Trends in
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Opinion

Reproductive resilience: putting pollen grains in two baskets

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To ensure reproductive success, flowering plants produce an excess of pollen to fertilize a limited number of ovules. Pollen grains mature into two distinct subpopulations – those that display high metabolic activity and elevated reactive oxygen species (ROS) levels immediately after hydration (high-ROS/active), and those that maintain an extended period of dormancy with low metabolic activity (low-ROS/inactive/arrested/dormant). We propose that the dormant pollen serves as a backup to provide a second chance for successful fertilization when the 'first wave' of pollen encounters an unpredictable growth condition such as heat stress. This model provides a framework for considering the role of dormancy in reproductive stress tolerance as well as strategies for mitigating pollen thermovulnerability to daytime and night-time warming that is associated with global climate change.

Highlights

The thermovulnerability of pollen in flowering plants poses a serious threat to food security as the average day- and night-time temperatures rise due to global warming.

Flowering plants have evolved 'a pollen in two baskets' strategy for increasing the chances of successful fertilization by producing pollen that is immediately active and ready to germinate after hydration, and a separate subpopulation of backup pollen that remains in an extended low-activity state of dormancy.

Night-time warming episodes might directly impact on reproductive success by disrupting the opportunity for backup pollen to utilize the relatively cool nights to engage in a second attempt to fertilize ovules.

FACS provides an experimental strategy to separate and study subpopulations of active and backup pollen.

Pollen innate heterogeneity

In many flowering plants the male gametophyte is considered to be the most sensitive tissue to high temperatures, with even a single hot day being able to cause male sterility [1,2]. Consequently, there has been a growing interest in studying the response of pollen to high-temperature stress over the past decade [3–9]. However, physiological and molecular analyses using purified pollen should take into account that pollen grains, even from inbred homozygous individuals, are not homogeneous. We recently showed that a population of freshly harvested pollen grains distribute into three distinct subpopulations according to their **reactive oxygen species (ROS)** (see Glossary) content – inactive or dead pollen with no ROS, and two viable **'low-ROS'** and **'high-ROS'** subpopulations that represent pollen with low and high metabolic activity, respectively [10] (Figure 1).

For many species including arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*), and lily (*Lilium longiflorum*), *in vitro* pollen germination assays show asynchronous pollen tube (PT) emergence (i.e., not all at the same time) [11,12]. It is likely that ROS play a role in this observation because low- and high-ROS pollen collected using fluorescence activated cell sorting (FACS) correlate with very low and high PT formation rates, respectively [10].

One non-heritable contribution to the overall heterogeneity of the pollen population likely arises during and/or following meiosis. Premeiotic microsporocytes (microspore mother cells) have a shared cytoplasmic continuity via cytomictic channels that is thought to synchronize their developmental progress, and this is cut off early in **pollen grain development** [13]. Variation in pollen gene expression and the physical location of individual pollen grains inside the anther may also be drivers of pollen asynchrony [14,15]. Desiccation of maturing pollen is relatively uniform, occurring simultaneously before anther dehiscence, even though pollen may be at different developmental stages [14]. Nevertheless, variations in the water content of desiccated pollen grains may reflect their relative positions within the locules of the anthers [16]. The propensity

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of a given mature pollen grain to germinate is therefore inevitably impacted by developmental variation during pollen maturation [17–21]. However, the observation that hydrated pollen grains can be categorized as **active** or inactive [10] suggests that pollen development is also programmed to produce at least two distinct types of pollen within an anther, regardless of other sources of variation.

Dormancy in pollen: analogies to and distinctions from seed dormancy

The arrested activity state of the low-ROS pollen is analogous to **dormancy** in seeds [22]. Pollen grains and seeds share similar physiological characteristics primarily with respect to changes in their water content status during the late stages of development and during germination. Like seeds, mature pollen is divided into two major categories according to the **pollen hydration** status, depending on the plant species: (i) 'orthodox', partially dehydrated pollen (<30% water) which is desiccation tolerant, and (ii) 'recalcitrant', partially hydrated pollen (30–50% water) which is desiccation sensitive [16]. Orthodox seeds and pollen undergo intense desiccation during their maturation, which slows down their metabolism as they become 'dormant' [16,21] (Figure 2).

