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Roles for NO and ROS signalling in pollen germination and pollen-tube elongation in *Cupressus arizonica*

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Abstract

Roles for nitric oxide (NO) and reactive oxygen species (ROS) during pollen-tube growth have been well established in angiosperms, but there remains lack of information regarding their potential signalling roles in pollen tubes in gymnosperms. Here, the pollen-tube elongation of Arizona cypress (*Cupressus arizonica* Greene) was investigated. Nitric oxide, ROS, and actin were detected using their respective fluorescent probes. Both NO and ROS were observed in the nuclei of generative cells and pollen-tube cells, and in the cytoplasm in the tip region. An intracellular NO content in the pollen cells was lowered using an NO scavenger or an NO-synthase inhibitor. Similarly, an endogenous ROS content in the pollen cells was lowered using an NAD(P)H oxidase inhibitor. These treatments reduced pollen germination and pollen-tube growth, and induced severe morphological abnormalities. Inhibition of NO and ROS accumulation also severely disrupted the actin cytoskeleton in the pollen tubes. These data indicate that NO and ROS had signalling roles in pollen germination and pollen-tube formation in cypress.

Additional key words: actin, cypress, cytoskeleton, hydrogen peroxide.

Introduction

During sexual plant reproduction in gymnosperms, pollen grains germinate on female structures and produce the tip of a growing pollen tube that penetrates a micropyle to deliver male gametes to ovules. During this process, the pollen of *Cupressus* is subjected to changes in water content (Chichiriccò and Pacini 2008), rupture, and exine opening, which is caused by rapid intine expansion (Chichiriccò *et al*. 2009, Danti *et al*. 2011). Successful pollen germination and pollen-tube production is essential for ovule fertilisation.

The emergence and elongation of pollen tubes is a typical example of polarised plant-cell expansion. In such cells, a complex signalling network which includes actin and Ca^{2+} is required for polar growth mechanisms (Hepler *et al*. 2001). Apart from such well-characterised factors, other molecules are likely to be important in the polarisation of pollen tubes, such as nitric oxide (NO) and reactive oxygen species (ROS). Both are key components of diverse signalling networks in many processes in plants including pollen-tube growth (Prado *et al*. 2004, Potocký *et al*. 2007).

The roles of NO in plants has been less characterised compared to animals. The enzymes that catalyse NO synthesis include nitrate reductase and a nitric oxide synthase (NOS)-like enzyme AtNOS1 (Moreau *et al*. 2010, Misra *et al*. 2011). Nitric oxide is involved in several processes in plants including seed germination, flower development, programmed cell death (Misra *et al*. 2011), vesicle trafficking (Kasprowicz *et al*. 2009), and regulation of pollen-tube growth (Prado *et al*. 2004). In plant reproductive processes, intracellular NO is produced by pollen primarily in peroxisomes (Prado *et al*. 2004), and it is involved in pollen-pistil interactions (Prado *et al*. 2008).

Reactive oxygen species are toxic products of oxygen metabolism that have important roles in the regulation of major cellular processes which include cell growth and development, programmed cell death, and responses to

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Abbreviations: BK medium - Brewbaker and Kwack liquid medium; $[Ca^{2+}]_i$ - intracellular Ca^{2+} concentration; cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM - 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; $DCFH_2$-DA - 2'7'-dichlorodihydrofluorescein diacetate; DPI - diphenyleneiodonium chloride; L-NNA - N_ω-nitro-L-arginine; NOS - nitric oxide synthase; ROS - reactive oxygen species.
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biotic and abiotic stresses (Bhattacharjee 2012). Plants produce a high amount of the ROS as part of their normal metabolism, *e.g.*, during modification of cell-wall components (Smirnova *et al*. 2014) or growth of root hairs (Carol and Dolan 2006). During pollen-tube growth, the ROS are involved in pollen germination (Speranza *et al*. 2012), polarised growth (Potocký *et al*. 2007), pollen-tube elongation (Lassig *et al*. 2014), guidance and ovule targeting (McInnis *et al*. 2006), and pollen-tube burst during fertilisation (Duan *et al*. 2014). The ROS in plant cells are mainly formed in chloroplasts, mitochondria, and cytoplasm (Mittler *et al*. 2004) through the actions of various oxidases and peroxidases, such as the well-known NAD(P)H oxidases (Sagi and Fluhr 2006).

In combination with other factors, such as an intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, NO and the ROS are hypothesised to modulate the cytoskeleton of pollen tubes. In angiosperms, $[Ca^{2+}]_i$ is known to affect the organisation of actin filaments through the activities of actin-binding proteins (Ren and Xiang 2007). On the other hand, NO and the ROS are candidates for modulators of $[Ca^{2+}]_i$ (Potocký *et al*. 2007). Most information on relationships between the ROS and $[Ca^{2+}]_i$ come from studies on root hairs, where the ROS appear to be involved in the regulation of $[Ca^{2+}]_i$ through activation of Ca^{2+}-permeable channels. In addition, $[Ca^{2+}]_i$ can regulate the activity of ROS-producing enzymes (Šamaj *et al*. 2004). Consequently, cytoskeleton dynamics is likely to be regulated by NO/ROS-dependent mechanisms (Kasprowicz *et al*. 2009). As result, the combinatory roles of NO/ROS and $[Ca^{2+}]_i$ are believed to control many cytoskeleton-dependent processes, such as organelle movement and vesicle accumulation. Nevertheless, it is not known how NO and the ROS precisely control cytoskeleton dynamics in pollen tubes.

