

RESEARCH PAPER

Peroxynitrite mediates programmed cell death both in papillar cells and in self-incompatible pollen in the olive (*Olea europaea* L.)

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Abstract

Programmed cell death (PCD) has been found to be induced after pollination both in papillar cells and in self-incompatible pollen in the olive (*Olea europaea* L.). Reactive oxygen species (ROS) and nitric oxide (NO) are known to be produced in the pistil and pollen during pollination but their contribution to PCD has so far remained elusive. The possible role of ROS and NO was investigated in olive pollen–pistil interaction during free and controlled pollination and it was found that bidirectional interaction appears to exist between the pollen and the stigma, which seems to regulate ROS and NO production. Biochemical evidence strongly suggesting that both O_2^- and NO are essential for triggering PCD in self-incompatibility processes was also obtained. It was observed for the first time that peroxynitrite, a powerful oxidizing and nitrating agent generated during a rapid reaction between O_2^- and NO, is produced during pollination and that this is related to an increase in protein nitration which, in turn, is strongly associated with PCD. It may be concluded that peroxynitrite mediates PCD during pollen–pistil interaction in *Olea europaea* L. both in self-incompatible pollen and papillar cells.

Key words: Nitric oxide, *Olea europaea* L., peroxynitrite, programmed cell death, pollen–pistil interaction, reactive oxygen species.

Introduction

In spite of the apparent paradox, cell death is crucial for the growth and development of eukaryotic cells because it maintains tissue and organ homeostasis (Van Breusegem and Dat, 2006). Programmed cell death (PCD) in plants is an active process leading to the selective elimination of unneeded or damaged cells during many developmental processes such as embryogenesis, tapetum degeneration, pollen selection due to self-incompatibility, organ senescence, and tracheary element differentiation and also during growth under stress conditions (Gechev *et al.*, 2006; Rogers, 2006). It is genetically controlled and, in plants, includes a variety of types of cell death, although some of them share common morphological and biochemical features with animal cell apoptosis, such as DNA cleavage into internucleosomal

fragments and shrinkage of the cytoplasm (Wang *et al.*, 1996).

In recent years, a dual role for reactive oxygen and nitrogen species (ROS and RNS) has been recognized in plant biology (Mittler *et al.*, 2004; Neill, 2005; del Rio and Puppo, 2009). The function of ROS is finely regulated by a ROS-producing and -detoxifying balance that adjusts their levels under different developmental or environmental conditions. ROS homeostasis may be cell-specific and even intracellular-specific (Mittler *et al.*, 2004). ROS and RNS were once considered to be toxic by-products of aerobic metabolism, leading to destructive modifications in proteins, DNA, and lipids, and that plants therefore developed a strong antioxidant system to remove any excess of ROS

(Halliwell, 2006). Recently, however, they have been recognized as signalling molecules that fine-tune such processes in plant biology as defence, hormone signalling, and development (Mittler *et al.*, 2004; del Rio *et al.*, 2006; Gapper and Dolan, 2006). In addition, nitric oxide (NO) appears to play a key role as a signalling molecule in plants (Romero-Puertas and Delledonne, 2003; Delledonne, 2005; Neill, 2005). As a developmental regulator it promotes germination, leaf extension, and root growth, and delays leaf senescence and fruit ripening (Neill *et al.*, 2003). It has recently been shown to be a key molecule that interacts with ROS in a number of ways, leading to cell death or signalling in response to different physiological and stress conditions (Clarke *et al.*, 2000; de Pinto *et al.*, 2002; Zaninotto *et al.*, 2006). Recent genetic studies have demonstrated the crucial role of ROS at different stages of plant PCD in *Arabidopsis thaliana* (Mittler and Rizhsky, 2000; Lorrain *et al.*, 2003), although initial evidence for this had already been obtained in cultured soybean cells (Levine *et al.*, 1994), and in animals it has been well established that H₂O₂ and O₂⁻ co-operate with NO to induce PCD (Jabs, 1999; Van Breusegem and Dat, 2006). Our understanding of the genetic mechanisms that trigger and regulate plant PCD is, however, somewhat limited. This, together with the diversity of cell-death types described in plant biology, suggests that, despite some similarities, the signalling pathways may well be different.

Self-incompatibility is the most widespread mechanism in the prevention of inbreeding via the rejection of self-pollen and genetically related pollen (de Nettancourt, 1997) and it has been demonstrated that PCD occurs as a result of incompatible pollination (Rogers, 2006). The involvement of PCD in the rejection of self-incompatible pollen in an *in vitro* system in *Papaver* is the best characterized to date. It has been observed in this species that elongation of the pollen tube belonging to self-pollen is inhibited within minutes of its landing on the stigma, a process known as stigmal gametophytic SI (Franklin-Tong and Franklin, 2003). PCD has also been described in *Pyrus pyrifolia*, where pollen-tube growth is arrested in the style (stylar gametophytic SI) (Wang CL *et al.*, 2009). More recently, Serrano *et al.* (2010) have demonstrated that PCD is involved in pollen selection in *Olea europaea* L., since self-incompatible pollen landing on the stigma triggers PCD. Self-incompatibility has been identified in most olive cultivars, including Picual, one of the most important for olive-oil production (Lavee *et al.*, 1999; Moutier, 2000; Wu, 2002; Díaz *et al.*, 2006). Nevertheless, difficulties in experimentation with this species (it flowers only once a year for a very short time) have meant that the olive tree has, in general, been studied from an agronomical point of view and that molecular and cell studies are very scarce.

It has been shown that stigmas from diverse angiosperms accumulate H₂O₂ and that pollen from a wide range of plant species produces NO (Prado *et al.*, 2004; McInnis *et al.*, 2006; Bright *et al.*, 2009; Wang Y *et al.*, 2009; Zafra *et al.*, 2010; Wilkins *et al.*, 2011). The involvement of ROS and NO in PCD caused by self-incompatibility has not been studied to date in an *in vivo* system, although very recently

it has been shown *in vitro* that ROS and NO mediate PCD in pollen tubes during self-incompatibility responses in *Papaver* (Wilkins *et al.*, 2011). In this study, the involvement of ROS and NO in PCD prompted by self-incompatibility in the olive was investigated using an *in vivo* system of controlled and freely pollinated flowers from *Olea europaea*, L.. The results reveal a ROS- and RNS-mediated interaction between pollen and pistil during pollination and a peroxynitrite-dependent PCD signalling pathway both in papillar cells and self-incompatible pollen grains after pollination.

Materials and methods

Perfect flowers, with both pistillate and staminate parts, were gathered periodically before and after pollination during the months of May and June 2007–2010 from *Olea europaea* L. cv. Picual trees growing in Granada and Córdoba (Spain). Within the phenologically mixed populations of flowers, care was taken to select exactly the different stages chosen for this study: stage I, white flower buds before pollination; stage II, open flowers after pollination; stage III, flowers after fertilization which had lost their petals and showed enlarged ovaries (Fig. 1A–C).

For controlled pollination, white flower buds before pollination were either enclosed in bags for self-pollination (self-incompatible pollination) or emasculated and cross-pollinated by hand with pollen of cv. Arbequina (compatible pollination) before being enclosed in bags. Flowers inside the bags were collected for analysis when non-bagged flowers of reference inflorescences on the same branch reached stage III.

ROS, NO, and peroxynitrite detection by fluorescence microscopy

Reactive oxygen and nitrogen species were detected according to Rodriguez-Serrano *et al.* (2006). To detect nitric oxide, controlled and freely pollinated pistils excised from olive flowers at the three different stages studied were incubated for 1 h at 25 °C in darkness with 10 μM 4,5-diaminofluorescein diacetate (DAF-2 DA, Calbiochem, San Diego, CA, USA) in 10 mM TRIS-HCl (pH 7.4). The same method was used for the detection in pollen grains germinated *in vitro*. O₂⁻ and H₂O₂ were detected by incubating samples for 30 min at 37 °C with 10 μM dihydroethidium (DHE) and 25 μM 2',7'-dichlorofluorescein diacetate (DCF-DA) respectively, in 10 mM TRIS-HCl (pH 7.4). To detect peroxynitrite pistils excised from controlled and freely pollinated flowers, they were incubated for 1 h at 25 °C in darkness with 25 μM HKGreen-2, kindly provided by Dr Dan Yang (Sun *et al.*, 2009). Controls of ROS, NO, and peroxynitrite detection were made by pre-incubating samples with 2 mM carboxy-2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) (a NO specific scavenger), 2 mM tetramethyl piperidinoxy (TMP) (an O₂⁻ scavenger), 2 mM ascorbate (ASC) (a H₂O₂ scavenger), and 2 mM epicatechin (a ONOO⁻-specific scavenger) (Pannala *et al.*, 1997). All the samples were then washed, squashed, and their fluorescence examined under a confocal laser scanning microscope (Leica TCS SL, Leica Microsystems, Wetzlar, Germany). DAF-2 DA (excitation 495 nm and emission 515 nm) and DCF-DA (excitation 485 and emission 530 nm) probes emit green fluorescence, whereas DHE (excitation 490 nm and emission 520 nm) and HKGreen-2 (excitation 520 nm and emission 539 nm) emit red fluorescence. A total of nine pistils were analysed for each stage and fluorochrome in three independent experiments with three replicates every year for four years.

