



Brief Report

# The Role of Reactive Oxygen Species in the In Vitro Germination and Growth of the Petunia (*Petunia hybrida* E. Vilm.) Male Gametophyte

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**Abstract:** The in vitro growth of the pollen tube (PT), an object of comprehensive and intensive research, is a model for studying the mechanisms of sexual reproduction in higher plants. We have studied the potential role of reactive oxygen species (ROS) in the in vitro germination and growth maintenance of the petunia (*Petunia hybrida* E. Vilm.) male gametophyte. The exogenous treatment with H<sub>2</sub>O<sub>2</sub> influences the PT germination and polar growth in vitro. The addition of H<sub>2</sub>O<sub>2</sub> to culture medium increases both the percentage of pollen grain germination and the PT length in the case of long cultivation, but inhibits both processes during the first hour of cultivation. This suggests that endogenous ROS play a decisive role in the early stages of pollen germination, with the sensitivity to endogenous ROS emerging later over the course of their growth. The addition of diphenylene iodonium chloride (DPI), a NADPH oxidase inhibitor, considerably decreases both the germination and the growth of the petunia male gametophyte at low concentrations (0.1 μM), and completely arrests the growth at high concentrations (1 μM). ROS are necessary for polar growth of the petunia male gametophyte; they are secreted in the early stages of pollen grain activation and are further localized to the initiation of the PT, mainly in the PT apical part, during polar growth, as confirmed with the help of intravital fluorescence microscopy.

**Keywords:** pollen grain (PG) germination; pollen tube (PT) growth; reactive oxygen species (ROS); diphenylene iodonium chloride (DPI)



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## 1. Introduction

The understanding of basic processes in plant sexual reproduction opens up potentially new opportunities for solving various applied problems, for example, in the agricultural production of seed crops, as well as for the clarification of the key principles in the signal transduction of plant cells. Current studies of the sexual reproduction of plants are focused on the signals between cells based on the interaction between a small family of receptor-like kinases and their coreceptors and ligands (for review, see [1]). The known subsequent signaling responses comprise the production of reactive oxygen species (ROS), the generation of specific calcium signatures, and alterations in the cell wall.

The male gametophyte of angiosperms is the key stage in the life cycle, because it provides the delivery of immobile sperm cells to the egg cell. Recent data have shown that ROS are involved in regulating polarity and growth in tip-growing cells in plants [2–5]. Arabidopsis root hairs exhibit high apical levels of ROS, which are proposed to modulate root hair tip growth by activating a Ca<sup>2+</sup> channel [2]. ROS produced by a NADPH oxidase are localized with a tip-high gradient in pollen tubes [4,6,7].

Some data suggest the involvement of ROS in pollination and fertilization [8–10], which has advanced our understanding of different pollination stages, including the

interaction of pollen with the stigma, the PT with the transmitting tissues of the style, and the PT with the female gametophyte. However, there are still many gaps in our understanding of the role of ROS as signaling molecules in pollen–pistil interaction. ROS accumulate in different compartments of the plant cell, for example, in the cytosol, chloroplasts, mitochondria, and peroxisomes (for review, see [11,12]).

The aim of the present study is to investigate the effect of ROS on petunia male gametophyte germination *in vitro* and on the maintenance of growth. We have previously demonstrated that *in vitro* and *in vivo* PG germination is accompanied by a considerable change in the level of the endogenous phytohormones 3-indolyl acetic acid (IAA), abscisic acid (ABA), gibberellins (GA<sub>3</sub>), and cytokinins, and is sensitive to exogenous plant growth regulators [13–18]. In particular, we have shown that exogenous IAA, ABA, and GA<sub>3</sub> are able to stimulate pollen germination and PG growth, while cytokinins inhibit these processes at any concentrations.