Here, the effects of NO and the ROS on the distribution of actin filaments were investigated during pollen-tube growth in the Arizona cypress. Furthermore, to study the patterns of actin distribution, cytoplasmic organisation, and vesicle trafficking during pollen germination and tube growth, NO/ROS-modulating agents were applied.

Materials and methods

Mature pollen was collected from *Cupressus arizonica* Greene plants grown in a rural area around Perugia (central Italy) in January and February 2012. The pollen was aliquoted into 2-cm^3 sterile microcentrifuge tubes and stored dry at 4 °C until use.

Unless otherwise stated, all chemicals used were from *Sigma-Aldrich* (St. Louis, USA). An NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO) was dissolved in distilled water, and an NOS inhibitor N_ω-nitro-L-arginine (L-NNA) was dissolved in 0.5 M HCl. An NAD(P)H oxidase inhibitor diphenyleneiodonium chloride (DPI) was dissolved in dimethyl sulphoxide. Probes used to detect NO and the ROS in growing pollen tubes were 4-amino-5-methyl-amino-2',7'-difluorofluorescein diacetate (DAF-FM, *Molecular Probes,* Eugene, USA) and 2',7'-dichloro-dihydrofluorescein diacetate ($DCFH_2$-DA); both were dissolved in dimethyl sulphoxide. A probe to detect actin filaments was *Alexa 543-phalloidin* (*Molecular Probes*) which was dissolved in methanol. The working solutions of these inhibitors and probes were prepared just before use.

The pollen was cultured in a Brewbaker and Kwack liquid medium that contained 10 % (m/v) sucrose (BK medium). After rehydration at room temperature under an almost 100 % relative humidity, the pollen was spread on 24-well sterile plates containing 1.5 cm^3 of a sterile BK medium and incubated at 22 - 24 °C for at least 3 weeks.

To evaluate the effects of the NO and ROS inhibitors on germination and pollen-tube length, different concentrations of cPTIO (0, 50, 100, and 200 μM), L-NNA (0, 15, 30, and 60 μM) and DPI (0, 10, 30, and 50 μM) were added to a BK liquid medium at the time zero of the pollen cultures.

The germination percentage was measured after 7 d, and the pollen-tube lengths after 14 and 21 d in 3 independent experiments (at least 100 pollen tubes per treatment in each experiment). Pollen grains were considered as germinated when the pollen-tube length was greater than the diameter of the pollen grain. The morphology of the pollen tubes was examined under a microscope (*Axiophot*, *Zeiss*, Göttingen, Germany), and digital images were captured with a video camera (*AxioCam MRc 5*, *Zeiss*).

The viability of the re-hydrated control pollen and of the pollen treated with the inhibitors was determined with a fluorochromatic reaction with fluorescein diacetate, which indicates the integrity of the plasma membrane, as reported by Heslop-Harrison *et al*. (1984). To determine whether the used agents inhibited pollen viability, the highest concentrations for the inhibition of NO (100 μM cPTIO, 60 μM L-NNA) and the ROS (50 μM DPI) were tested. The fluorochromatic reaction was allowed to proceed in the dark for 10 min, and the samples were then observed under the fluorescence microscope (*Axiophot*) equipped with the video camera (*AxioCam MRc 5*). At least 300 pollen grains were examined per treatment.

Intracellular NO was visualised using a fluorescent NO indicator DAF-FM according to the method of Bright *et al*. (2009) with modifications. Pollen (1 mg) was incubated in a BK medium at 4 °C for 10 min, and then 10 μM DAF-FM was added. The samples were incubated in the dark for 30 min to allow the dye to enter the pollen. The pollen suspensions were then centrifuged at 6 500 *g*

for 1 min, and the supernatants were discarded. A fresh BK medium was then added to the pellets which were resuspended and left at room temperature for 20 min. Finally, 0.05cm^3 of the pollen suspension was placed onto a glass slide, covered with a glass coverslip, and examined under the fluorescence microscope (*AxioImager*) equipped with an *ApoTome* module (a 470 nm excitation and 525 nm emission). Detection of the ROS in the hydrated pollen grains was performed using a fluorescence ROS indicator $DCFH_2$-DA (2.5 μM), as previously reported (Pasqualini *et al*. 2011). The ROS were determined as the percentages of fluorescent pollen grains relative to the total pollen grains. Six slides were prepared, and at least 100 pollen grains were counted for each slide.

