in the nucellus (C). (D) Spectrophotometric assay indicating hydrogen peroxide production following a treatment for 2 h with 1 mM SNP with or without the NO scavenger c-PTIO. (E) H_2O_2 can be visualized in vivo by the specific fluorescent probe DCFH-DA. (F) H_2O_2 production is completely abolished in the presence of the NO scavenger c-PTIO. Bars in B, E = 200 μm. Bars in C, F = 500 μm. FU = fluorescence units. Error bars represent ± SE , n = 5.

Fig. 4. Viability staining of the nucellus with FDA and FM 4-64. Cell death is localized only in the P region of control untreated nucellus (A), while it is extended also to the D region after a treatment for 2 h with 3 mM H_2O_2 (B) or with 1 mM SNP (C). Addition of the NO scavenger c-PTIO together with SNP prevents cell death in the D region (D). Images of the fluorescein and of the FM 4-64 signals have been merged.

Fig. 5. Aspect of nuclei in control D region of the nucellus (A) and in treated D region (B, C). Compared to the control (A), nuclei treated for 2 h with 1 mM SNP (b) or 3 mM H_2O_2 (C) undergo profound changes in morphology; SNP treatment causes chromatin condensation and the appearance of half-moon-shaped nuclei. H_2O_2 treatment causes chromatin condensation only in a small percentage of nuclei, while others remain similar to the control or show granular condensation of chromatin at the periphery. Slides are stained with fast-red nuclear stain. Bar = 10 μm.

Fig. 6. DAPI (A,C,E) and TUNEL (B,D,F) staining of nucellar tissue sections from the D region. No TUNEL-positive nuclei are visible in the control, untreated D region (B), while TUNEL-positive nuclei are evident after treatment for 2 h with both 1 mM SNP (D) and 3 mM H_2O_2 (F). Some nuclei in the H_2O_2-treated nucellus do not show condensed chromatin and are TUNEL-negative (arrows in E).

Fig. 7. In vitro caspase-like activities towards the two synthetic substrates YVAD-pNA and DEVD-pNA measured in the D region, in the dying P region, in D region previously treated for 2 h with 3 mM H_2O_2, and in D regions previously treated for 2 h with 1 mM SNP (with or without c-PTIO 200 μM). Hydrolytic activity is compared to the one present in living D region, taken as the baseline. Error bars represent ± SE , n = 5.

Figures

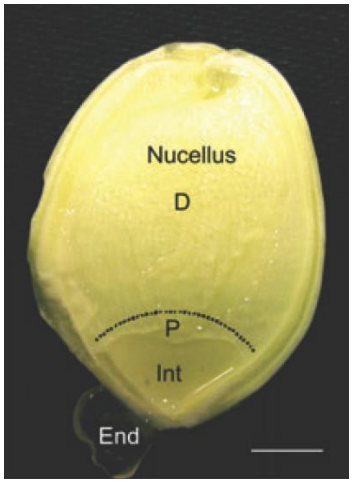
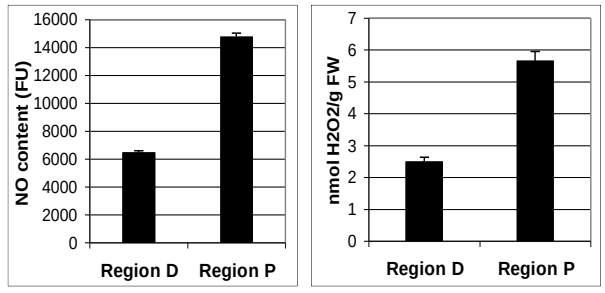
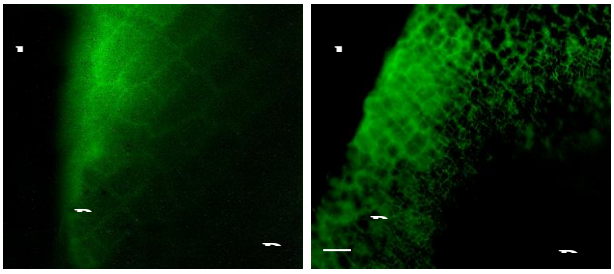