To check possible ROS and NO enzymatic sources, samples at stage III were studied after pre-incubation with 10 μM of diphenyl-iodonium (DPI) (a NADPH oxidase inhibitor), 10 mM of sodium azide (a peroxidase inhibitor), and 5 mM of NG-nitro-L-arginine

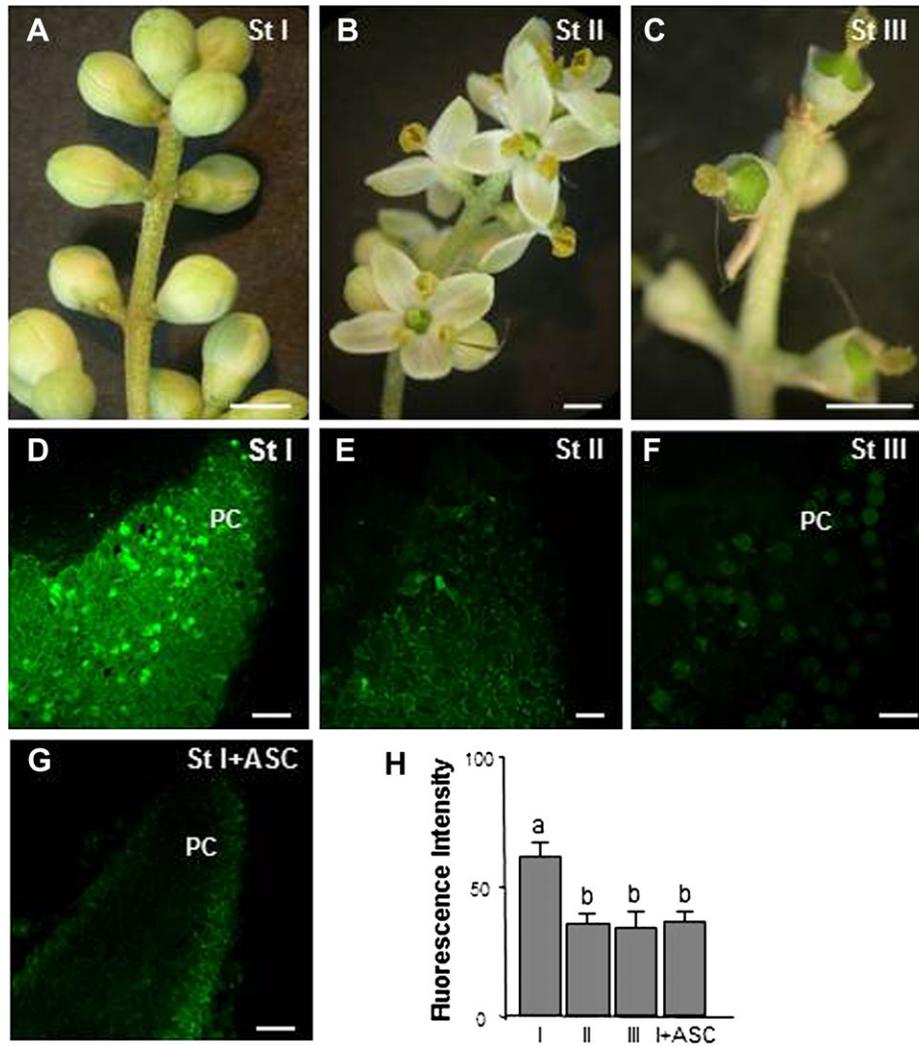


Fig. 1. Hydrogen peroxide in olive pistils excised from freely pollinated flowers detected by confocal laser scanning microscope. (A, B, C) Stereomicroscope images of olive flowers before pollination (St I), during pollination (St II), and after fertilization (St III). (D, E, F, G) Detection of H₂O₂ by DCF-DA in stigmas at different stages. Green fluorescence corresponding to H₂O₂ is detected in papillar cells mainly at stage I. (G) Pistil at stage I treated with ascorbate (ASC), a H₂O₂ scavenger. (H) Histogram showing fluorescence quantified in total images of the stigmal lobules in arbitrary units using LAS AF Leica software. Different letters indicate significant difference at *P* < 0.05 as determined by Duncan’s multiple-range test. PC, papillar cells. (A, B, C) Bars=2 mm; (D, E, F, G) bars=50 μm.

methyl ester (L-NAME) (a NOS inhibitor), in the same way as with the scavengers. A total of six pistils were analysed with each inhibitor in two independent experiments with three replicates every year for four years. Fluorescence intensity was quantified taking into account the whole image of the stigma lobule.

Pollen–pistil interactions *in vitro*

Pollen grains isolated from olive trees were germinated in a medium containing 20% w/v sucrose, 0.01% w/v CaCl₂, 0.01% w/v H₃BO₃, and 0.01% w/v KNO₃ in a humid chamber at 30 °C for 3 h for ROS and NO detection, and for 18 h for RNA and protein extraction. Pollen grains were germinated in *in vitro* cultures both in the presence and absence of fresh receptive pistils.

After removing the pistils, pollen grains and pollen tubes were used to detect reactive species as described above for pistils. For RNA and protein studies pollen grains and pollen tubes were centrifuged before extraction. Germination *in vitro* included three independent experiments with three replicates each.

To test the influence of NO upon the development of stigma-cell death, 12 flower buds (stage I) were emasculated, sprayed with sodium nitroprusside (SNP), a NO donor, and enclosed in bags to avoid the arrival of pollen grains to their surface. The samples were collected when the flowers in the reference inflorescence reached stage III. Parallel control experiments were carried out with pistils not sprayed with SNP. ROS were detected in two independent experiments with 12 samples each.

Cell-death detection

Our group have already shown in experiments using the TUNEL reaction, DNA degradation analysis, and caspase-like activity that cell death detected by trypan blue in olive stigmal papillar cells and pollen grains corresponded to PCD (Serrano *et al.*, 2010). Trypan-blue staining was used here because it is a very direct, simple, and rapid method to detect cells that are suffering cell death and allows a greater number of samples to be analysed compared with the techniques mentioned above. Fresh pistils were stained by boiling

them in alcoholic lactophenol (96% ethanol:lactophenol, 1:1 v/v) containing 0.1 mg ml⁻¹ trypan blue (Sigma) for 1 min, any excess of which was removed in a chloral hydrate solution (2.5 mg ml⁻¹) at room temperature overnight. After staining, the ovary was removed and the stigma and style were squashed and studied under a Zeiss Axioplan microscope. To gauge the influence of O₂⁻, NO, and ONOO⁻ upon cell death, pollinated flowers (stage II) were sprayed with 2 mM tetramethylpiperidinoxy (TMP), 2 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO), or 2 mM epicatechin and collected at stage III. A total of 12 flowers on four different branches were treated with each scavenger and the experiment was repeated twice, using the same number of flowers as control. A total of 1000 pollen grains were evaluated for each treatment (TMP, cPTIO, and epicatechin) to determine whether the differences found in the number of pollen grains positive to trypan-blue staining were significant. The significance of the differences was tested with a χ^2 test.

Detection of nitrated proteins in pollen germinated *in vitro*

To detect possible differences in the number of nitrated proteins produced as a consequence of PCD, protein extracts from pollen germinated *in vitro* in the presence and absence of pistils of the same cultivar were used. To test the influence of peroxyntirite upon the nitration of pollen proteins, protein extracts from pollen germinated *in vitro* in the presence of 2 mM epicatechin were also obtained. Immunoblotting experiments were carried out with anti-nitrotyrosine antibody (Sigma). 100 mg of each sample was ground to a fine powder in liquid nitrogen using a mortar and pestle. Proteins were then extracted in a homogenization medium (50% v/v phenol, 0.45 M sucrose, 5 mM EDTA, 0.2% v/v 2-mercaptoethanol, and 50 mM TRIS-HCl, at pH 8.8) as described elsewhere (Hajduch et al., 2005). Protein concentration was determined using a protein assay from Bio-Rad (Hercules, CA) based on the modified procedure of Bradford (1976). Protein samples were loaded in 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore Co., Bedford, MA, USA) following standard protocols (Sambrook and Russell, 2001). The membrane was stained with Ponceau red to check the equivalency in protein loading. Nitration was detected using the anti-nitrotyrosine antibody diluted 1:2000. Protein extracts were incubated with sodium dithionite 5 mM to ensure antibody specificity. Probing and detection of immunocomplexes were carried out as described for the ECL Plus detection system (Amersham Biosciences). This experiment was carried out twice, similar results being obtained on each occasion. The experimental procedures of the Supplementary figures are included in Appendix S1.