Following preliminary experiments to determine the effects of NO, we used the lowest concentration of the NO quencher cPTIO (50 μM) and the intermediate concentration of the NO inhibitor L-NNA (30 μM). To determine the effects of ROS inhibition, we used the lowest concentration of DPI (10 μM). The presence of NO in pollen tubes was monitored by incubating 14-d-culture pollen with 10 μM DAF-FM for 30 min in the dark to allow the dye to enter the pollen. Subsequently, the excess fluorophore was washed out. As negative control, samples without DAF-FM were run in parallel. Imaging was carried out using the fluorescence microscope (*AxioImager*). No fluorescence was detected in the absence of DAF-FM.

To determine the effects of NO inhibition, 50 μM cPTIO and 30 μM L-NNA were added to 14-d-culture pollen. After 1 h, the probe for NO localisation was added (see above), and the pollen was observed under the fluorescence microscope (*AxioImager*). The ROS were detected by incubating 14-d-culture pollen with 2.5 μM $DCFH_2$-DA for 1 min followed by a wash. Imaging was carried out using the fluorescence microscope (*AxioImager*). No fluorescence was detected in the absence of $DCFH_2$-DA, and at least 40 pollen grains per treatment in each of three independent experiments were measured using *ImageJ*.

Labelling F-actin was performed as previously described by Lovy-Wheeler *et al*. (2005) with modifications. Cultured pollen grains were simultaneously fixed and permeabilised for 30 min using a buffer composed of 100 mM piperazine-1,4-bis(2-ethanesulfonic acid (PIPES), 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.05 % (v/v) *Triton X-100*, 1.5 % (m/v) formaldehyde, and 0.05 % (m/v) glutaraldehyde, pH 9. The fixative was then washed two times with the same buffer, and 6.6 μM *Alexa 543-phalloidin* was added. The samples were examined 15 to 60 min after staining using the fluorescence microscope (*AxioImager*) equipped with the *ApoTome* module. To evaluate the effects of NO and ROS inhibition on actin filaments, the NO inhibitor (30 μM L-NNA) and the ROS inhibitor (10 μM DPI) were added to 14-d-cultured pollen. After 1 h, the inhibitors were washed out and the culture medium with the actin probe was added to the pollen.

To determine NAD(P)H oxidase activity and H_2O_2 content, pollen grains (400 mg) were hydrated in Petri dishes containing 5 cm^3 of a phosphate-buffered saline, 0.1 % (v/v) *Triton X-100*, and a protease inhibitor cocktail (10 mm^3 cm^{-3} in a phosphate-buffered saline) at 4 °C for 24 h with gentle shaking. The pollen suspensions were then centrifuged at 14 000 *g* for 30 min, and the H_2O_2 content, NAD(P)H oxidase activity, and soluble protein were assayed in the supernatants as reported by Pasqualini *et al*. (2011). An H_2O_2 content released from the pollen after hydration was measured without and with the NAD(P)H oxidase inhibitor (100 μM DPI). A soluble protein content in the supernatants was measured using the dye-binding method of Bradford (1976) with bovine serum albumin as standard.

One-way or two-way *ANOVA* was used to test the effects of the NO and ROS inhibitors on the germination rate, pollen-tube length, and NO and ROS relative fluorescence. *Post-hoc* multiple comparisons were conducted using the Duncan's tests to analyse differences among means. For other details see figure legends.

Results and discussion

Intracellular NO was monitored using the NO-sensitive probe DAF-FM in a fluorimetric assay (Kojima *et al*. 1998). Constitutive NO was detected in the protoplast of cypress pollen grains where the exine was broken (Fig. 1*C,D*). Without the probe added to the hydrated pollen grains, green autofluorescence was detected in the exine (Fig. 1*A,B*). These data indicate that the pollen grains from *C. arizonica* generated NO internally after the re-hydration.

Plasma-membrane-localised NAD(P)H oxidases catalyse the extracellular formation of the superoxide radical ($^{\bullet}O_2^-$) from molecular oxygen using NADH/NADPH as electron donor. This is in turn transformed into H_2O_2 by superoxide dismutase. As NAD(P)H oxidase activity is the major source of ROS in plants including pollen (Potocký *et al*. 2007), the H_2O_2 content in the hydration medium was measured after 24 h without (control) and with the addition of the NAD(P)H oxidase inhibitor DPI. Under these conditions in the control, 79.73 ± 4.26 nmol(H_2O_2) g^{-1}(pollen f.m.) was detected after the 24 h hydration (n = 6), whereas no H_2O_2 was detected with DPI in the hydration medium.

Although NAD(P)H oxidase activity in pollen grains of gymnosperms has been reported to be significantly lower than in pollen grains of angiosperms (Wang *et al*. 2009, Pasqualini *et al*. 2011), the NAD(P)H oxidase activity was also determined for the hydrated pollen grains using nitroblue tetrazolium reduction to formazan. This demonstrated the relative NADH and NADPH oxidase activities of 0.133 ± 0.003 and 0.142 ± 0.004 a.u.