Dormancy in seeds and pollen are conceptually comparable adaptive traits because they ensure that the embryo and the male gametophyte, respectively, are protected, able to disperse over long distances, and germinate at the appropriate time and environment. However, there is one fundamental difference between the two. Orthodox seeds can maintain their dry state for multiple years, keeping the embryonic tissues viable when buried in the soil, sometimes for centuries [23], whereas pollen from angiosperms typically has a limited window of time (hours to several days) to find a receptive stigma and initiate **pollen germination**.

The fact that mature pollen grains do not normally germinate within the anthers, and only do so when exposed to suitable conditions, indicates that dormancy and germination in pollen are tightly regulated (Figure 2). The identification of an arabidopsis *raring-to-go* (*rtg*) mutant whose pollen precociously germinates within the anther under relatively high humidity conditions provided clear evidence that regulatory mechanisms inhibiting germination exist in pollen [24]. This phenotype is analogous to vivipary mutations associated with seeds in which deficiencies in the synthesis or response to abscisic acid (ABA) cause seeds to precociously germinate within the pod, spike, or ear. This analogy raises a question of whether ABA might also play a role in preventing precocious germination in pollen grains.

ABA and other phytohormones are known to play important roles in anthers and pollen development that affect the reproductive fitness of plants [25–28]. In tomato anthers, ABA levels are lowest at the microsporocyte stage before meiosis, peak during pollen vacuolation following mitotic divisions, and thereafter decrease gradually during pollen maturation until dehiscence [25] (Figure 2). As in mature desiccating seeds, ABA-dependent and ABA-independent genes are exclusively activated during the late stages of pollen/anther development, as shown in lily [29]. Repression of jasmonic acid synthesis by the conserved pollen-specific WD40 repeat protein JINGUBANG (JGB) is required for inhibiting promiscuous germination inside the anthers [30,31]. Additional deficiencies resulting in promiscuous pollen germination in arabidopsis include the inositol polyphosphate 5-phosphatase 12 (5PT12) that regulates the levels of inositol trisphosphate [Ins(1,4,5)P3]/Ca^{2+} in an ABA-independent manner [32], and cellular processes related to the nuclear Dbf2-related protein kinases NDR2/4/5 and their interacting proteins MOB1A/1B [33]. Together, genetic evidence indicates that establishment and release from dormancy is more than a simple change in hydration and is highly regulated to ensure that germination occurs at the appropriate place and time (Figure 2).

Glossary

Active pollen: hydrated pollen ready to germinate a pollen tube.
Backup pollen: hydrated pollen with low metabolic activity still retaining dormancy.
Dormancy: (i) the state of having normal physical functions suspended or slowed down for a period of time, deep sleep; (ii) a genetically programmed process that inhibits germination of seeds or pollen grains.
Double fertilization: the process in which each of the sperm cells transported by the pollen tube to the ovule fuse with the egg cell or the two central cells within the ovule to produce the embryo and endosperm, respectively.
Excessive-ROS: a pollen state characterized by toxic levels of ROS that ultimately lead to cell death.
High-ROS: a pollen state characterized by high metabolic activity. Importantly, high ROS may not be a stress state but rather optimal metabolic activity.
Low-ROS: a pollen state characterized by low metabolic activity.
Pollen grain development: pollen development begins in the anther locules when a diploid microspore mother cell undergoes meiosis to produce a tetrad microspore. Each microspore divides mitotically once or twice (depending on the species). This is followed by a maturation stage when the pollen desiccates in preparation for dehiscing of the pollen sac and release of the mature pollen.
Pollen hydration: a passive process in which desiccated pollen takes up water, typically after landing on a receptive stigma or in appropriate *in vitro* culture media.
Pollen fitness: the relative ability of pollen to successfully fertilize an ovule. Pollen fitness can be decreased by factors such as heat stress or genetic deficiencies.
Pollen germination: following hydration, the pollen grain produces a tube which breaks through the outer pollen wall (the exine) and grows through the pistil towards the ovule.
Reactive oxygen species (ROS): partially reduced species of oxygen that are chemically unstable and able to react with a wide range of organic molecules, causing them to oxidize, often leading to their degradation. Despite their toxic activity as oxidizing molecules, ROS can also function as signaling molecules

Pollen dormancy release

As with dry orthodox seeds, simple hydration of orthodox pollen is not necessarily sufficient to trigger a release from dormancy [34]. With orthodox seeds, dormancy breaks down slowly and asynchronously during an 'after-ripening' aging period while the seeds are still dry [35]. By analogy, we propose that dormancy release in orthodox pollen might occur while pollen is still dry (during the 'after-desiccation' period), leading to the observed dichotomy of two pollen subpopulations (i.e., high and low ROS) at the time of hydration – those that are immediately active and ready to germinate, and those that remain in an extended period of dormancy, despite being hydrated [10,36].