Results

ROS and NO presence throughout pollination

ROS and NO production during free pollination was analysed in pistils during three different stages: before pollination (white flower buds, stage I), after pollination (open flowers, stage II), and after fertilization (pistils with enlarged ovaries, stage III) (Fig. 1A, B, C). Since an *in vivo* system was used, it must be borne in mind that the time of flowering and the various stages depended to a great extent upon the environmental conditions, which is why the results of exogenous treatments were analysed not after any fixed time but when the flowers in the reference inflorescence reached the appropriate stage. All the experiments were repeated a significant number of times and similar results were obtained, thus proving that the environmental conditions

had not affected the study. Observations with confocal laser scanning microscopy after pistil staining with specific fluorescent probes for ROS and NO (Figs 1, 2) revealed that changes in these molecules took place during the progamic phase (from pollination to fertilization) both in pollen and papillar cells. Fluctuations in H₂O₂ during the different stages were detected with DCF-DA. H₂O₂-dependent green fluorescence was mainly to be seen in the stigmal papillae of pistils during stage I (Fig. 1D, H) but decreased after pollination in the stigmal lobules during stages II and III (Fig. 1E, F, H). The level of fluorescence observed during stage II was due on the whole to the exudate present among papillar cells, whilst during stage III it was mainly due to pollen grains. Ascorbate was used as a negative control in stage I (Fig. 1G). DHE was used to visualize the superoxide anion (O₂⁻). A green fluorescent signal was detected in the stigmal papillar cells of pistils during stage I (Fig. 2A), which increased during stages II and III, appearing not only in the papillar cells but also in the areas surrounding them containing exudate, where pollen grains were germinating. Most pollen grains and tubes showed fluorescent signal (Fig. 2B, C, D). TMP was used as a negative control for stage III (Fig. 2E). DAF-2DA was used to visualize NO. An increase in fluorescence of around 50% was observed after pollination (stage II), compared with fluorescence during stage I (Fig. 2G, I). Most striking was that, after fertilization (stage III), both the papillar cells and most of the pollen grains were fluorescent (close to 70%) (Fig. 2H, J). cPTIO was used as negative control in stage III (Fig. 2I, J).

To establish clearly whether the fluctuations in ROS and NO in the papillar-cell fluorescence depended upon the kind of pollen landing on the stigma (compatible or incompatible), their production was analysed after controlled pollination with pollen of the same self-incompatible cultivar or with pollen of a compatible cultivar (Fig. 3). When fluorescence corresponding to H₂O₂ during stage III was analysed, a similar fluorescence was seen to occur after both compatible and incompatible pollination (Fig 3A, D, G). O₂⁻-dependent green fluorescence increased by around 30% after incompatible pollination compared to compatible pollination (Fig. 3B, E, G). This increase was more obvious with NO-dependent green fluorescence, it being more than three times higher after incompatible pollination (Fig. 3C, F, G).

To locate H₂O₂ and O₂⁻ more efficiently, DAB and NBT stainings were used in fresh pistils during free pollination. The pistils were observed directly under the microscope after they had been squashed and also fixed and embedded in Unicryl to obtain sections in which the presence of these molecules could be studied cytochemically. A brown staining corresponding to H₂O₂ was detected after DAB treatment in the stigmas during stage I (see Supplementary Fig. S1A, B at *JXB* online), which decreased after pollination during stages II and III (see Supplementary Fig. S1F, G, K, L at *JXB* online), confirming the results obtained with the fluorescent probes. In the sections, H₂O₂-dependent brown staining was visible in the cytoplasm of papillar cells at stage I (see Supplementary Fig. S2A at *JXB* online). O₂⁻ was

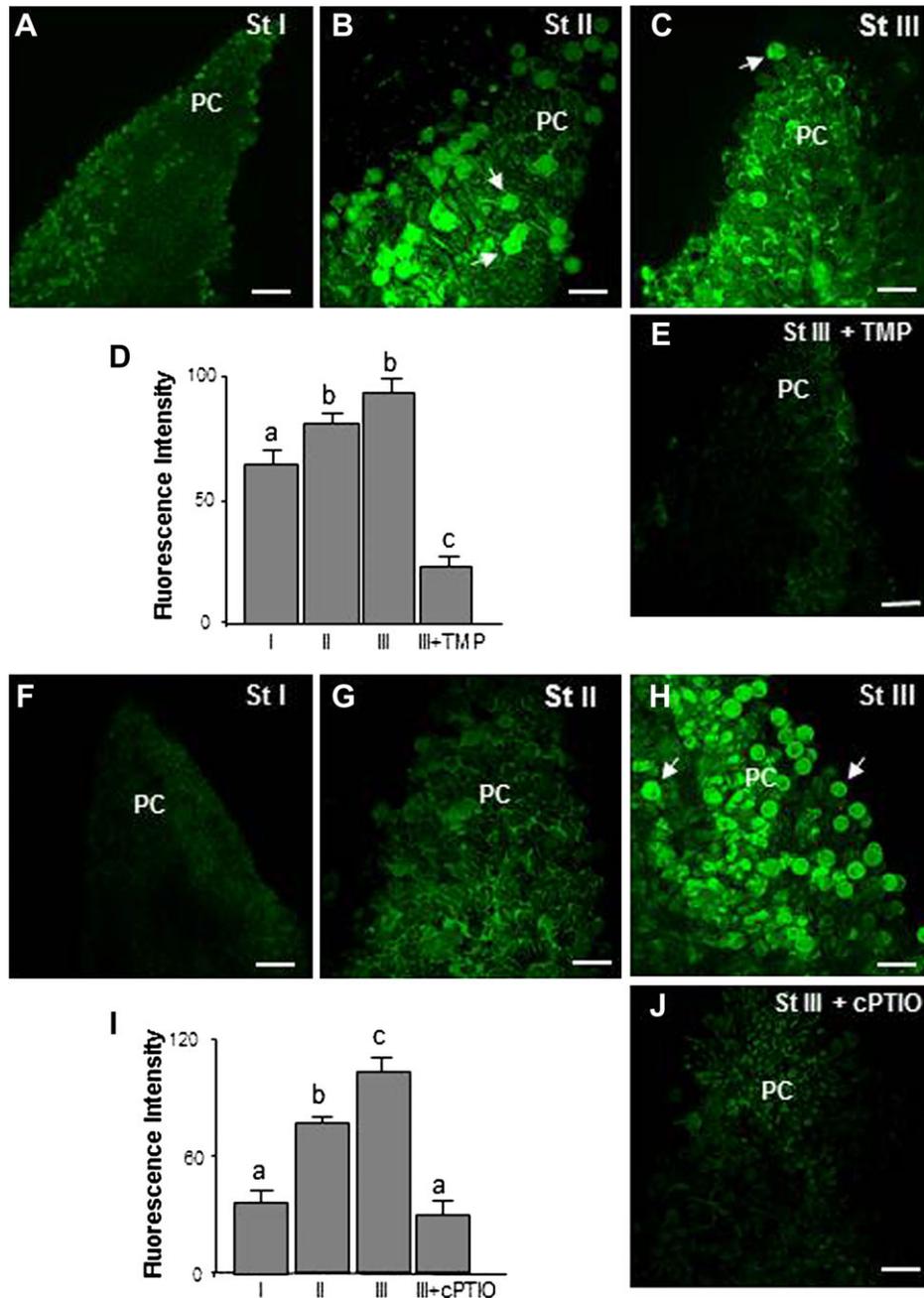


Fig. 2. Superoxide and NO detection in olive pistils excised from freely pollinated flowers by confocal laser scanning microscope. (A, B, C, E) Detection of O_2^- by DHE in pistils at different stages. Green fluorescence corresponding to O_2^- is visible during all three stages in papillar cells and pollen grains after pollination (arrows). (E) Pistil at stage III treated with TMP, an O_2^- scavenger. (F, G, H, J) Detection of NO by DAF2-DA in pistils at different stages. Green fluorescence corresponding to NO was detected both in papillar cells and pollen grains (arrows) at stages II and III. (J) Pistil at stage III treated with cPTIO, a NO scavenger. (D, I) Histograms showing fluorescence, quantified in total images of the stigmal lobules in arbitrary units using LAS AF Leica software. Different letters indicate significant difference at $P < 0.05$ as determined by Duncan's multiple-range test. PC, papillar cells; pollen grains (arrows). Bars=50 μ m.

present in the stigmas during stage I (see Supplementary Fig. S1C, D at *JXB* online). O_2^- -dependent staining remained after pollination during stages II and III (see Supplementary Fig. S1H, I, M, N at *JXB* online). O_2^- was located in the outer part of the papillar cells in the pistil sections, which supports the data obtained with the

corresponding fluorescent dyes (see Supplementary Fig. S2B at *JXB* online). Furthermore, some pollen grains and tubes showed O_2^- -dependent staining (see Supplementary Fig. S2B at *JXB* online). When no DAB or NBT was added to the incubation buffer no signal was visible (see Supplementary Fig. S1E, J, O at *JXB* online).