Fig. 1





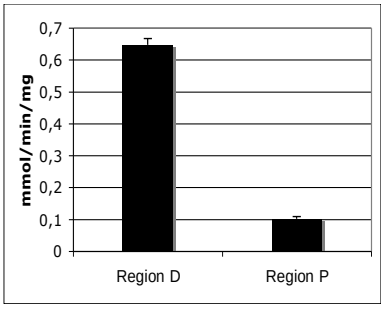


Fig. 2

Fig. 3

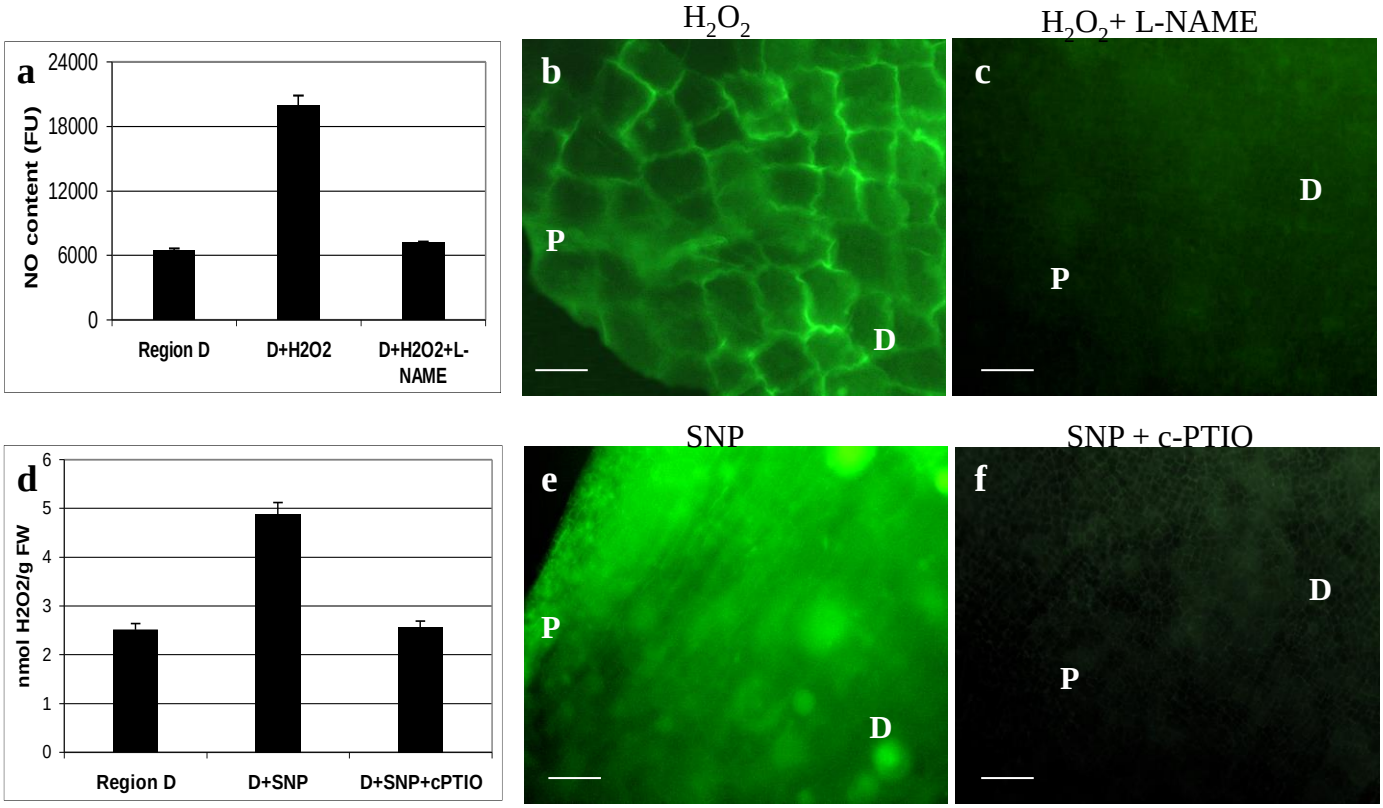

a
NO content (FU)
24000
18000
12000
6000
0
Region D
D+H2O2
D+H2O2+L-NAME
H2O2
b
P
D
H2O2+ L-NAME
c
D
P
d
nmol H2O2/g FW
6
5
4
3
2
1
0
Region D
D+SNP
D+SNP+cPTIO
SNP
e
P
D
SNP + c-PTIO
f
D
P