For seeds, germination is gated by an 'oxidative window of germination' (OWG), where seeds that have aged too long lose viability because of an accumulation of oxidative damage, whereas freshly desiccated seeds cannot germinate because they have not yet reached a critical threshold in which oxidative degradation of key proteins and mRNA trigger a switch to a pro-germination program [37–41]. During dry storage, ROS is mainly produced via nonenzymatic autoxidative reactions that occur spontaneously, such as lipid peroxidation and the Amadori–Maillard reaction [38,40]. However, for pollen, the after-desiccation activation period might be considerably shorter than that of seeds simply because pollen has a smaller size, a larger surface-to-volume ratio, and less protection from the environment [42]. Although there might be considerable variation in timing, eventually these aging-related ROS-induced changes will exceed the OWG range, and both seeds and pollen lose viability [10,43–46].

The division of pollen grains into active and dormant subpopulations contributes to reproductive resilience

The division of viable hydrated pollen grains between two subpopulations with low or high metabolic activity raises several issues (see Outstanding questions) regarding the role each subpopulation may fulfill. Clues to these roles can be deduced from the different germination rates of each pollen subpopulation and their metabolic activities under control and heat stress (HS) conditions – which correlates with the production of ROS (Figure 3).

Although every cell needs to balance the signaling functions of ROS with its potential for oxidative damage, this challenge is especially relevant in pollen grains and tubes, which have relatively short lifespans, and which represent some of the most metabolically active cells in plants. Pollen development, germination, and the fast-growing PT are highly ATP-consuming processes relying on cellular respiration, glycolysis, and fermentation [47,48]. Pollen has up to 20-fold more mitochondria [49] and a 10-fold higher respiration rate [50] than vegetative tissues. In mitochondrial respiration, complexes I and III of the electron transport chain (mtETC) are major sites for electron leakage leading to ROS production [51]. During abiotic stress conditions, including HS, increased mitochondrial respiration and the rate of ROS production may exceed the antioxidative capacity of the cell, leading to the accumulation of oxidative damage and even cell death [52]. Therefore, even a short and moderate *in vitro* HS (30 minutes, 35°C) may increase the ROS level of the active pollen grains above the maximum threshold of the OWG (i.e., **excessive-ROS**; Figure 3), resulting in unrecoverable damage and a dramatic decrease in the relative proportion of the high-ROS active pollen that remain viable. Interestingly, HS also increased the average ROS levels in the low-ROS pollen population. Although it is not clear whether subtle heat-triggered ROS increases might contribute to a release from dormancy, mild HS has been used as a method to increase the rate of germination and tube growth [12]. Regardless, these findings inspired our 'pollen in two baskets' model. According to this risk management hypothesis, plants use the dormant pollen grains as a reserve ('backup') for a contingency plan B in situations such as hot days in which the subpopulation of active pollen is more vulnerable to the stress and fails to initiate or maintain PT

mediating, inducing, or propagating, processes including development, response to stimuli, and cell-to-cell communication.
Vapor pressure deficit (VPD): the difference between the actual water vapor pressure in the air and the vapor pressure when air is saturated with moisture at a given temperature.
Yield: the harvested part of the plant that generates economic return (e.g., seed, fruit, flower, leaf, stem, tuber).