A hyperpolarizing effect of IAA was simulated with hydrogen peroxide; moreover, the shift in membrane potential induced by H<sub>2</sub>O<sub>2</sub> was inhibited by the same agents that suppressed the IAA-induced hyperpolarization of the pollen plasmalemma. We inferred that the IAA-induced hyperpolarization of the plasma membrane in petunia male gametophyte was determined by an increase in the electrogenic activity of the ATP-dependent pump in the presence of this phytohormone, and assumed that the effect of IAA was mediated by a transient increase in the level of cytosolic Ca<sup>2+</sup> and ROS formation [16,19,20].

It is still unclear how redox regulation is accomplished in the progamic phase of fertilization in angiosperms. Our study, focused on this most relevant issue, has demonstrated the involvement of ROS in the germination of PGs and the maintenance of PT growth *in vitro* conditions; it shows that hydrogen peroxide is a necessary component for pollen germination and PT growth, accelerating the germination, and most likely facilitating successful reproduction.

## 2. Materials and Methods

### 2.1. Research Object

The object of our study is the male gametophyte (pollen and germinating PTs) of the self-compatible (SC) and self-incompatible (SI) petunia (*P. hybrida* E. Vilm.) clones from laboratory collection. Micropropagation of SC and SI petunia clones was performed in tubes on agar-solidified Murashige and Skoog basal medium without plant growth regulations in a WLR-351H (Sanyo, Tokyo, Japan) climate chamber under 25/23 (day/night) ± 1 °C, with fluorescent light (65 μmol m<sup>-2</sup> s<sup>-1</sup>) during the long-day photoperiod (16 h light/8 h dark). Rooted microshoots were adapted to the soil conditions and grown in the boxes with soil (Agrobalt, St Petersburg, Russia) under natural illumination in the greenhouse conditions at 22–25 °C (day) and 18–19 °C (night) and 60–70% humidity.

### 2.2. Pollen Cultivation

Freshly harvested pollen from SC and SI clones was cultivated for 3 h in a thermostat at a temperature of 25 °C. Pollen samples (2 mg) were placed into a 15 mL flask with 2 mL of the medium containing 0.3 M sucrose and 1.6 mM H<sub>3</sub>BO<sub>3</sub>; H<sub>2</sub>O<sub>2</sub> and NADPH oxidase inhibitor, and DPI (diphenylene iodonium chloride; Sigma), which was added to culture medium immediately before pollen. The following variants were used for pollen cultivation: H<sub>2</sub>O<sub>2</sub> at a concentration of 1, 5, or 10 μM and DPI at a concentration of 0.5 μM, 1 μM, 0.1 mM, or 1 mM (the H<sub>2</sub>O<sub>2</sub> concentration was adjusted with culture medium). These ROS and DPI concentrations were used based on previously performed experiments [19].

Results were recorded on an hourly basis during 3 h of pollen cultivation. We monitored every hour of cultivation in order to monitor the dynamics of PT growth and investigate the time dependence of ROS exposure on PT germination and PT growth. The degree of germination was assessed based on the number of germinated pollen grains randomly selected and examined in four microscope (Zeiss Axioplan, Carl Zeiss, Jena,

Germany) fields ( $n = 200$ ). The PT lengths were measured using AxioVision 4.8 (Carl Zeiss, Germany) software.

### 2.3. Detection of ROS Generation (Intravital Fluorescence Microscopy)

During the cultivation in nutrient medium, pollen was sampled after 30 min, 1 h, 2 h, and 3 h of germination. PTs were harvested via filtration through a net (mesh,  $<10 \mu\text{m}$ ) and transferred to 25 nM) carboxy- $\text{H}_2\text{DFFDA}$  aqueous solution (Thermo Fisher Scientific, Waltham, MA, USA) for intravital ROS visualization in cells after a 30 min incubation. The PTs were then washed three times with sterile water. A drop of the water with pollen was placed on a glass slide, sealed with a cover glass, and examined under an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan) at a magnification of  $\times 10$  at a wavelength of 490 nm. Images were captured using a Color View II digital camera (Soft Imaging System, Munster, Germany). At least 200 pollen tubes were examined in each variant of the experiment.

### 2.4. Statistical Processing

The experiments were performed in three to five replicates with three recordings in each. The data in the figures are shown as the means and their standard errors. The significance of differences was estimated with Student's  $t$ -test at  $p \leq 0.05$ .