Fig. 4

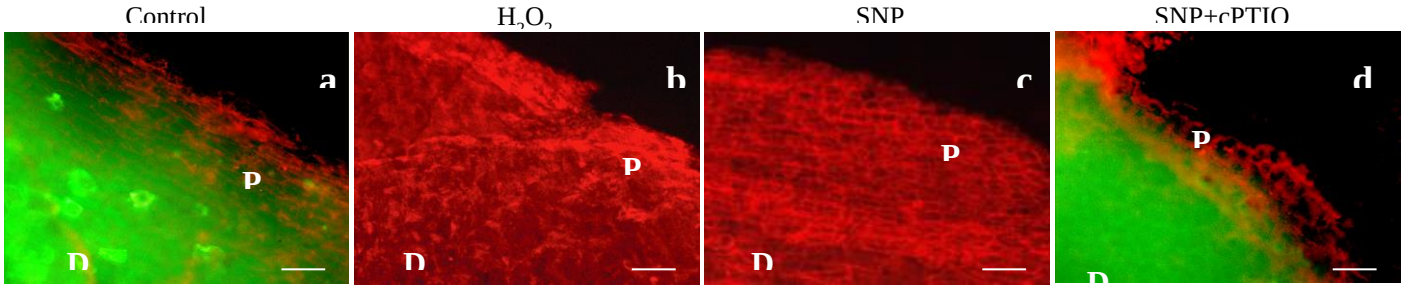

Control
a
P
D
H2O2
b
P
D
SNP
c
P
D
SNP+cPTIO
d
P
D

Fig. 5

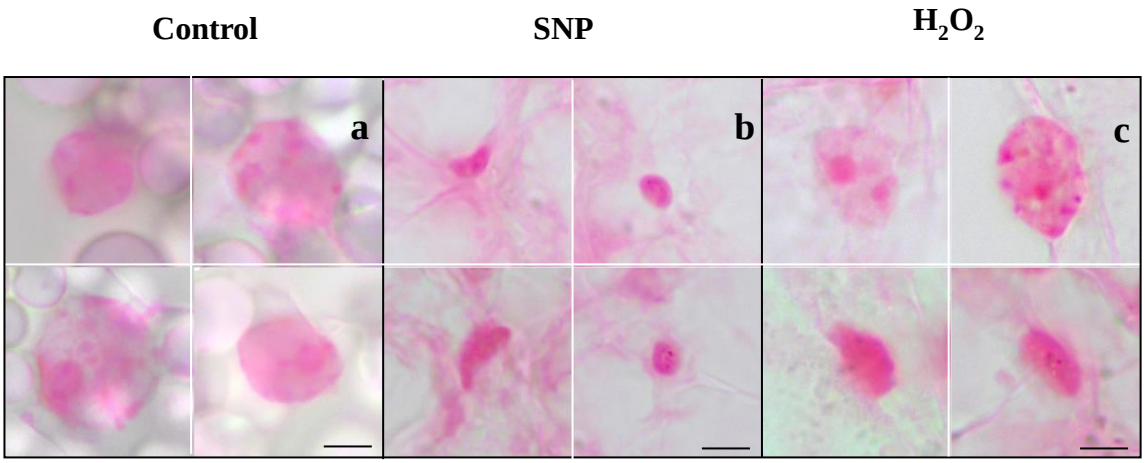


Fig. 6

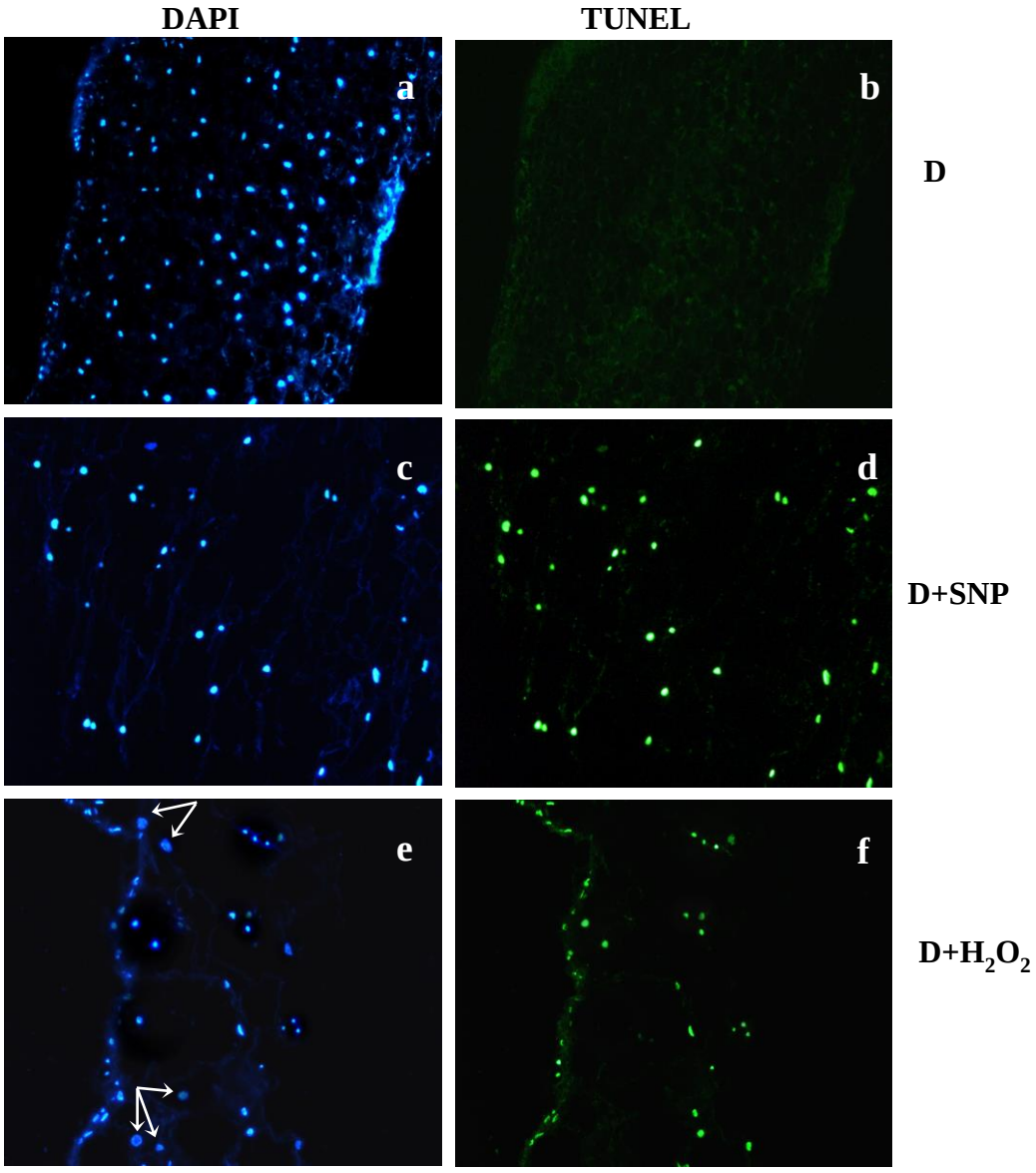