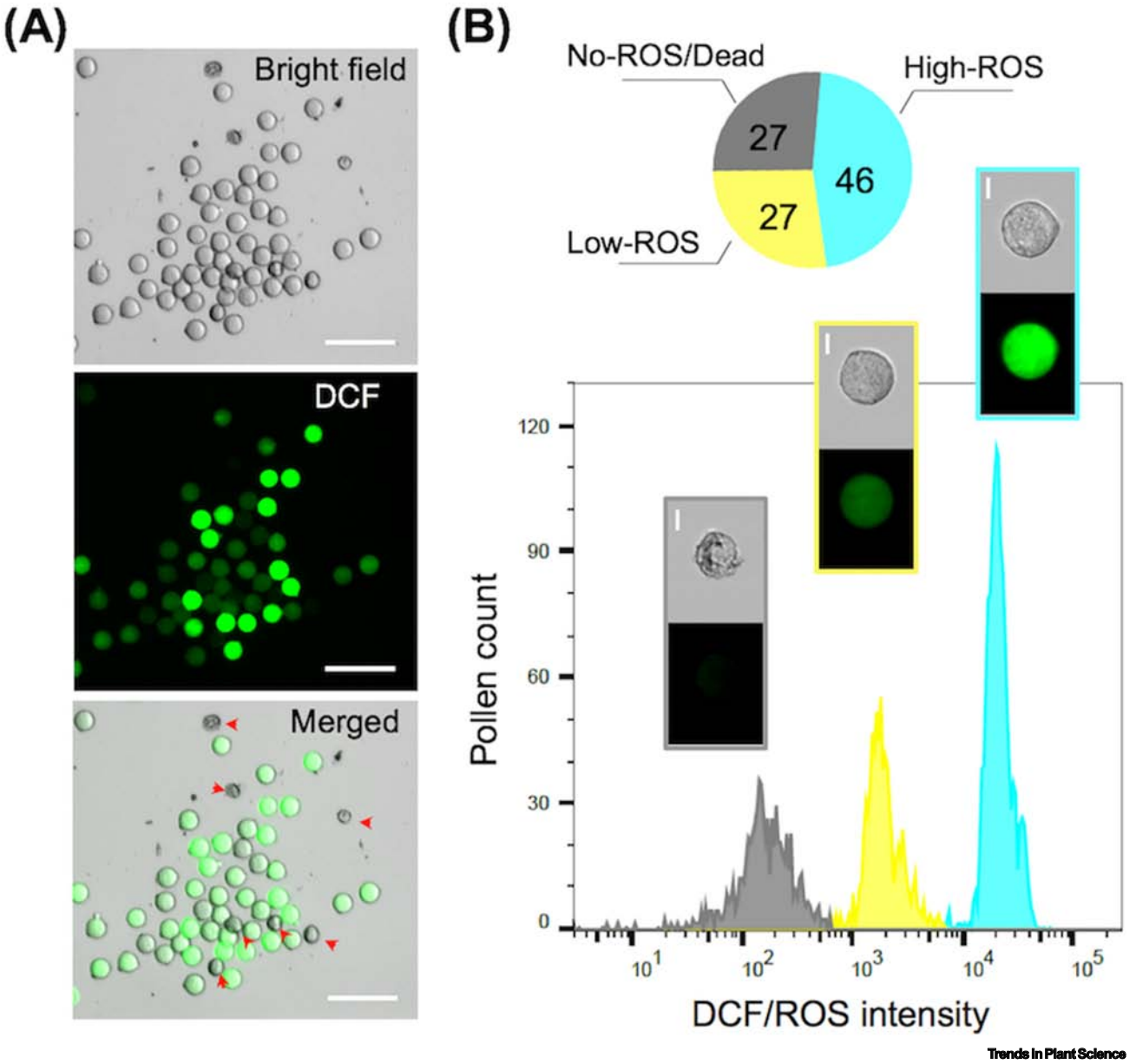

Figure 1. Hydrated pollen grains can be divided into three distinct subpopulations with different production rates of reactive oxygen species (ROS). (A) Microscope images of tomato pollen stained with the ROS indicator dichlorodihydrofluorescein diacetate (H2DCFDA). The non-fluorescent H2DCFDA is retained in live cells following cleavage of an acetate group by intracellular esterases, and is converted to highly fluorescent dichlorofluorescein (DCF) upon subsequent oxidation by ROS. Red arrowheads indicate DCF-negative pollen grains. Scale bar, 100 µm. Images adapted from [10]. (B). A representative example of quantification of DCF-stained tomato pollen by flow cytometry revealing the typical three distinct subpopulations of pollen: DCF-negative (gray, dead pollen), low-ROS (yellow, low metabolic activity/backup pollen) and high-ROS (light-blue, high metabolic activity/active pollen). High-resolution imaging flow cytometry of the pollen above the corresponding peaks represent typical pollen grains of each subpopulation. Scale bar, 10 µm. The pie chart represents the distribution (%) of the pollen subpopulations under normal conditions. The low-ROS pollen may constitute up to ~40% of viable pollen in *Arabidopsis thaliana* and tomato, respectively [10,36]. Charts and images adapted from [36].

growth. Thus, the **backup pollen** is kept in reserve for a second chance at germination when conditions are better, such as during the night when temperatures normally cool (Figure 4, Key figure).