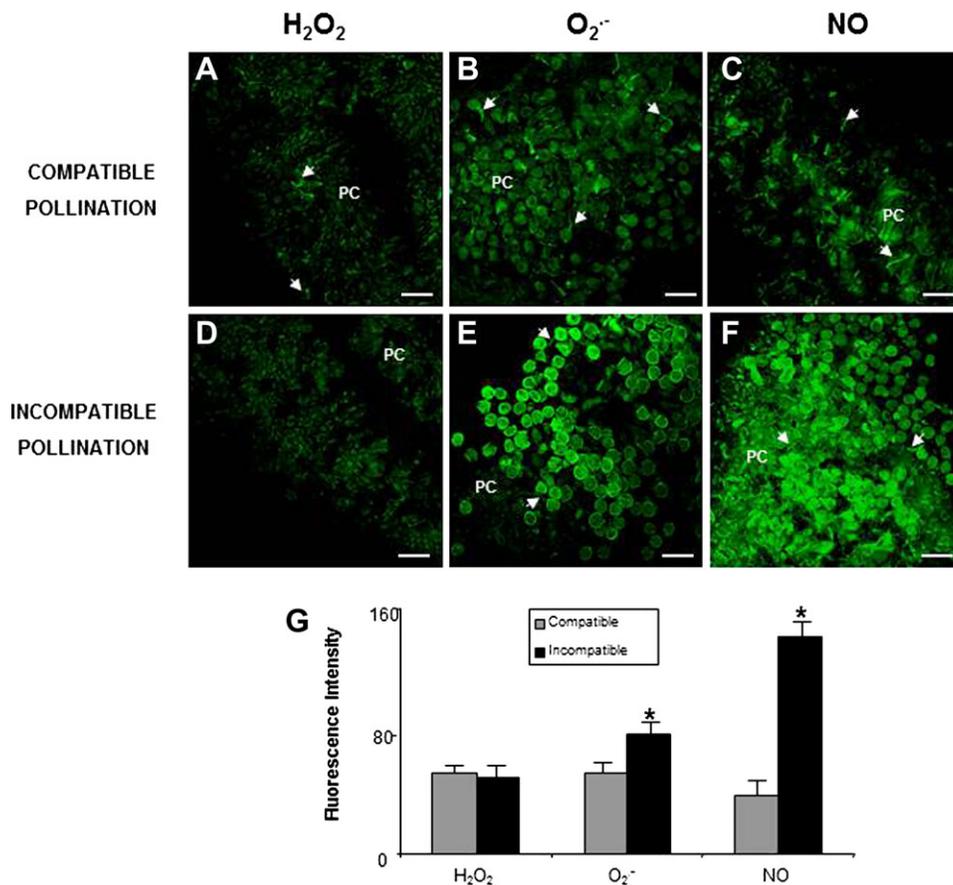


Fig. 3. ROS and NO detected in olive pistils excised from controlled-pollinated flowers. Confocal laser scanning microscope images of stigmas from pistils at stage III. (A, B, C) Stigmas after compatible pollination. (A) H₂O₂ detection by DCF-DA. Faint fluorescence is observed. (B) Detection of O₂⁻ by DHE. Green fluorescence corresponding to O₂⁻ is visible only in some pollen tubes (arrows). (C) Detection of NO by DAF2-DA. Fluorescence detectable only in some pollen tubes (arrows). (D, E, F) Stigmas after incompatible pollination. (D) H₂O₂ detection by DCF-DA is similar to that in compatible pollination. (E) Detection of O₂⁻ by DHE. Papillar cells, pollen grains, and pollen tubes (arrows) show green fluorescence. (F) Detection of NO by DAF2-DA. Green fluorescence is visible in pollen grains and pollen tubes (arrows) and in papillar cells (PC). (G) Histogram showing relative fluorescence intensities quantified in total images of the stigmal lobules and corresponding to H₂O₂, O₂⁻, and NO, reflecting differences between compatible and incompatible pollination. Asterisk indicates significant difference at $P < 0.05$ according to Duncan's multiple-range test. Bars = 50 μm.

ROS and NO sources after free pollination

In the light of the fact that O₂⁻ and NO were the two main reactive species detected in papillar cells and pollen tubes in stages II and III, during which pollen–pistil interaction takes place, it was decided to study the enzymatic activities responsible for their production. When pistils at stage II were incubated with diphenyliodonium (DPI), and sodium azide, O₂⁻-dependent fluorescence decreased during stage III both in papillar cells and pollen grains and tubes (Fig. 4A, B, C, F), suggesting that both NADPH oxidase and peroxidase activities could be involved in O₂⁻ production during free pollination in the olive. Furthermore, an increase in the expression of the different NADPH oxidase isoforms was observed in pollen grains germinated in the presence and absence of pistils (see Supplementary Fig. S3 at *JXB* online).

In addition, after incubating the pistils during stage I with L-NAME, a nitric oxide synthase (NOS) inhibitor, NO-dependent fluorescence was reduced by about 50% in pollen grains in the stigma (Fig. 4D, E, G), suggesting that

a NOS-like activity is involved in NO production during free pollination.

Pollen–pistil interaction controls ROS and NO production

To check whether ROS and NO production in pollen were influenced by their interaction with the pistil, their accumulation was studied in pollen germinated *in vitro* in the presence and absence of pistils from the same cultivar. In the absence of pistils H₂O₂ was detected by DCF-DA both in pollen grains and tubes (Fig. 5A) but fluorescence clearly diminished when pollen grains were germinated in the presence of pistils (Fig. 5B). Staining with the fluorescent dye DHE showed that O₂⁻ was present in pollen grains but not in pollen tubes germinated *in vitro* in the absence of pistils (Fig. 5C). When pistils of the same cultivar were added to the germination medium, both pollen tubes and pollen grains showed an increase in O₂⁻-dependent fluorescence (Fig. 5D). In addition, NO-dependent fluorescence

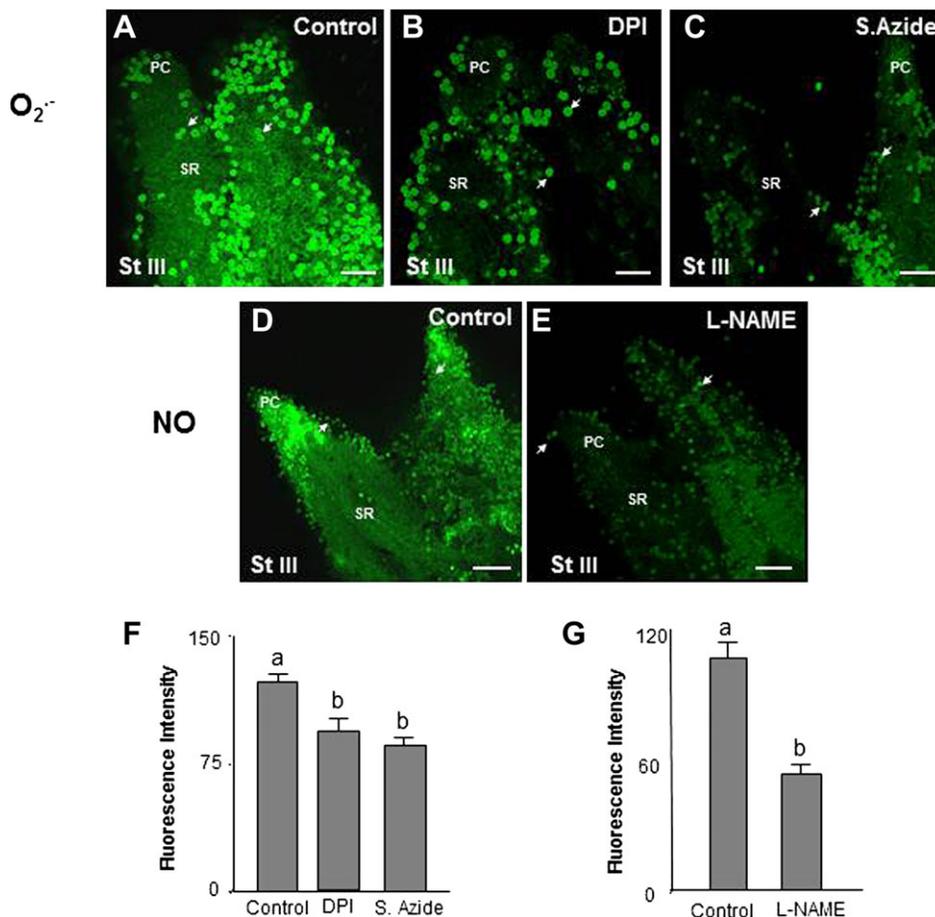


Fig. 4. ROS and NO sources during free pollination. Confocal laser scanning microscope images of stigmas from pistils at stage III incubated with specific enzyme activity inhibitors. (A) Detection of O_2^- by the specific probe DHE in an untreated stigma. (B) Stigmas pretreated with the NADPH oxidase inhibitor DPI before O_2^- detection. (C) Detection of O_2^- by the specific probe DHE in a stigma pretreated with the peroxidase inhibitor sodium azide. (D) Detection of NO by DAF2-DA in an untreated stigma. (E) Stigma pretreated with the specific nitric oxide synthase inhibitor L-NAME before NO detection. (F, G) Histograms showing relative fluorescence intensities quantified in total images of the stigmal lobules and corresponding to O_2^- and NO, reflecting the effect of enzyme-activity inhibitors. Different letters indicate significant difference at $P < 0.05$ according to Duncan's multiple-range test. PC, papillar cells; SR, subpapillar region. Pollen grains (arrows). Bars=50 μ m.

was observed in pollen tubes only when the pollen grains were germinated in the presence of pistils (Fig. 5F). These results suggest that the presence of pistils modifies pollen ROS and NO accumulation, inhibiting H_2O_2 and inducing O_2^- and NO production.