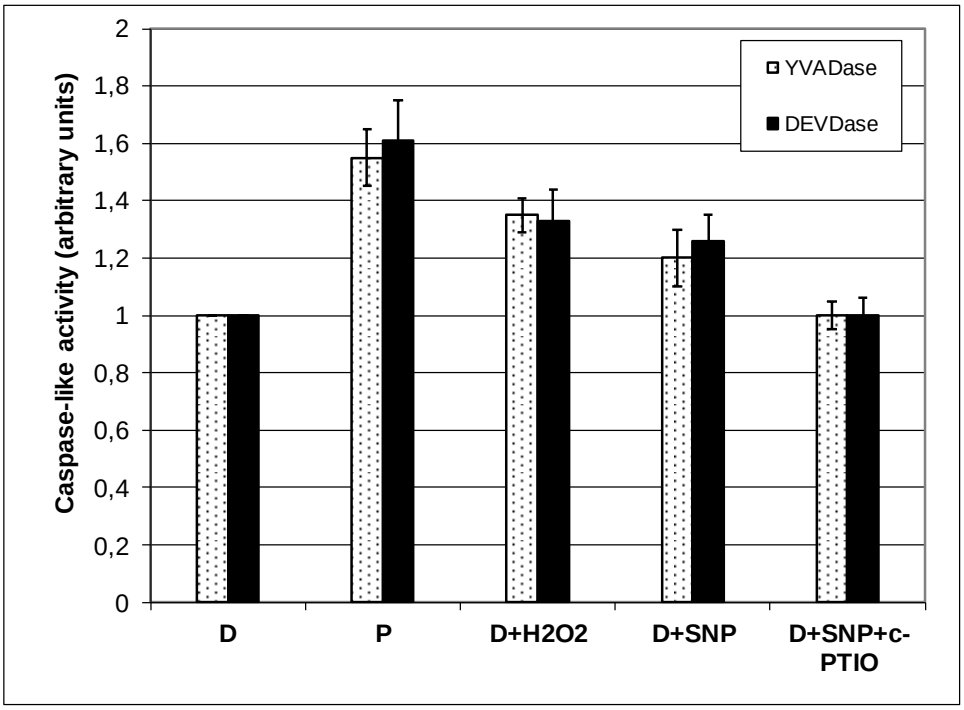


Fig. 7


Caspase-like activity (arbitrary units)
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
YVADase
DEVDase
D
P
D+H2O2
D+SNP
D+SNP+c-PTIO