(n = 6), respectively, in the hydration medium after 24 h.

Using fluorescent microscopy and the ROS-sensitive fluorescent probe $DCFH_2DA$, the ROS inside pollen were detected. This probe lacks specificity among ROS and reacts only slowly with H_2O_2 and $^{\bullet}O^-_2$ and rapidly with the hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$; Setsukinai *et al*. 2003). It thus follows that this DCF fluorescence assay determines a generalised oxidative stress rather than that of any particular ROS. Our analysis shows that 22.7 ± 1.69 % (at least 100 pollen grains examined in each of 6 slides prepared) of the pollen grains showed ROS fluorescence.

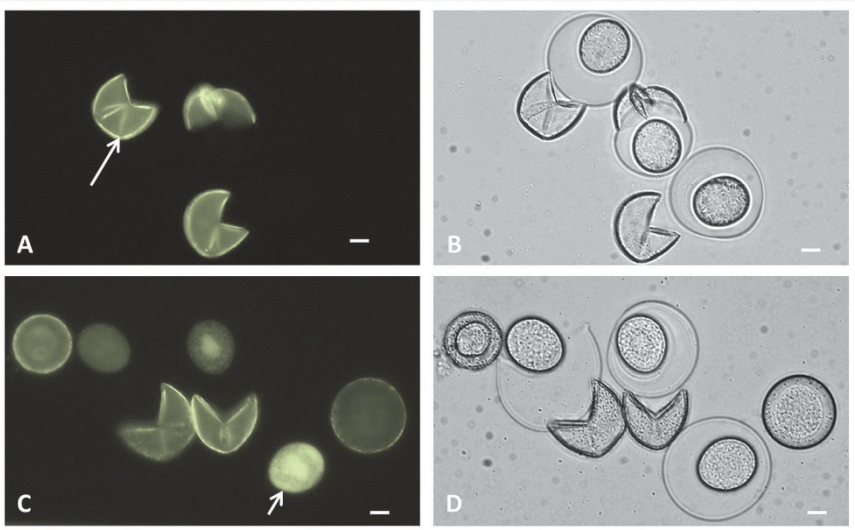

Fig. 1. Visualisation of NO production in hydrated pollen grains. *A*,*B* - Pollen without the addition of NO probe DAF-FM showing autofluorescence of the exine (*A*, *arrow*), and corresponding image of the pollen under bright field microscopy (*B*). *C*,*D* - Pollen with the addition of the NO probe DAF-FM showing fluorescence in the pollen protoplast (*C*, *arrow*), and the corresponding image of the pollen under bright field microscopy (*D*). *Bars* are 10 μm.

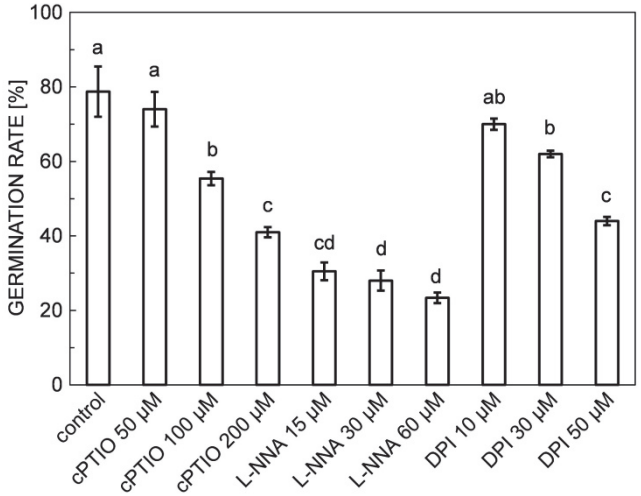

Fig. 2. Effects of different concentrations of NO-inhibitors cPTIO and L-NNA and of a ROS inhibitor DPI on the germination rate of 7-d-cultured pollen. Data are means ± SE of three independent experiments with at least 100 pollen grains per treatment in each experiment. Different letters indicate statistically significant differences at $P \leq 0.01$ according to the Duncan's multiple range tests.

The amount of soluble proteins released from these cypress pollen grains into the hydration medium during 24 h was low [2.78 ± 0.13 mg g^{-1}(f.m.); n = 6] when compared with the protein released from pollen of an angiosperm *Ambrosia artemisiifolia* [14 mg g^{-1}(f.m.); Pasqualini *et al*. 2011]; however, a low protein release from hydrated cypress pollen was also reported [0.5 mg g^{-1}(f.m.); Arilla *et al*. 2004].

In-vitro germination of the cypress pollen was very slow (a tube elongation rate of 0.38 μm per hour) as expected and reported for other conifer pollen (Fernando *et al*. 2005). The pollen thus took many days to germinate. However, the percentage of germinated pollen was high, with a mean pollen viability of 81.1 ± 5.6 %, as measured with the fluorescein diacetate probe.