## 3. Results and Discussion

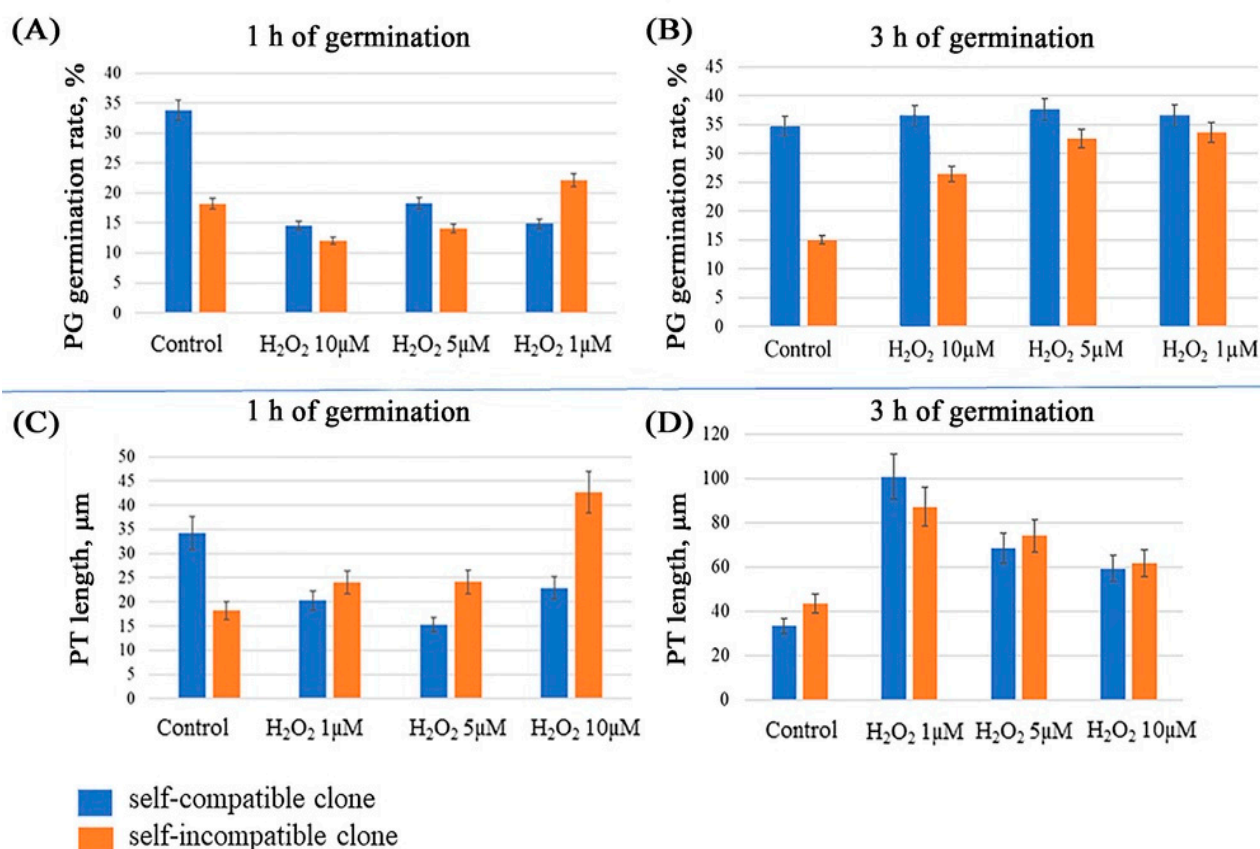
### 3.1. Effect of Hydrogen Peroxide on the In Vitro Germination Rate of Pollen Grains of Self-Compatible and Self-Incompatible Petunia Clones

In our work, we used hydrogen peroxide because it is the most stable ROS and, presumably, the most important in terms of physiological activity [21,22]. The following effects were observed when adding hydrogen peroxide at different concentrations to the culture medium (Figure 1).