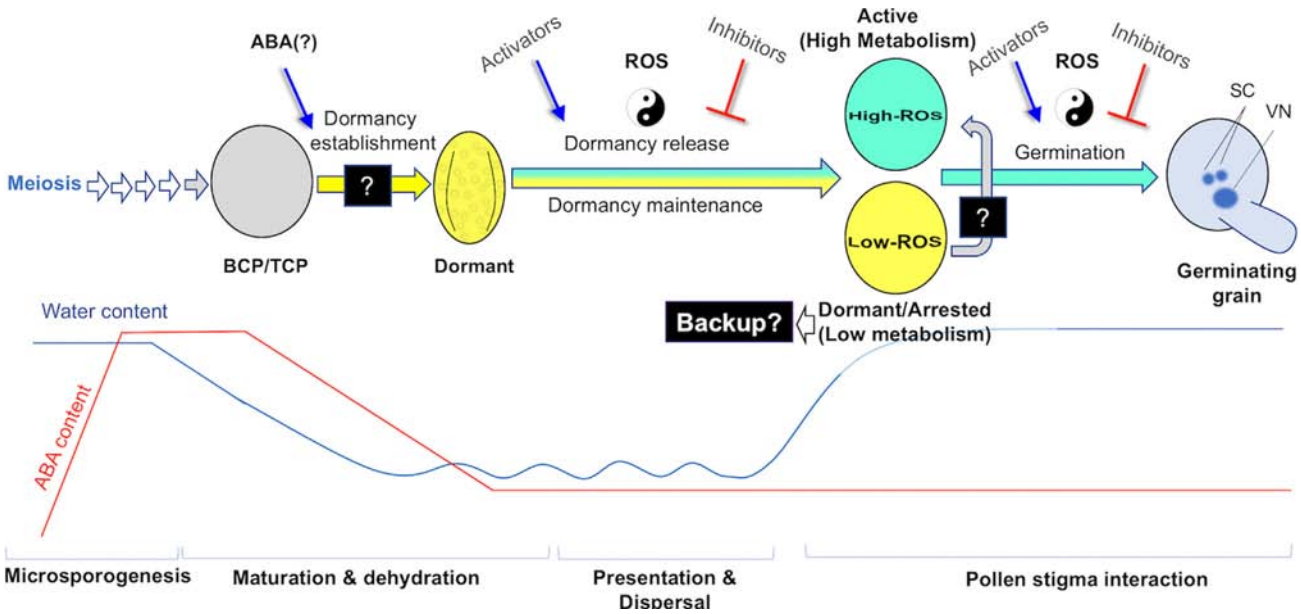

Figure 2. Schematic representation of pollen development and conceptual regulation of dormancy and germination. The corresponding water content (blue line) and ABA concentration in anthers (red line) are based on [16,25], respectively. Microsporogenesis (empty arrows) begins with meiosis of the microsporocyte to generate a haploid microspore tetrad, and continues with vacuolation, a first haploid mitosis, and a second mitosis (in the case of TCP-shedding plants). Maturation (gray arrow) follows mature BCP/TCP grain desiccation and the establishment of dormancy. Dormancy release may begin upon anther dehiscence, gradually progressing with the accumulation of ROS during the 'after desiccation' period. However, we cannot rule out that differences in the water content of the mature dry pollen might impact the degree of dormancy. Although some dry pollen grains break dormancy, others maintain it. With hydration, the nondormant pollen become immediately active and ready to germinate, whereas pollen maintaining an extended period of dormancy may serve as 'backup' for situations such as high temperature in which the active pollen fails to fertilize. 'Activators' and 'inhibitors' represent known transcription factors and regulatory proteins mentioned in the text. Black boxes indicate the total absence of knowledge of regulatory components involved in the indicated stages. The delicate balance in ROS homeostasis represented by the yin and yang symbols refer to (i) the 'after desiccation' period in which ROS level increase in the dry mature pollen grain, and (ii) the oxidative window of germination. In both cases, ROS level must be maintained within a physiological range below which the process cannot progress and above which there is a risk of oxidative damage and cell death. Abbreviations: ABA, abscisic acid; BCP, bicellular pollen; ROS, reactive oxygen species; SC, sperm cell; TCP, tricellular pollen; VN, vegetative nucleus.