After checking the influence of the pistil ROS and NO production in pollen, it was decided to ascertain whether pollen arrival was responsible for the alterations in ROS content observed in the papillar cells. To this end, pistils in which pollination was avoided by emasculating and bagging the flower buds (stage I) were studied. Since pollen is known to produce NO, an external source of NO (SNP) was applied to these emasculated flowers during stage I to find out whether it was able to mimic the effect of pollen arrival. Flowers were bagged after SNP treatment to avoid the entrance of pollen. After SNP treatment, O_2^- was detected (Fig. 6B) and signs of cell death were found in the stigma during stage III (Fig. 6C). In addition, the level of H_2O_2 detected by DFC-DA was similar to that detected in

pollinated pistils (Fig. 6A). When pistils from emasculated and bagged flower buds (stage I) were analysed at stage III without applying SNP, no fluorescence signal corresponding to H_2O_2 or O_2^- was observed (Fig. 6D, E), and no signs of cell death were found in papillar cells (Fig. 6F). These results highlight the fact that SNP mimicked the effect of pollination in papillar cells, thus supporting the idea of pollen–pistil cross-talk during pollination.

ROS and RNS are crucial for programmed cell death during pollination

To characterize the possible contribution of ROS and RNS to PCD during free pollination in the olive, specific scavengers of these signalling molecules were applied directly to pistils during stage II, and cell death was then investigated at stage III by trypan-blue staining (Fig. 7). Practically all the stigma cells in untreated flowers were dead, as were a high percentage of the pollen grains germinating in them (80%).

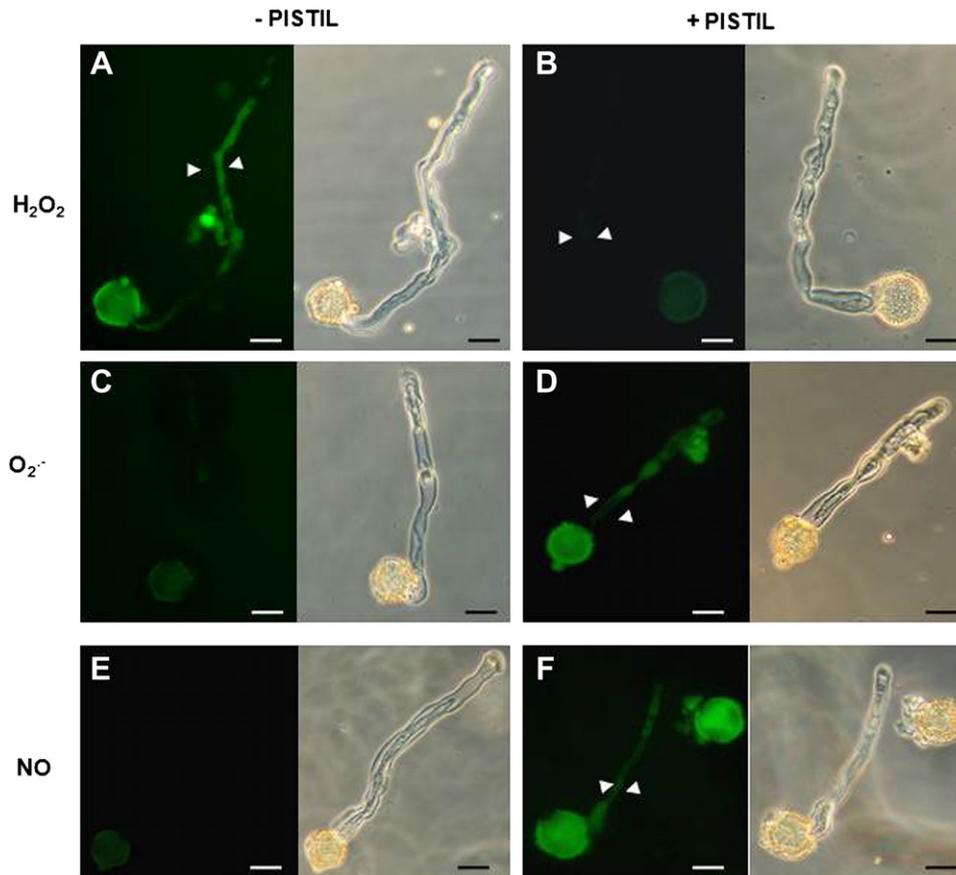


Fig. 5. Detection of ROS and NO in pollen grains germinated *in vitro*. Pollen grains were germinated *in vitro* in the presence or absence of receptive olive pistils of the same cultivar. (A) Detection of H_2O_2 by DCF-DA in pollen tubes (arrowheads) growing in the absence of pistils. (B) Detection of H_2O_2 in pollen grains and pollen tubes (arrowheads) growing in the presence of pistils. (C) Detection of O_2^- by DHE in pollen grains germinated in the absence of pistils. (D) Detection of O_2^- in pollen tubes (arrowheads) of pollen grains germinated in the presence of pistils. (E) Detection of NO by DAF2-DA in pollen grains germinated in the absence of pistils. (H) The same pollen grain under a light. (F) Detection of NO in the pollen tubes (arrowheads) of pollen grains germinated in the presence of pistils. The same pollen grain observed under a light microscope is shown in all cases. Bars=10 μ m.

At the same stage, stigmas from pistils that had been sprayed with TMP during stage II showed a reduction in the staining of papillar cells with trypan blue as well as a 55% decrease in the number of pollen grains stained (Fig. 7A, B, E). Similarly, papillar cells remained practically unstained in stigmas sprayed with cPTIO, whereas there was a 30% decrease in the number of dead pollen grains compared with control samples (Fig. 7A, C, E). Papillar cells from stigmas sprayed with epicatechin were not stained with trypan blue and the number of pollen grains stained underwent a decrease of around 30%, similar to that observed with cPTIO (Fig. 7A, D, E). These results suggest that O_2^- , NO, and peroxynitrite are key signal molecules for PCD in papillar cells and self-incompatible pollen grains in the olive after free pollination.

Since H_2O_2 levels fell significantly in pistils after pollination and O_2^- and NO coexisted in pollinated pistils the possible production of peroxynitrite ($ONOO^-$) as a consequence of O_2^- and NO reaction was studied. The HKGreen-2 fluorescent probe was used to examine $ONOO^-$ production during stage III during free and controlled pollination

(compatible and incompatible; Fig. 8). Fluorescence corresponding to $ONOO^-$ was detected in papillar cells, pollen grains, and pollen tubes during free pollination (Fig. 8A). A 20% increase in the signal was detected in controlled pollination with incompatible pollen (Fig. 8B, E) whilst $ONOO^-$ -dependent fluorescence was reduced by 30% after controlled pollination with compatible pollen (Fig. 8C, E) compared with free pollination. Epicatechin, a $ONOO^-$ scavenger, was used as a negative control, fluorescence intensity being reduced both in papillar cells and pollen during incompatible pollination (Fig. 8D, E).

Because $ONOO^-$ causes tyrosine nitration (Radi, 2004), its presence was also studied by immunocytochemistry with an anti-nitrotyrosine antibody to locate $ONOO^-$ -dependent nitration in pistil sections. Nitrotyrosines were detected after pollination in papillar cells and pollen (see Supplementary Fig. S4 at *JXB* online). When pistils were treated with TMP or cPTIO, a reduction in immunoreactivity was observed (see Supplementary Fig. S4A, B, C, E at *JXB* online). Furthermore, when nitrotyrosine detection and the TUNEL reaction were carried out in serial sections it was found that