The SC clone in the control medium displayed the highest percent rate (34%) of germinated pollen after 1 h of in vitro cultivation (Figure 1A) versus the SI clone, with the rate of germinated PGs not exceeding 20%. After the addition of hydrogen peroxide to the medium, the rate of germinated PGs of SC clone after 1 h of cultivation is 4% higher as compared with the SI clone for two  $\text{H}_2\text{O}_2$  concentrations (10  $\mu\text{M}$ ); however, this increase is statistically insignificant.

This experiment demonstrates that the addition of hydrogen peroxide to the medium has a positive effect on the percent rate of PG germination in the case of longer pollen cultivation. This is evident in Figure 1B, where all characteristics of the treated pollen are higher as compared with the control. This is rather insignificant for the SC pollen, but displays an almost two-fold increase for SI.

A low germination rate has been characteristic of the SI clone PGs in all our long-term experiments [23]. However, PGs effectively germinate and further grow in the pistil-transmitting tissues of SC clones, and reach the ovary, followed by fertilization and seed setting in the case of cross-pollination in vivo. The PGs of most plant species are able themselves to activate and support the regulatory network that controls polar growth in the absence of the signals produced by the female gametophyte and sporophyte [24]. Correspondingly, PGs often successfully germinate and implement the program of polar cell growth in nutrient medium in vitro. For this purpose, the nutrient medium in the majority of cases must contain boric acid and a source of heterotrophic nutrition, usually sucrose. The composition of medium is additionally optimized to reach a larger germination rate and a higher growth rate. Naturally, in vitro PT growth does not completely match the in vivo processes [4]. However, this experimental model is actively and beneficially used when studying the regular patterns in the polar directed cell growth.



**Figure 1.** Effects of hydrogen peroxide on in vitro germination rate and pollen tube length of self-compatible and self-incompatible petunia clones. The rate (%) of PG germination after (A) 1 h and (C) 3 h of germination and the lengths (μm) of PTs after (B) 1 h and (D) 3 h of germination.

The patterns of the PT growth are detailed in numerous reviews [2,25]; some reviews consider various aspects in more detail, such as the ionic regulation of growth [5,26]. The identification of intracellular signaling systems of the male gametophyte has considerably advanced; in particular, the important roles of Rop GTPases [1,27,28], the intracellular Ca<sup>2+</sup> signaling network [29], and anion transport [30] have been discovered. The role of ROS in the processes underlying polar growth is also actively debated in the relevant literature [9].

### 3.2. Effect of Hydrogen Peroxide on the Pollen Tube Length of Self-Compatible and Self-Incompatible Petunia Clones Cultivated In Vitro

The growth patterns of the PTs on culture medium (0.4 M sucrose and 1.6 mM H<sub>3</sub>BO<sub>3</sub>) supplemented with H<sub>2</sub>O<sub>2</sub> at a concentration of 1, 5, or 10 μM differ in a statistically significant manner according to the PT length, depending on the exposure (Figure 1C,D).

Similar to the influence of H<sub>2</sub>O<sub>2</sub> on the percent rate of PG germination, we did not observe any strong inhibitory effect on the PT length. By the first hour of cultivation, the differences in the PT growth displayed by two clones are evident (Figure 1C). In the case of the SC clone, the PT growth at all H<sub>2</sub>O<sub>2</sub> concentrations is inhibited. However, the opposite effect—the stimulation of PT growth—is observed in the case of the SI clone; moreover, the lowest concentration causes the highest increase.

At 3 h of germination, the length of PTs of all treated pollen exceeds the control by 40% (Figure 1D). The maximum result was attained for the SC pollen cultivated in the medium with 1 mM H<sub>2</sub>O<sub>2</sub>.

Analysis of the results suggests that the patterns are similar to the data on the percent rate of PG germination; namely, a stimulatory effect of the tested H<sub>2</sub>O<sub>2</sub> concentrations

in a long cultivation is evident for both the length of treated PTs and the percent rate of germination.