The pollen in 'two baskets' model is supported by experiments using arabidopsis and tomato plants where a HS-dependent decrease in the number of viable pollen grains correlated with an asymmetric decrease in the proportion of surviving high-ROS grains compared with low-ROS grains, and low-ROS grains emerged as the predominant subpopulation [10,36]. This observation suggests that dormant pollen is better able to survive a HS. In thermosensitive tomato varieties (cultivars Money maker, Manapal) the selective HS-hypersensitivity of high-ROS pollen decreased this subpopulation by as much 50-fold compared with the low-ROS pollen. By comparison, several of the more thermotolerant varieties showed a less than a twofold decrease in the ratio of high-ROS to low-ROS grains. This raises the possibility that quantification of heat-triggered changes in ratios of high-ROS to low-ROS subpopulations might provide a robust experimental predictor of the reproductive thermotolerance of a plant.

Given the apparent role of dormancy as a mechanism of stress tolerance in pollen, additional research will be necessary to better understand how dormancy is established and broken, and how to use that knowledge to improve reproductive HS tolerance. ABA is an obvious candidate for orchestrating the HS-induced enhancement of pollen dormancy (Figure 2). Evidence showing that HS increases ABA concentrations in desiccating anthers and pollen of lily and wheat [53,54], and that exogenously applied ABA to rice (*Oryza sativa*) spikelets reduced HS-induced pollen

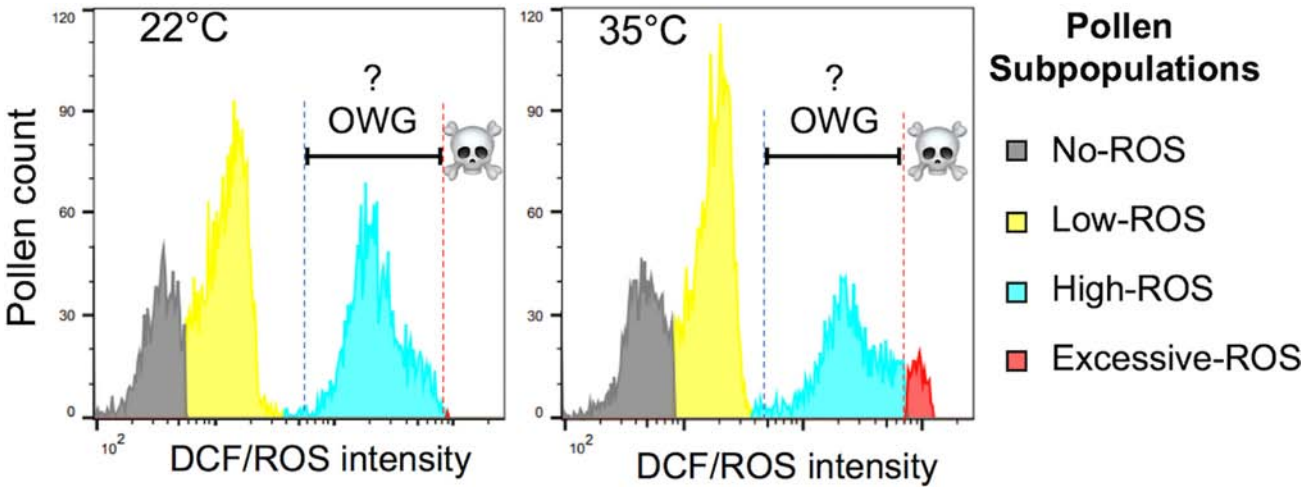

Figure 3. The oxidative window of germination in pollen. High-resolution imaging flow cytometry of dichlorofluorescein (DCF)-stained *Arabidopsis thaliana* pollen showing the distribution of pollen grains in each subpopulation according to their reactive oxygen species (ROS) levels under control conditions and following moderate short *in vitro* heat stress. Flow cytometry plots adapted from [10]). Vertical dashed blue and red lines indicate the minimum and the maximum threshold of the oxidative window of germination (OWG). Heat stress leads to excessive accumulation of ROS above the OWG range, causing excessive oxidative damage in some of the active pollen that can lead to cell death. Dead pollen (gray), backup pollen (yellow), active pollen (light blue), and stressed pollen (red).

sterility [55], supports ABA involvement in protecting maturing pollen, potentially by promoting pro-dormancy pathways. Nevertheless, the role of ABA and other factors in pollen dormancy await further investigations.

Pollen vulnerability to night-time warming contributes to yield loss

The increase in the mean temperature resulting from global warming is expected to severely decrease crop **yields** worldwide. Interestingly, mean night-time temperatures are increasing 1.4-fold more than daytime temperatures [56,57]. Persisting night-time warming can extend over larger geographic regions than daytime warming, and might pose a more significant impact than daytime temperatures on global food security [58]. The global warming trend is also associated with 'atmospheric drying' caused by increases in **vapor pressure deficits (VPDs)** which show an increasing frequency of events of much higher average VPD values at night [59,60].