To determine whether NO and the ROS are essential

for pollen germination, the NO and ROS content was manipulated. To ascertain whether NO quencher or inhibitor and ROS inhibitor affect *per se* pollen viability, the highest concentrations of the NO quenchers (100 μM cPTIO, 60 μM L-NNA), and the ROS inhibitor (50 μM DPI) were tested. Pollen viabilities remained at

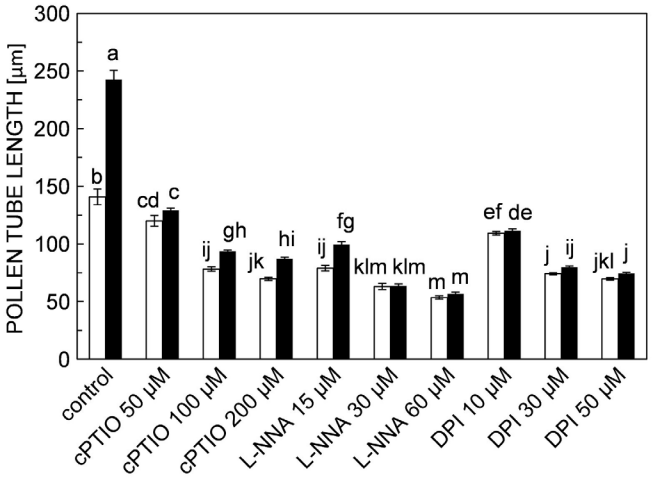

Fig. 3. Effects of cPTIO, L-NNA, and DPI on the pollen-tube length after 14 and 21 d after inhibitor addition. Data are means ± SE of three independent experiments with at least 100 pollen tubes per treatment in each experiment. Different letters indicate statistically significant differences at $P \leq 0.01$.

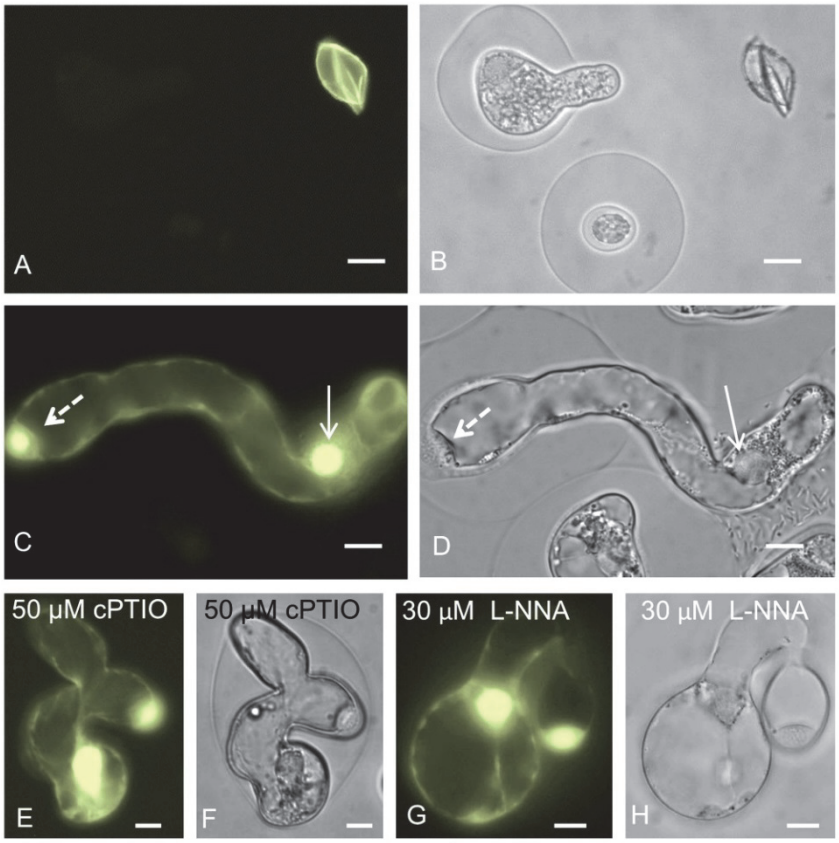

Fig. 4. Visualisation of NO production in 14-d-germinated pollen. *A*,*B* - Pollen without the addition of an NO probe DAF-FM showing autofluorescence of the exine (*A*), and the corresponding image of the pollen under bright field microscopy (*B*). *C*-*H* - Pollen with the addition of the NO probe DAF-FM (*C*,*E*,*G*), with the corresponding images of the pollen under bright field microscopy (*D*,*F*,*H*). *C*,*D* - NO in the germinated pollen without NO inhibitors; the *solid arrow* shows NO in the nucleus of a generative cell and the *dashed arrow* NO in the nucleus of a tube cell. *E*-*G* - NO in the germinated pollen with NO inhibitors as indicated. *Bars* are 10 μm.

78.2 ± 3.2 % with cPTIO, 80.5 ± 4.2 % with L-NNA, and 79.6 ± 1.2 % with DPI with respect to 81.1 ± 5.6 % of the control without inhibitors.