The experiments by Podolyan et al. [31] demonstrate the stimulation of the lily PT growth after treatment with H<sub>2</sub>O<sub>2</sub>. The same effect was observed by Maksimov et al. [12] in the PTs of gymnosperms. Smirnova et al. [32] demonstrated the stimulation of tobacco pollen germination by H<sub>2</sub>O<sub>2</sub> at low concentrations, whereas the high concentrations slowed down this process but did not decrease pollen viability. The authors explain that this effect is due to the fact that H<sub>2</sub>O<sub>2</sub> and hydroxyl radical (•OH) can regulate the germination of tobacco pollen by changing the mechanical properties of pollen intine (inner layer of the pollen wall). The •OH radical caused excessive intine loosening over the entire PG surface, thereby interfering with the induction of polar growth. On the contrary, H<sub>2</sub>O<sub>2</sub> compacted the wall, thereby preventing the PT from emerging. Presumably, this is the cause underlying the PG germination during the first hours of cultivation.

### 3.3. Effect of DPI on the In Vitro Germination of the *P. hybrida* Male Gametophyte

It is known that DPI inhibits NADPH oxidase, which is regarded as the main ROS producer in cells. The effect of DPI on the percent rate of PG germination was assessed using four concentrations, namely 0.5 µM, 1 µM, 0.1 mM, and 1 mM. High DPI concentrations (0.1 and 1 mM) completely arrested PG germination, while PGs still succeeded in germinating at lower concentrations (0.5 and 1 µM; Table 1).

**Table 1.** Effect of DPI on the germination percent rate of SC and SI petunia clones in in vitro culture.

Variant	Rate (%) of Germinated PGs of SC Clone	Rate (%) of Germinated PGs of SI Clone
	1 h cultivation	
Control	33.8 ± 0.5	18.2 ± 1.0
DPI, 1 µM	14.9 ± 0.7	9.8 ± 0.6
DPI, 0.5 µM	20.0 ± 0.7	18.7 ± 0.7
	2 h cultivation	
Control	45.3 ± 1.2	22.5 ± 1.5
DPI, 1 µM	31.8 ± 2.4	10.8 ± 0.3
DPI, 0.5 µM	40.4 ± 4.6	19.6 ± 1.8
	3 h cultivation	
Control	50.7 ± 4.1	37.2 ± 2.8
DPI, 1 µM	32.0 ± 2.4	27.8 ± 2.4
DPI, 0.5 µM	43.0 ± 3.7	28.7 ± 1.9

The cultivation of PGs in the medium containing DPI at two concentrations (0.5 and 1 µM) revealed a distinct dependence. DPI at a concentration of 1 µM caused a very strong inhibitory effect on the PG germination rate of both SC and SI clones. In all cases, the germination rate decreased approximately two-fold, and this pattern was retained over the entire period of PG germination. A DPI concentration of 0.5 µM had a less dramatic effect, although it also decreased the rate of PG germination for both clones, in most cases.

### 3.4. Effect of DPI on the In Vitro Growth of *P. hybrida* Male Gametophyte

The experiment when DPI was added to the medium during petunia pollen cultivation revealed a decrease in the PT lengths, similar to the inhibition of PG germination percent (Table 2).

**Table 2.** Effect of DPI on the pollen tube length of SC and SI petunia clones in in vitro culture.

Variant	PT Length of SC Clone, $\mu\text{m}$	PT Length of SI Clone, $\mu\text{m}$
	1 h cultivation	
Control	34.2 $\pm$ 19.4	5.0 $\pm$ 0.8
DPI, 1 $\mu\text{M}$	14.4 $\pm$ 7.4	6.3 $\pm$ 2.0
DPI, 0.5 $\mu\text{M}$	25.0 $\pm$ 18.4	10.0 $\pm$ 5.2
	2 h cultivation	
Control	55.4 $\pm$ 8.6	10.1 $\pm$ 4.5
DPI, 1 $\mu\text{M}$	20.7 $\pm$ 9.4	10.1 $\pm$ 6.6
DPI, 0.5 $\mu\text{M}$	33.0 $\pm$ 8.6	62.0 $\pm$ 51.2
	3 h cultivation	
Control	110.6 $\pm$ 29.7	69.4 $\pm$ 28.8
DPI, 1 $\mu\text{M}$	33.4 $\pm$ 10.1	34.7 $\pm$ 9.1
DPI, 0.5 $\mu\text{M}$	93.0 $\pm$ 38.4	56.9 $\pm$ 12.9

The addition of DPI at a concentration of 1  $\mu\text{M}$  to the medium for PT cultivation caused inhibition of the length of the germinating male gametophyte. The PT length considerably varied in each experiment, and the differences between PT lengths in the variants were not always statistically significant.