Yield decreases associated with night-time warming are likely caused by several factors, including a net reduction in the amount of photoassimilate available for vegetative growth, seed filling, and fruit development [61–65]. The underlying mechanisms include increased night-time respiration, feed-forward downregulation of photosynthesis during the day, reduced translocation from sink organs, and increased transpiration leading to water loss [59,60]. These systemic processes also negatively affect anther and pollen development [66].

However, another night-time warming problem that needs to be considered has a more direct impact on **pollen fitness**. After a daytime HS episode that might kill the active pollen, the cooler nights could provide a crucial opportunity for dormant pollen to germinate and complete fertilization before the heat increases during the following day (Figure 4). The ability of dormant pollen to respond to and take advantage of the cooler nights might be delayed or severely inhibited by night-time warming. Two scenarios might directly impact the effectiveness of the backup

Key figure

The reproductive resilience of plants during hot days takes advantage of dividing pollen into two 'baskets'

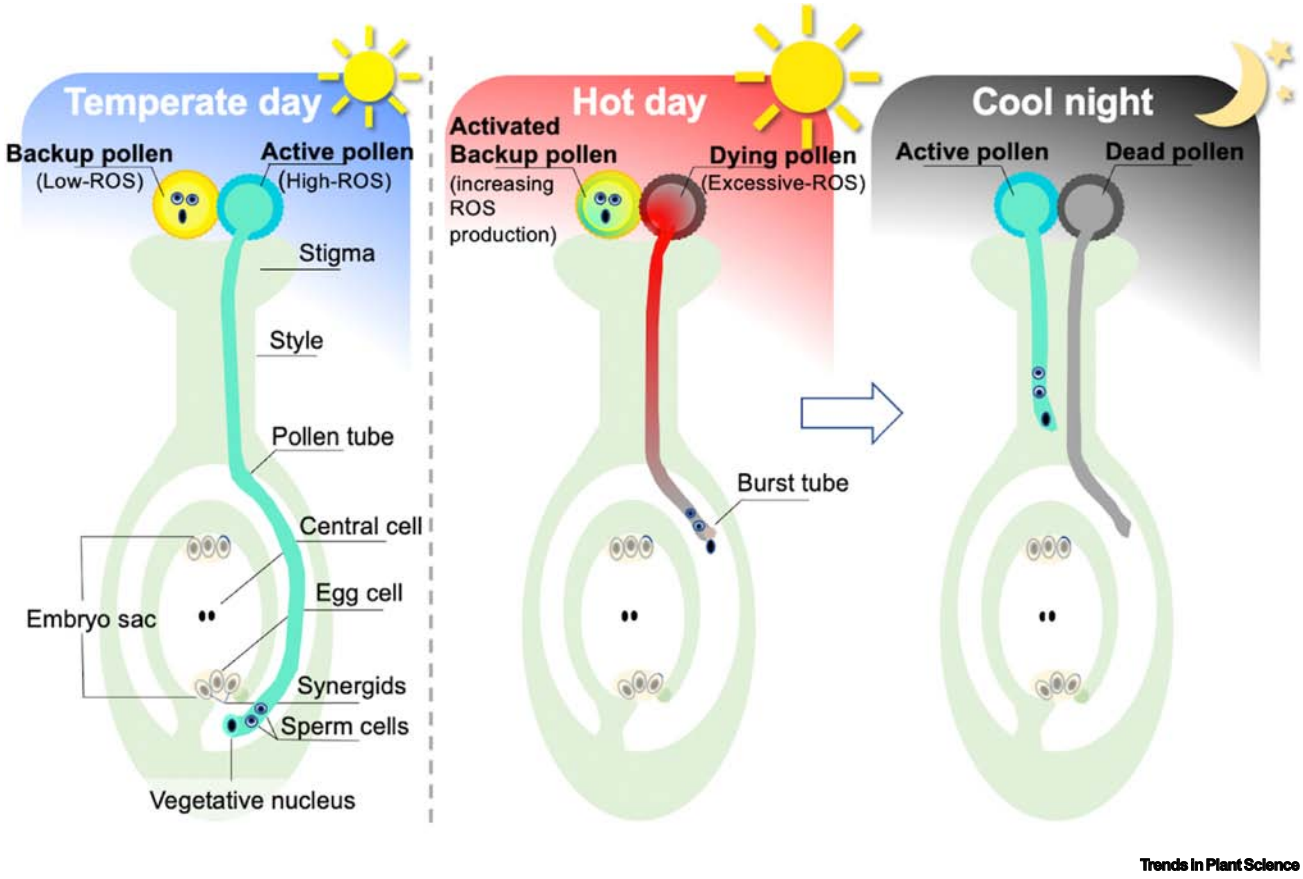

Figure 4. Under moderate temperature conditions the active pollen (light blue) that is ready to germinate can grow pollen tubes without interruptions towards the ovules inside the pistil and engage in **double fertilization**, while the backup pollen is still waiting for activation stimuli. However, during hot days the active pollen suffers from massive oxidative stress (red) due to overproduction of reactive oxygen species (ROS), often leading to failure to grow tubes, or for the tubes to burst, and eventually to premature death, while at the same time many of the backup pollen survive the heat stress owing to their low metabolic activity, and may even start to break dormancy (yellow–light blue). Following the hot day, the backup pollen can regain full activity (light blue) by taking advantage of the cool night-time window of opportunity to grow tubes.

fertilization during warmer nights. In the first, the backup pollen germinates and succumbs to the same negative impacts of stress that are encountered by the early-germinating active pollen [67], even though the nights are still cooler than the day. In a second scenario, the dormant pollen might be delayed or fail to break dormancy, and thereby miss the appropriate window of opportunity to become active, germinate, and fertilize the waiting egg cells. This failure to activate the backup pollen may involve accumulation of ABA in the anthers and pistil, respectively, caused by high VPD [59,68,69]. Thus, our 'pollen in two baskets' model (Figure 4) offers an additional explanation to account for the negative impacts on crop yields that are associated with night-time warming.

Concluding remarks and future perspectives