However, inhibition of NO accumulation following addition of cPTIO and L-NNA influenced the germination rate (Fig. 2) and pollen-tube growth (Fig. 3) in a dose-dependent manner. Indeed, inhibition of the pollen-tube growth by NO and the ROS was even more evident 21 d after the the treatments (Fig. 3): in the control, there was an increased pollen-tube length of ~70 % from 14 to 21 d, whereas after NO/ROS inhibition, there was no increase in the pollen-tube length over this period.

The fluorescent probe DAF-FM is a membrane-permeable derivative of an NO-sensitive fluorophore 4,5-diaminofluorescein (DAF-2), and it was used to determine the content of NO in 14-d-germinated pollen. Without the probe added, brilliant green autofluorescence was detected only in the exine (Fig. 4*A*,*B*). After addition of the NO probe (Fig. 4*C*-*H*), NO was distributed throughout nearly the entire pollen tube (Fig. 4*C*,*D*). Bright green fluorescence was observed in the nucleus of generative cells and pollen-tube cells and also in the cytoplasm in the tip region (Fig. 4*C*). This fluorescence in the nuclei was confirmed by DAPI staining (data not shown). Also, in pollen treated with cPTIO and L-NNA, NO was localised in these nuclei (Fig. 4*E*,*G*) although fluorescence was lower compared with the control cells (Fig. 5). Thus, both the NO quencher and the inhibitor of NO synthesis dramatically reduced pollen-tube elongation (Fig. 3) and produced severe morphological abnormalities including branched (Fig. 4*E*,*F*) and bent pollen tubes with swollen tips (Fig. 4*G*,*H*).

The ROS were detected by incubating 14-d-culture pollen with 2.5 μM $DCFH_2$-DA. In pollen tubes, the strongest fluorescence was at the tips (from the subapical domain to the apex), and in the cytoplasm and nucleus of a generative cell (Fig. 6*A*,*B*). To determine the effects of the ROS on pollen-tube growth, the NAD(P)H oxidase inhibitor DPI was added. As expected, DPI blocked pollen-tube growth (Fig. 3) and significantly reduced ROS fluorescence (Fig. 5), which indicates that the ROS were produced by NAD(P)H oxidases. In addition, this treatment dramatically changed the ROS localisation as the pollen tubes showed uniform fluorescence (Fig. 6*C*), which indicates that the ROS no longer accumulated in the tips of the pollen tubes.

To determine the status of the actin cytoskeleton during pollen-tube elongation, the actin cytoskeleton was visualised through staining with *Alexa 543-phalloidin*. The actin filaments crossed the whole length of the pollen tube from the pollen grain to the apex of the tube (Fig. 7*A*,*B*). Dense and bright fluorescence was also associated with the surface of the generative cell nucleus and with the nucleus of the pollen-tube cell.

To determine whether inhibition of NO synthesis affects actin filament distribution, 30 μM L-NNA was added. Fig. 7*C*,*D* shows a less clear and less defined distribution of actin filaments compared to the control pollen (Fig. 7*A*) where the filaments were arranged along the entire length of the pollen tube. In Fig. 7*C*, it can be seen that the distribution of the filaments developed from the region below the nucleus of the pollen grain whereby the filaments appeared to surround the nucleus with distribution just below the cell membrane. The distribution was not uniform for the entire length of the pollen tube, and there were no actin filaments at the apex. The perpendicular septum separating the two cells can also be seen in the development phase (Fig. 7*C*).

After the NAD(P)H oxidase inhibition by DPI (Fig. 7*E*,*F*), the arrangement of the actin filaments was again less precise and less defined with respect to the control pollen tube (Fig. 7*A*). Figure 7*E* shows a developing tube that is still contained in the mucilaginous layer of the intine. Here, the filaments were distributed in a disordered manner both in the region immediately below the grain nucleus and in the growing tube. In Figure 7*F*, the vacuolated cytoplasm can be seen; it cannot therefore be excluded that with the growth of the pollen tube, the actin filaments were distributed

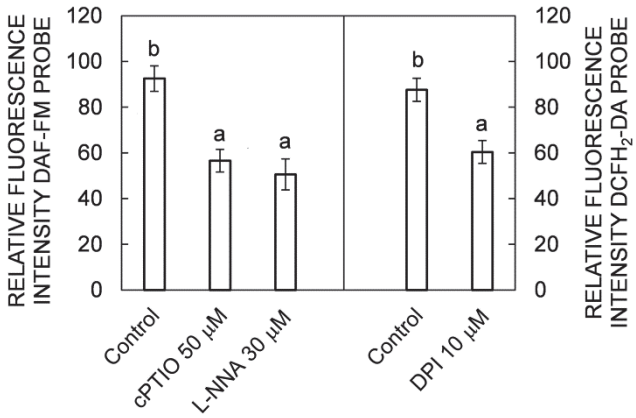

Fig. 5. Relative fluorescence from specific probes for NO (DAF-FM) and ROS (DCFH2-DA) in 14-d-germinated pollen tubes without (control) and with inhibitors. Data are means ±SE of three independent experiments with at least 40 pollen tubes per treatment in each experiment. Different letters indicate statistically significant differences at $P \leq 0.01$.