Earlier papers [12,31,32] demonstrate that DPI at high concentrations (0.1 mM) blocks the PG germination of both angiosperms and gymnosperms. Lassing et al. [33] discovered decreased  $\text{K}^+$  currents in the PTs of the Arabidopsis mutants with NADPH oxidase deficiency; the same effect was observed when treating non-mutant plants with DPI. Podolyan et al. [31] propose a putative mechanism of ion zoning and the sensitivity of ion transport to ROS. ROS are able to modulate the activity of cation channels and, as a consequence, the efficiency and rate of PT germination.

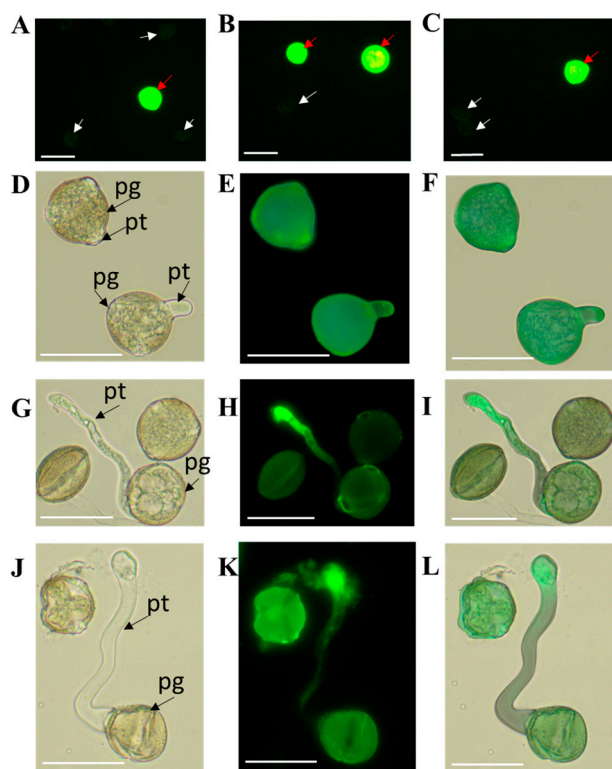
As is assumed,  $\text{Ca}^{2+}$  release and the subsequent inhibition of  $\text{H}^+$ -ATPase in the sub-apical PT region can mediate the efficiency of  $\text{H}_2\text{O}_2$ . Thus, DPI can simulate  $\text{K}^+$ - and  $\text{Ca}^{2+}$ -conducting channels, although with somewhat lower efficiency [31].

It is assumed that ROS generation is determined by NADPH oxidase activity on the membrane. According to our data, the two main phytohormones that stimulate PG germination and PT growth are IAA and ABA. Our data are interpretable in the view that ROS are necessary and mediate the pathways mediated by IAA and ABA as in the other plant systems [34–37]. In our experimental system, we can assume that IAA induces the activation of NADPH oxidase, responsible for ROS generation, which is presumably caused by an increase in the  $\text{Ca}^{2+}$  cytosolic level, when  $\text{Ca}^{2+}$  arrives from extracellular medium at the stage of IAA sensing by pollen grain cells. Note that the  $\text{Ca}^{2+}$ -dependent NADPH oxidase activation is a well-known specific feature of this enzyme in eukaryotic cells [38].