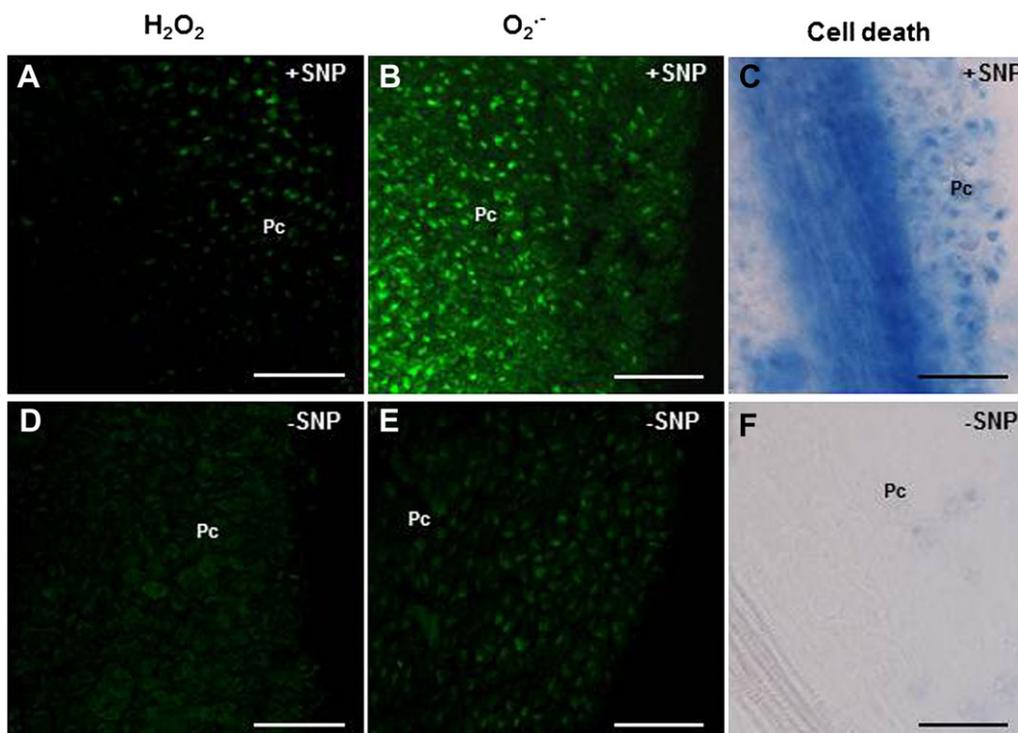


Fig. 6. NO involvement in stigma development. (A, B, C) Flower buds were emasculated during stage I, sprayed with a NO donor (SNP), and bagged to avoid the arrival of pollen grains onto their surface, after which samples were collected at stage III. (D, E, F) Flower buds were emasculated and bagged during stage I without adding SNP, after which samples were collected at stage III. (A, D) H_2O_2 was detected by DCF-DA. (B, E) $\text{O}_2^{\cdot-}$ was detected by DHE. (C, F) Cell death was detected by trypan blue staining. Pc, papillar cells. Bars=50 μm .

pollen containing the nitrotyrosine signal was also positive to TUNEL reaction, whereas the opposite was true with grains containing no nitrotyrosine signal (see Supplementary Fig. S4D at *JXB* online). It is likely, therefore, that ONOO^- -dependent nitration is involved in PCD signalling taking place during incompatible pollination. These results affirm that there is a strong correlation between ONOO^- -dependent nitration and PCD signalling that takes place during incompatible pollination.

In addition, protein nitration was analysed in pollen extracts before and after germination. Western blot analysis using an anti-nitrotyrosine antibody revealed an increase in the number of nitrated proteins in germinated compared with non-germinated pollen (Fig. 9). When *in vitro* germination was carried out in the presence of pistils of the same self-incompatible olive cultivar four new nitrated proteins appeared, two of about 30 kDa and another two of about 50 kDa (Fig. 9), suggesting that pollen–pistil interactions are required to trigger the signalling pathway leading to PCD during olive pollination. These results were confirmed by the absence of nitrated proteins when epicatechin was applied in pollen grains germinating in the presence of Picual pistils (Fig. 9).

Discussion

It is accepted that some types of PCD in plants share features with that in animals, such as DNA fragmentation,

cytochrome *c* leakage from mitochondria to the cytosol and caspase-3 activity (Krishnamurthy *et al.*, 2000; van Doorn and Woltering, 2005; Reape and McCabe, 2008). One example of PCD in plants is the death of the pollen tube during self-incompatible interaction. Within this context, a Ca^{2+} -dependent signalling pathway has been detected in *Papaver*, involving MAPK, cytochrome *c*, and caspase-3-like activity (Thomas *et al.*, 2003, 2004, 2006; Wilkins *et al.*, 2011). It has also been demonstrated that, after the *in vitro* germination of self-incompatible pollen of *Pyrus pyrifolia*, S-RNases induce mitochondria and nuclear DNA alterations (Wang CL *et al.*, 2009) suggesting that PCD is involved in a self-incompatibility response in this species as well. Recently, Serrano *et al.* (2010) reported that PCD is activated in pollen and stigmal papillae during free pollination *in vivo* in olive trees (*Olea europaea* L.). Trypan-blue staining was used to detect the stages during which cell death occurred and, subsequently, TUNEL reaction, caspase-like enzyme activity, and DNA fragmentation showed that this cell death was in fact PCD. In this study it was also found that PCD began after pollination in papillar cells (irrespective of the type of pollen arriving at the stigma: compatible, incompatible or even sterile) and that about 80% of pollen undergoes PCD in freely pollinated pistils (Serrano *et al.*, 2010).

During recent years a large body of evidence has suggested that ROS can activate cell-death programmes (Jabs, 1999; Van Breusegem and Dat, 2006). In plants,

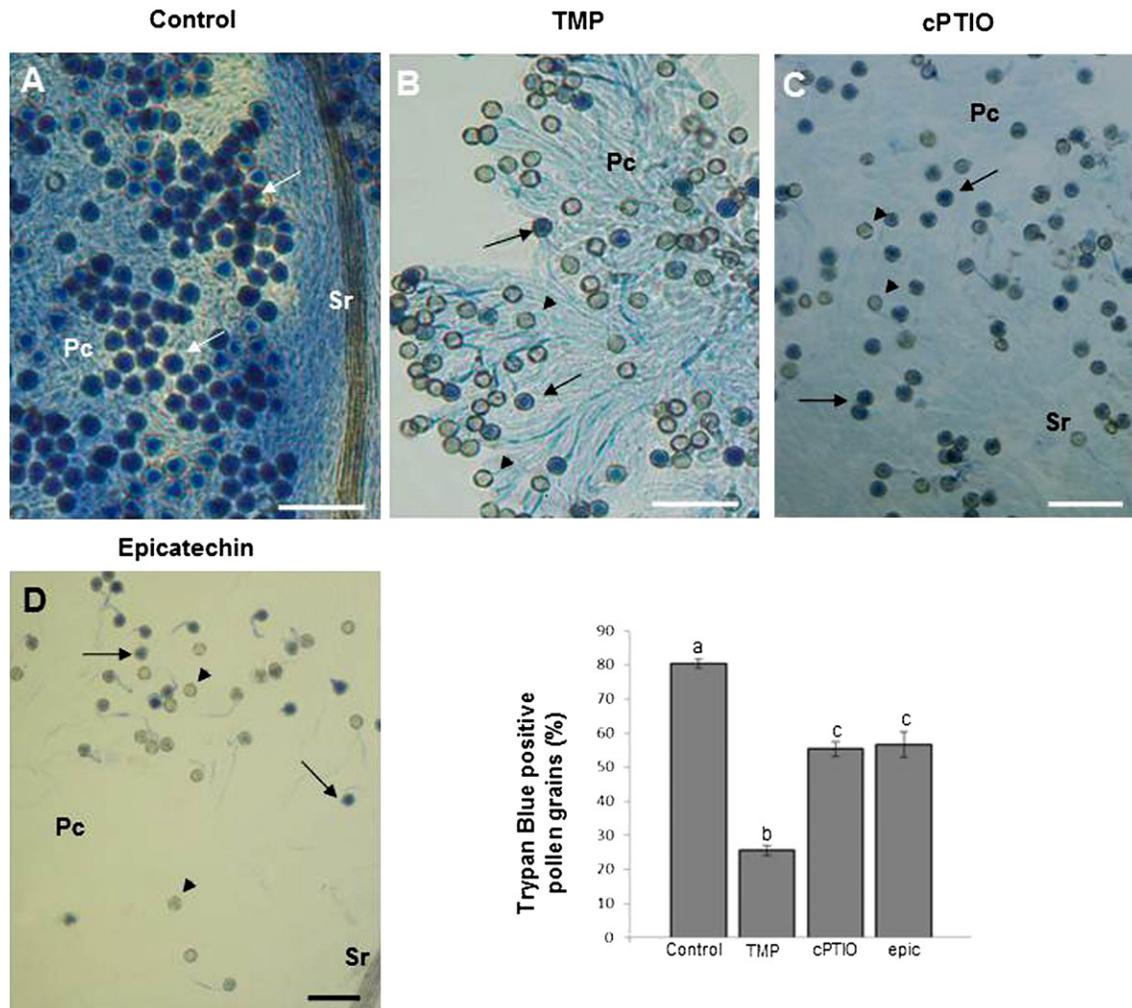


Fig. 7. ROS and RNS function in PCD after free pollination in olive trees. Flowers at stage II were sprayed with TMP (an O_2^- scavenger), cPTIO (a NO scavenger) or epicatechin (a $ONOO^-$ scavenger) and then collected at stage III, in which cell death was assayed by trypan-blue staining. (A) Stigma without pre-treatment. (B) Stigma after TMP pre-treatment. (C) Stigma after cPTIO pre-treatment. (D) Stigma after epicatechin treatment. (E) Histogram showing the number of dead pollen grains after the different pre-treatments. Different letters indicate significant difference at $P < 0.0001$, tested with a χ^2 test. Pc, papillar cells; Sr, subpapillar region. Blue pollen grains (arrows), non-stained pollen grains (arrowheads). Bars=100 μ m.

complementary data come from two different approaches: exogenous treatments with ROS or antioxidants, and transgenic plants with perturbed levels of ROS (Gechev and Hille, 2005; Zago *et al.*, 2006; Gao *et al.*, 2008). Experimental evidence exists to show the specificity in the signalling pathway of different ROS in their triggering of PCD (Gadjev *et al.*, 2006; Gechev *et al.*, 2006; Van Breusegem and Dat, 2006). Finely balanced concentrations of NO and ROS co-operate to trigger PCD (Delledonne *et al.*, 2001; Zago *et al.*, 2006). As far as is known, except for some very recent work carried out in *Papaver* pollen tubes *in vitro* (Wilkins *et al.*, 2011), there are no published data on *in vivo* studies into the involvement of ROS and NO in self-incompatibility PCD. For this reason an analysis of H_2O_2 , O_2^- , and NO production during free and controlled pollination in the olive tree was undertaken and it has been found for the first time that an interplay, both spatial and temporal, of O_2^- and NO is essential to the triggering of

PCD both in papillar cells and self-incompatible pollen grains.