Desiccation tolerance and dormancy in pollen are essential adaptive traits that delineate the durability and resilience of the male gametophyte under abiotic challenges during pollen development, dispersal, and hydration on the pistil. Despite its importance in contributing to the evolutionary success of flowering plants together with seed dormancy, pollen dormancy has been largely ignored. This is not surprising because the genetic basis of pollen dormancy is more difficult to study than that of seeds, in part owing to the difficulty of conducting large genetic screens to identify mutants with impaired dormancy phenotypes [30].

The ability to distinguish between and isolate different pollen subpopulations using FACS creates new opportunities for investigating the genetic, molecular, physiological, and metabolic basis of dormancy in pollen. For example, experiments can be designed to test different conditions/treatments that affect dormancy establishment, maintenance, and release. Because different subpopulations can be isolated by FACS, different omic comparisons are now possible between active and backup pollen. In addition, FACS-based genetic screens provide an opportunity to identify genes that regulate pollen dormancy. For instance, pollen from M1-selfed ethyl methanesulfonate (EMS)-mutated plants could be screened for insensitivity to dormancy-promoting treatments. We expect that such insensitive pollen grains would show a high-ROS phenotype, whereas all other nonrelated mutants would be low-ROS, as determined by dichlorodihydrofluorescein diacetate (H2DCFDA) staining. However, the success of such FACS-based screens will depend on the ability to identify the causative point mutation by single-cell whole-genome amplification and sequencing [70], or the ability to use FACS-isolated mutant pollen to fertilize pistils and generate progeny for further genetic and physiological analyses. To the best of our knowledge, these two procedures have not yet been employed in plants.

The observation of plants that produce two distinct subpopulations of pollen, high-ROS (active pollen) and low-ROS (dormant backup pollen), invites further speculation and research. Continued surveys are warranted to examine pollen from a diversity of crops and wild plant varieties to look for correlations between the regulation of ROS homeostasis and reproductive HS tolerance. Research on the role of pollen dormancy in reproductive resilience might stimulate new approaches for breeding programs aimed at increasing crop yields in the age of global warming (see Outstanding questions).

Outstanding questions

What are the genetic and physiological programs that regulate dormancy in pollen, and how is backup pollen activated?

How do dormancy mechanisms differ between seeds and pollen?

Does backup pollen remain viable for a longer period of time and thereby increase opportunities for pollen to disperse and outcross over a wider geographic range?

Do gymnosperms utilize backup pollen?

Can pollen dormancy programs be modified to improve reproductive success under hot or cold stress conditions?

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Declaration of interests

The authors declare no conflicts of interest.

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