in a chaotic manner. Recently, NO and the ROS have emerged as important endogenous signalling molecules in plants with regulatory roles in many developmental and physiological processes and with an interesting interplay of NO and the ROS in growth of the tips of polarised cells (Bell *et al*. 2009, Prado and Feijo 2009). Nitric oxide is involved in growth regulation and reorientation of lily and *Arabidopsis* pollen tubes (Prado *et al*. 2004, 2008) and in pollen-stigma interactions (McInnis *et al*. 2006). It has also been reported that a

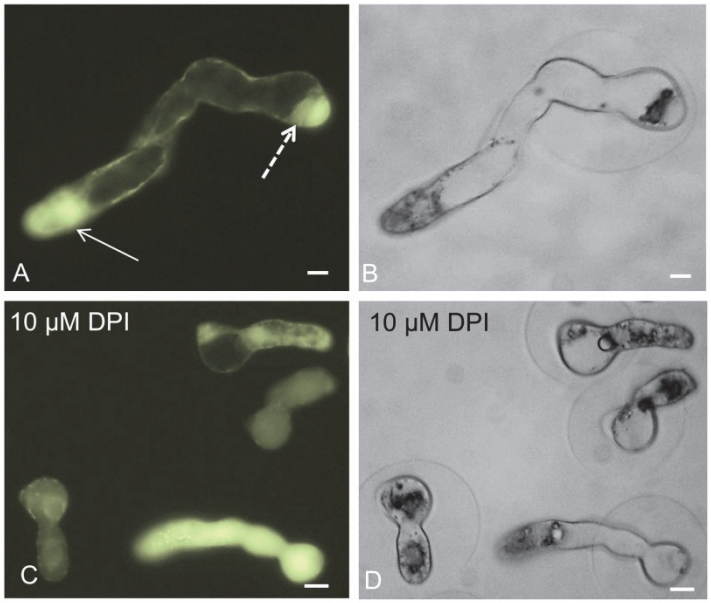

Fig. 6. Visualisation of ROS production using a ROS probe $DCFH_2$-DA in 14-d-germinated pollen. *A*,*B* - ROS fluorescence was strongest at the tip (from the subapical domain to apex; *solid arrow*) and in the cytoplasm and nucleus of a generative cell (*dashed arrow*) (*A*), and the corresponding image of the pollen under bright field microscopy (*B*). *C*,*D* - ROS in germinated pollen incubated with a ROS inhibitor DPI (*C*), with the corresponding image of the pollen under bright field microscopy (*D*). *Bars* are 10 µm.

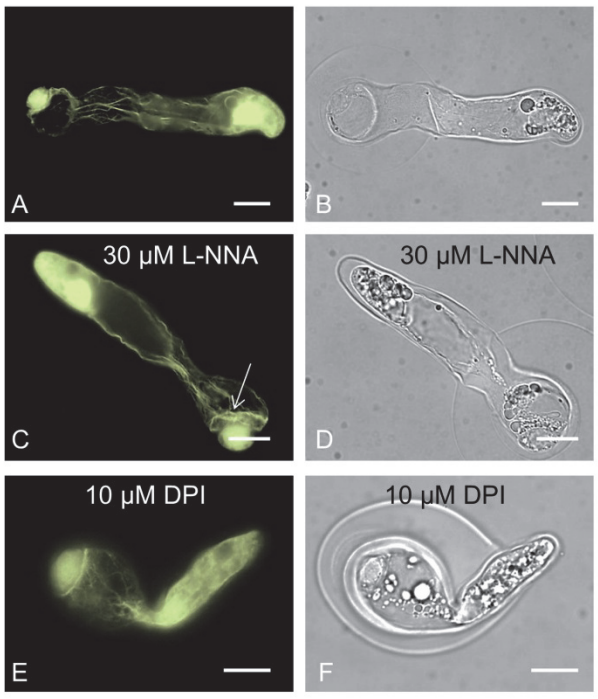

Fig. 7. Visualization of actin using an *Alexa 543-phalloidin* probe in 14-d-germinated pollen without (*A*) and with NO (*C*) and ROS (*E*) inhibitors, and the corresponding images of the pollen under bright field microscopy (*B*,*D*,*F*, respectively). *C* - *arrow* shows a perpendicular septum separating two cells. *Bars* are 10 µm.

ROS gradient that increases at the tip is produced by NAD(P)H oxidase activities, and it is required for pollen-tube tip growth (Potocký *et al*. 2007, Wang *et al*. 2010, Boisson-Dernier *et al*. 2013, Kaya *et al*. 2014, Lassig *et al*. 2014) and is essential for pollen-tube rupture (Duan *et al*. 2014).