Firstly, a study into the presence of H_2O_2 during pollination was undertaken and it was found that H_2O_2 was present in stigmal papillae from pistils before pollination (Fig. 1). Within this context, it has been suggested that H_2O_2 may play some part in the defence of the stigma against pathogens before pollination (Carter and Thornburg, 2004; McInnis *et al.*, 2006) and it may also be related to stigma receptivity in different species (Dafni and Maués, 1998). As has been reported elsewhere, after free pollination H_2O_2 decreases substantially in stigma lobules in the olive (Zafra *et al.*, 2010). The reduction in H_2O_2 after pollen arrival suggests that it plays no direct role in triggering PCD in response to self-incompatibility in the olive in contrast to what occurs with HR in *Arabidopsis* (Delledonne *et al.*, 2001).

Consequently, two other possible agents involved in PCD, NO, and O_2^- , were investigated (Fig. 2). It was found

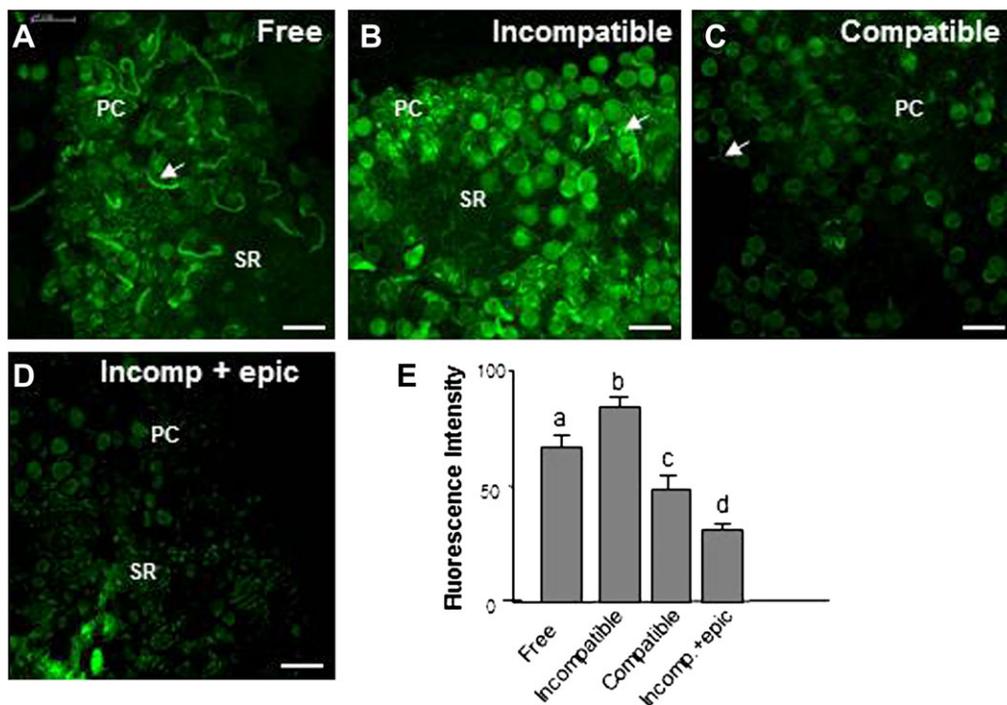


Fig. 8. Peroxynitrite detection by HKGreen-2 in controlled and freely pollinated pistils. Confocal laser scanning microscope images of stigmas from pistils at stage III. (A) Stigma after free pollination. Green fluorescence corresponding to ONOO⁻ is detected mainly in pollen grains and pollen tubes. (B) Stigma after incompatible pollination. An increase in green fluorescence is detected in papillar cells, pollen grains, and pollen tubes (arrows) compared with freely pollinated pistils. (C) ONOO⁻ detection after compatible pollination. Green fluorescence is reduced compared to freely pollinated pistils. (D) ONOO⁻ detection in pistils after incompatible pollination treated with the ONOO⁻ scavenger epicatechin. A reduction in green fluorescence is detected compared with incompatible pollinated pistils (B). (E) Histogram showing relative fluorescence intensities quantified in total images of the stigmal lobules and corresponding to peroxynitrite detection under different conditions. Different letters indicate significant difference at *P* < 0.05 according to Duncan's multiple-range test. PC, papillar cells; SR, subpapillar region. Bars=50 μm.

that papillar cells produced O₂⁻ before pollination (stage I) and its level increased during the pollination and fertilization processes (stages II and III). Furthermore, O₂⁻ was also observed in pollen grains and pollen tubes on the stigma. Papillar cells do not, however, produce NO before pollination, but they do so after the arrival of pollen and until the end of pollination. Furthermore, NO was found in pollen grains and tubes during free pollination. NO has in fact been found in pollen grains and tubes in experiments made in different species (Prado *et al.*, 2004, 2008; McInnis *et al.*, 2006; Bright *et al.*, 2009; Zafra *et al.*, 2010) and it has recently been suggested that it might function as an external signalling molecule, reducing H₂O₂ in *Senecio squalidus* and *Arabidopsis thaliana* stigmal papillae (McInnis *et al.*, 2006). Thus this possibility was checked in our system and, when SNP was added to bag-emasculated flowers during stage I, H₂O₂ that was already present in stigmal papillae had disappeared from the pistils by stage III (Fig. 6), as it also had from pollinated pistils. This suggests that NO from pollen regulates ROS production in papillar cells. When pollination was impeded, no H₂O₂ was detected in the papillar cells of stigmas during stage III. This might be related to changes in receptivity due to alterations in H₂O₂ and peroxidase activity, which do not depend upon pollination, as has been described in various

different species (Dafni and Maués, 1998; Honsho *et al.*, 2007).

To discover whether the stigma might, in the same way, exert any influence upon pollen ROS and NO levels, ROS and NO production were analysed in pollen germinated *in vitro* both in the presence and absence of pistils (Fig. 5). The presence of H₂O₂ in germinating pollen tubes has been reported in *Nicotiana* and *Papaver* in *in vitro* systems (Potocky *et al.*, 2007; Wilkins *et al.*, 2011) and the same has been found in some olive pollen grains germinating *in vivo* (Zafra *et al.*, 2010), but under our fluorescence-detection conditions this signal was only strong enough to be detected when pollen was germinated *in vitro* in the absence of pistils. In our assays, no clear differences were founded *in vitro* in ROS and NO in Picual pollen grains germinated in the presence of pistils of cv. Arbequina but, when the pistil added was from the same cultivar, H₂O₂ production was suppressed, indicating that H₂O₂ production is closely controlled when self-incompatible pollen comes into contact with the pistil. Nevertheless, the results of our experiments show clearly that the presence of self-incompatible pistils increased O₂⁻ and NO production in pollen grains and stimulated it in pollen tubes. They also indicate that O₂⁻ and NO may act as key signalling molecules in the interaction between incompatible pollen and pistils, this being

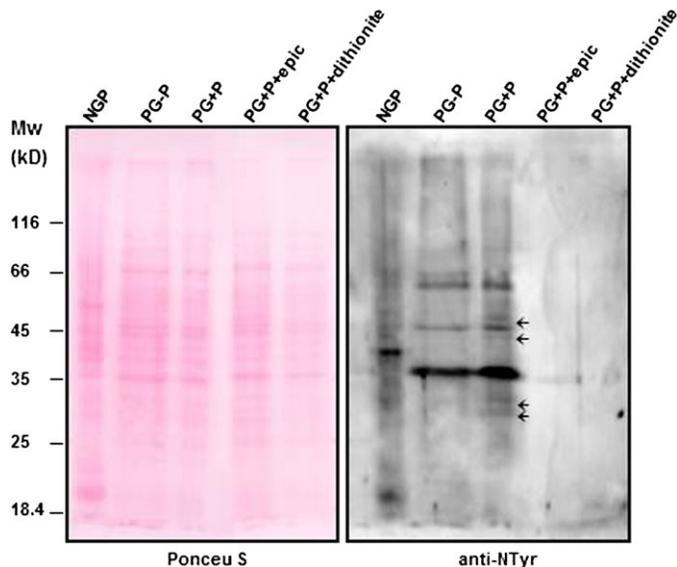


Fig. 9. Tyrosine nitration during *in vitro* pollen germination. Proteins extracted from non-germinated pollen grains (NGP), germinated *in vitro* in the absence (PG-P) or presence (PG+P) of pistils of the same self-incompatible cultivar with epicatechine (PG+P+epic) or not (PG+P), and protein extracts incubated with sodium dithionite were subjected to protein-gel-blot analysis with an anti-nitrotyrosine antibody. The polyvinylidene difluoride membrane was stained with Ponceau red after protein transfer and the stained blot is shown as a loading control. Arrows indicate new bands that appear in the presence of olive pistils.

confirmed in controlled pollinations, in which O_2^- and NO were mainly produced when incompatible pollen was used for pollination.