Our results demonstrate that IAA induces hyperpolarization of the plasmalemma in the germinating male gametophyte, which is inhibited by EGTA and verapamil, the agents preventing the input of extracellular calcium to cells. Of particular interest is the fact that a hyperpolarizing effect of IAA is also blocked by DPI, an inhibitor of NADPH oxidase, and, in contrast, is simulated by the addition of hydrogen peroxide. Note that  $\text{H}_2\text{O}_2$  shifts the membrane potential, which is inhibited by the same agents as IAA, namely orthovanadate [20].

### 3.5. Detection of ROS Generation

Figure 2 shows the ROS staining in the male gametophyte of petunia. The petunia PGs secrete ROS as early as the beginning of activation (Figure 2A–C). Bright fluorescence of the viable PGs (red arrows) is observed, whereas the fluorescence of nonviable PGs is very weak, and almost invisible (white arrows). Maksimov et al. [12] demonstrated that ROS were also secreted in the pollen of blue spruce at the early activation stage.



**Figure 2.** Distribution of ROS in the pollen and pollen tubes grown in vitro. (A–C) Petunia pollen grains after 30 min of germination. Bright fluorescence of viable pollen grains (red arrows) and very weak, almost invisible fluorescence of nonviable PGs (white arrows). (D–F) Petunia PGs after 1 h of germination. Fluorescence of overall PG and the pollen tube apex. (G–I) Male gametophyte of a petunia self-compatible clone after 3 h of germination. Bright fluorescence of the PT apex. (J–L) Male gametophyte of a petunia self-compatible clone after 3 h of germination. Bright fluorescence of the PT apex. (A,D,G,J) Light-field microscopy; (B,E,H,K) fluorescence microscopy, staining with carboxy- $H_2DFFDA$  aqueous solution (wavelength, 490 nm); and (C,F,I,L) overlay of light and fluorescence microscopy images (pg, pollen grain and pt, pollen tube; bar = 50  $\mu$ m).

According to relatively recent data from Maksimov et al. [12], endogenous ROS, including the species produced by NADPH oxidase, are necessary for the germination of spruce pollen, and regulate the membrane potential in PTs, while  $H_2O_2$  is nonuniformly distributed along PT. The ROS location in PT initials and PTs themselves suggests  $H_2O_2$  accumulates in the PT apex. In our experiments, we also observed bright fluorescence in the PT apices after 1 h (Figure 2D–F) and 3 h (Figure 2G–L) of in vitro germination. The PG rather brightly fluoresces at the moment of hydration and the first hour of germination; then, the bright PG fluorescence disappears after 3 h of germination, while the bright fluorescence at the PT apex remains. Cardenas et al. [39] observed both the apical and subapical ROS accumulation in the (*Lilium formosanum*) PTs.

Speranza et al. [40] demonstrated that Chinese gooseberry PTs released hydrogen peroxide into culture medium. Presumably, this explains certain green fluorescence near the PT apices in Figure 2H,K.

According to published data [41], ROS can be involved in the regulation of PT integrity via the metabolism of the cell wall components such as pectin and callose.

It is known that the stigma exudate contains ROS [9,42,43] synthesized on the stigma of various flowering plants. The moderate activity of some enzymes that regulate ROS, in particular, peroxidases, has been shown. In line with the modern view of the exudate as the medium of pollen–stigma interaction, the authors postulate that one of the functions of ROS on the stigma is the support and/or stimulation of pollen germination.

#### 4. Conclusions

ROS have already been detected during the process of hydration and the beginning of PG germination. Subsequently, ROS accumulation is detected in the PT initials. With further growth of the PT, ROS become distributed unevenly in the PT. Additionally, inhibition of ROS generation by DPI negatively affects the PG germination and PT growth, while the addition of H<sub>2</sub>O<sub>2</sub> to the culture medium has a positive effect on these processes, especially during long-term (3 h) cultivation. These facts convincingly demonstrate that ROS are a necessary component in regulating the polar growth of the male gametophyte; however, ROS targets (in particular, those of hydrogen peroxide) in the growing PT are still unclear.

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**Data Availability Statement:** Data are contained within the article.

**Conflicts of Interest:** The authors declare no conflict of interest.

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