Our data show that after 60 min of hydration of cypress pollen, there was NO seen in the protoplast of the pollen with a high ROS content in ~23 % of cells. When left for 24 h in the hydration medium, the pollen showed a significant release of H_2O_2. As inhibition of NAD(P)H oxidase activity with DPI completely inhibited the release of H_2O_2 into the hydration medium, this indicates that the NAD(P)H oxidases were responsible for the production of $^{\bullet}O_2$ and H_2O_2. These NAD(P)H oxidases were active in the hydration medium of the pollen, which was similar to what happens in nature.

This NO and ROS production had marked effects on the germination of the pollen as shown by a significant reduction in germination when these inhibitors or quenchers of NO and the ROS were added. Nitric oxide and the ROS were also present during elongation of the pollen tube where their actions are essential, as inhibition of NO with cPTIO and L-NNA, and of the ROS with DPI led to drastic reductions in pollen-tube elongation. In addition, there were visible changes to the morphology of the pollen tubes (*i.e*., bifurcation and an enlarged apex). In summary, our data indicate that both NO and the ROS had roles as signalling molecules in cypress pollen germination and pollen-tube growth similarly as reported by Wang *et al*. (2009) in *Pinus bungeana*.

As shown by the fluorescent probes used here, both NO and the ROS were present in the tip and in the cytoplasm and nucleus of generative cells. The presence of NO and the ROS in pollen has been reported but to date never in the nuclei of growing pollen (Wang *et al*. 2009, 2010, 2012). However, there is evidence that NAD(P)H oxidase can be found in the nucleus, which thus indicates its involvement in redox-responsive gene expression (Ushio-Fukai 2006). For NO, Ribeiro *et al*. (1999) used an *anti-macNOS* fluorescent antibody to show intense labelling around the nucleus, and Foissner *et al*. (2000) used a DAF-2DA probe to show intense fluorescence in the nuclei of epidermal cells that were challenged with cryptogein (an elicitor from *Phytophthora cryptogea*).

The actin cytoskeleton has a crucial role in growth and polarity of the pollen tube. It has long been appreciated that together with myosin, actin micro-filaments generate cytoplasmic streaming and thus provide mechanisms that transport vesicles containing cell-wall precursors to the tip, where they can fuse and participate in cell elongation. More recently, evidence has emerged that actin polymerisation participates in pollen-tube growth, and several studies have shown longitudinally oriented filaments of actin in the shank of pollen tubes. In contrast, in the apex, there appears to be no specific consensus despite numerous studies to resolve this issue (Vidali *et al*. 2009, Fu *et al*. 2010, Qu *et al*. 2015).

The organisation of the actin filaments in the pollen tube of gymnosperms differs in part to that seen for angiosperms (Lovy-Wheeler *et al*. 2005). This is not surprising as pollen-tube development in conifers differs from that of their counterparts in angiosperms in various ways such as an extended period of growth, a relatively slow growth rate, and extremely delayed gametogenesis (Fernando *et al*. 2005, Lazzaro *et al*. 2005). In angiosperms, the actin filaments are distributed according to the longitudinal axis of the pollen tube, with most arranged in bundles where supports organelle trafficking. In gymnosperms, the organisation of the actin filaments appears to be more complex. Even if the actin filaments are still organised in bundles, their distribution is not as linear as that in angiosperms. Moreover, in angiosperms, there are no actin filaments in the apex, where there is instead a cortical actin fringe.

In the control cypress pollen, the actin filaments were distributed throughout the whole pollen tube, in a net axial array that was oriented mainly parallel to the direction of pollen-tube elongation. These actin filaments formed a continuous network that originated from the grain and emanated into the tube, with a relatively structured network of filaments in the apical zone of the pollen tube. Dense and bright fluorescence for actin was associated with the surface of the generative cell nucleus and with the nucleus of the pollen-tube cell, which suggests an active role of actin filaments in the movement of the nucleus (Heslop-Harrison and Heslop-Harrison 1989, Åström *et al*. 1995). When NO synthesis was inhibited by L-NNA, the density of the actin filaments was clearly reduced, and their distribution appeared not to include the entire pollen tube but only some regions, such as the compartment that contained the nucleus of the pollen grain, with actin apparently absent in the growing apex. The ROS inhibition by DPI also significantly affected the actin filament distribution. In ROS-inhibited pollen a more chaotic distribution of actin was observed such that the individual actin filaments could not be clearly defined. In the light of this, it appears that inhibition of these routes of NO and ROS synthesis results in changes in $[Ca^{2+}]_i$ which is considered a key factor in the control of the dynamics of the cytoskeleton in general, and more specifically of actin filaments (Mori and Schroeder 2004, Carol and Dolan 2006).

In summary, the present study shows that NO and the ROS had roles in the regulation of pollen germination and pollen-tube growth in cypress. Nitric oxide was generated, at least in part, from NOS-like activity which could be inhibited by L-NNA. The NAD(P)H oxidases in the plasma membrane produced the ROS, particularly in terms of H_2O_2. This excess of H_2O_2 promoted NO synthesis, which induced the mobilisation of intracellular $Ca^{2+}$and in turn regulated actin filament organisation.

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