The induction of O_2^- observed in germinating pollen grains led us to search for a possible source of this particular ROS. Several mechanisms have been proposed to explain the generation of ROS by plants, but it would seem that a plasma membrane NADPH oxidase, analogous to that which generates the respiratory burst in mammalian phagocytes, is a likely source (Lamb and Dixon, 1997; Torres *et al.*, 2002). *In vivo* assays showed a reduction of O_2^- in papillar cells, pollen grains, and tubes pretreated with DPI (Fig. 4), an inhibitor of the neutrophil NADPH oxidase, which blocks the plant oxidative burst (Doke and Ohashi, 1988), suggesting that this enzyme may well be a possible source of O_2^- in pollen. Semi-quantitative RT-PCR results showed an increase in NADPH oxidase 2 expression in germinating olive pollen in the presence of pistils of the same cultivar (see Supplementary Fig. S3 at *JXB* online), revealing the possible involvement of this isoform in self-incompatible O_2^- production. Furthermore, pretreatment with sodium azide, a peroxidase inhibitor, also resulted in a reduction in O_2^- production. NOS-like activity is one of the sources of NO since the treatment of pistils with L-NAME (Sandalio *et al.*, 2008) drastically reduced NO production in both pollen and papillar cells. Alternative sources for both NO and O_2^- cannot be ruled out, i.e. the

possible role of peroxisomes in ROS and NO production via xanthine oxidase and plant NOS-like activities respectively, which have been investigated in pollen and pistils of different species (Tezuka *et al.*, 1997; Prado *et al.*, 2004) and, in the same way, the involvement of nitrate reductase in the production of NO cannot be excluded, as has previously been suggested (Mittler *et al.*, 2004; Neill *et al.*, 2008).

Cell death during stage III in pistils that had previously been emasculated and bagged during stage I was checked to investigate further the involvement of pollen-dependent NO production in papillar PCD. In this case, no cell death was observed in stigmal papillar cells, confirming what has been previously reported (Serrano *et al.*, 2010). Our results also showed that cell death appeared during stage III when emasculated bagged pistils had been pretreated during stage I with the NO donor SNP (Fig. 6). These results suggest that NO may play a role in papillar PCD. To confirm this, PCD was studied *in vivo* in pollen–pistil interactions taking place in the absence of NO. cPTIO eradicated PCD in papillar cells and reduced pollen PCD (Fig. 7). When pistils were pretreated with SNP during stage I and no pollen was allowed to land, both an accumulation of O_2^- and PCD were detected. Thus the possible involvement of O_2^- in PCD during pollen–pistil interaction was also investigated. The inhibition of superoxide accumulation by using TMP reduced PCD detected both in papillar cells and pollen grains by 55%, indicating that during pollen–pistil interaction both NO and O_2^- were required to activate PCD.

Our results showed that NO and O_2^- coexisted after pollination and both molecules were needed to activate PCD and so the possible role of ONOO⁻ in self-incompatibility PCD was further investigated. NO and O_2^- rapidly form ONOO⁻ in mammalian native immune cells such as macrophages and neutrophils in order to kill pathogens (Fang *et al.*, 1997) and it is considered to be the main toxic reactive nitrogen species in animal cells (Stamler *et al.*, 1992). Although high external concentrations of ONOO⁻ (1 mM) have been reported to induce some necrotic lesions in *Arabidopsis* (Alamillo and Garcia-Olmedo, 2001), exposure to this high concentration does not seem to be essential to the induction of PCD (Delledonne *et al.*, 2001; Romero-Puertas *et al.*, 2007), probably due to the ability of plants to detoxify ONOO⁻ under normal physiological conditions. Even so it is likely that ONOO⁻ plays an important role in signalling functions in plants, since it was found that SIN-1, an ONOO⁻ donor, induces genes related to defence against pathogenesis, such as PR-1 in tobacco leaves (Durner *et al.*, 1998) and ONOO⁻ induces protein nitration in soybean and tobacco (Delledonne *et al.*, 2001; Saito *et al.*, 2006). Furthermore, an increase in nitrated proteins during the progression of the HR has been shown *in vivo*, as well as an inhibition of the plant antioxidant system involved in ONOO⁻ detoxification (Romero-Puertas *et al.*, 2007). Experiments with HKGreen-2 showed that during free pollination, both papillar cells and pollen produce ONOO⁻ during stage III (Fig. 8). PCD is induced in papillar cells after pollen landing irrespective of their origin (compatible, incompatible, and even sterile pollen; Serrano *et al.*, 2010).

No cell death was observed in papillar cells treated with epicatechin, indicating that ONOO⁻ is involved in papillar cell PCD. In addition, ONOO⁻ production was found in self-incompatible pollen, which undergoes PCD. Furthermore, when open flowers during stage II were treated with epicatechin, a reduction of about 30% was observed in pollen grains in the process of PCD (Fig. 7). These results suggest that ONOO⁻ is a key signal molecule in the PCD of incompatible pollen. The increase in ONOO⁻ during stage III reveals that O₂⁻ is reacting with NO, which could also explain why a parallel increase in H₂O₂ production was not observed.

In addition, when protein tyrosine nitration was looked into as being an effect of ONOO⁻ reactivity (Radi, 2004; Szabo *et al.*, 2007), nitration was found both in stigmal papillar cells and in pollen undergoing cell death. A positive nitrotyrosine signal was found in self-incompatible pollen undergoing PCD (see Supplementary Fig. S3 at *JXB* online). To confirm that protein nitration is important during pollen germination, pollen extracts before and after germination were studied in the presence and absence of pistils of the same cultivar. Immunoblotting revealed an increase in protein nitration in germinating pollen and four new nitrated proteins were detected when germination occurred in the presence of pistils of the same self-incompatible cultivar (Fig. 9). Tyrosine nitration decreased when TMP, cPTIO or epicatechin were applied, supporting the idea that the tyrosine nitration observed is ONOO⁻-dependent. Protein tyrosine nitration, the covalent attachment of a nitro (-NO₂) group to the phenolic ring of Tyr, is considered to be a functionally important form of physiological NO-dependent post-translational modification (Schopfer *et al.*, 2003). It has been shown that Tyr nitration can interfere with phosphorylation, a well-known signalling mechanism involved in a wide range of cell transduction pathways (Greenacre and Ischiropoulos, 2001; Schopfer *et al.*, 2003). In particular, an important role in self-incompatibility has been shown for MAPK signalling via p56-MAPK, which participates in initiating the early PCD signalling cascade, leading to the death of the pollen tube (Li *et al.*, 2007). Interestingly, a physiological concentration of ROS has been proposed in the enhancement of mammal sperm capacity by regulating phosphorylation pathways (Ford, 2004). In fact, in human sperm, superoxide regulates the phosphorylation of the Thr-Glu-Tyr motif in low-molecular-weight proteins (16–33 kDa), as well as the ERK pathway (de Lamirande and Gagnon, 2002). In addition, O₂⁻ may react with NO to produce ONOO⁻, which may go on to induce Tyr nitration of sperm proteins as well as Tyr phosphorylation (Herrero and Gagnon, 2001), suggesting an exciting parallel with sperm–egg interactions in mammal systems.

In conclusion, it would seem that there is sufficient evidence to assign a signalling role to ROS and RNS during pollen–pistil interactions in the olive, showing that reactive oxygen and nitrogen species are key components of pollen–stigma interaction leading papillar cells and self-incompatible pollen to PCD. These results reinforce the concept of a model of ROS/NO balance to decide cell fate, in particular,

with regard to self-incompatibility. The link observed between Tyr nitration and PCD in self-incompatibility paves the way for future studies into possible interaction in nitration-phosphorylation signalling pathways.

Supplementary data

Supplementary data can be found at *JXB* online.

Appendix S1. Supplementary experimental procedures.

Supplementary Fig. S1. Detection by stereomicroscopy of ROS in olive pistils at stages I, II, and III.

Supplementary Fig. S2. Detection by light microscopy of ROS in olive pistils at stages I, II, and III.

Supplementary Fig. S3. NADPH oxidase expression during pollen germination.

Supplementary Fig. S4. Tyrosine nitration in *in vivo* pollen germination.

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