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Methodologies and Considerations in Evaluating Heat Stress Response and Thermotolerance of Pollen Grains

Gad Miller[1] | Max Frencken[2] | Helena Sapeta[3] | Fabienne Gidding[2] | Danny Geelen[4] | Pawan Kumar[5] | Ivo Rieu[2] | Michal Lieberman-Lazarovich[5]

[1]The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel | [2]Department of Plant and Animal Biology, Radboud Institute for Biological and Environmental Sciences, Radboud University, Nijmegen, the Netherlands | [3]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal | [4]HortiCell, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium | [5]Department of Vegetables and Field Crops Research, Institute of Plant Sciences, Agricultural Research Organization – Volcani Institute, Rishon LeZion, Israel

Correspondence: Michal Lieberman-Lazarovich (michall@volcani.agri.gov.il)

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ABSTRACT

Elevated temperatures severely disrupt pollen function, posing a major threat to agricultural productivity. While research into pollen thermotolerance is rapidly expanding, the quest to identify and develop heat-tolerant crops is challenged by a lack of consistent methodological considerations and experimental design principles. This review critically examines the experimental pipeline for assessing pollen quality and function under heat stress conditions, pinpointing where methodological variability most affects data reliability and comparability. We emphasize that accurate assessment begins with a careful experimental design, including the selection of appropriate methods to test thermotolerance, precise staging of pollen development, and effective sampling strategies to ensure comparable pollen populations. We then detail how different thermal stress parameters, such as duration, intensity, and timing, should be appropriately applied to accurately capture physiological responses, including the induction of thermotolerance. Finally, we provide a structured overview of current phenotypic and molecular assays, emphasizing the importance of high-throughput techniques in uncovering underlying mechanisms of pollen thermotolerance. By offering clear guidance and recommendations at each stage, from experimental setup to data analysis, this review offers a consistent and rigorous approach to pollen heat stress studies, aiming at enhancing the reproducibility and impact of future discoveries in this vital field.

1 | Introduction

Pollen development in flowering plants is a highly regulated and sensitive process essential for successful fertilization and seed production, a critical determinant of crop yield and reproductive fitness.

Microsporogenesis begins when diploid microsporocytes undergo meiosis to produce tetrads of haploid microspores. During microgametogenesis, each microspore forms a large vacuole, followed by asymmetric mitosis to generate the vegetative and generative cells. The generative cell then divides once more to produce the two sperm cells, yielding the mature tricellular pollen grain. Concurrently, the pollen wall (exine and intine) is elaborated through tightly coordinated sporophytic and gametophytic programs, culminating in a metabolically poised, desiccation-tolerant mature pollen ready for dispersal (Suzuki 2009).

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This complex process occurs through distinct stages: microsporogenesis, microgametogenesis, and pollen maturation (Gómez et al. 2015; Halbritter et al. 2018), each of which must proceed without disruption to produce functional pollen grains. However, the delicate nature of pollen development makes it exceptionally vulnerable to environmental stresses (Chaturvedi et al. 2021; Zinn et al. 2010).

With the increasing severity and frequency of climate irregularities, heat stress has emerged as a major abiotic factor severely compromising crop productivity. Pollen is often considered the most susceptible reproductive tissue to high temperatures, with heat stress leading to a cascade of negative effects, including anther deformation, reduced pollen viability and germination, and ultimately, a drastic decrease in fruit and seed set (Sato et al. 2006, 2001, 2000). For example, temperatures above 30°C during meiosis can disrupt pollen development in rice (Hu et al. 2021), and heat stress in tomato leads to a significant reduction in pollen number and germination (Firon et al. 2006; Pressman et al. 2007).

While the detrimental effects of heat stress on pollen have been known for decades, a notable research focus in recent years reflects the growing urgency to understand and mitigate this threat to food security. However, designing heat stress experiments targeted to the reproductive phase is challenging due to a myriad of variables, including the timing, duration, and intensity of the stress, as well as species-specific responses. These complexities mean that achieving a single, universal standard for testing pollen thermotolerance is likely out of reach.

Therefore, the primary goal of this review is not to establish rigid standards but to increase awareness of this complexity and provide guidance for designing rigorous and comparable experiments that will yield informative data. We aim to offer a consolidated framework that will help researchers navigate the numerous methodological considerations and provide practical considerations for the effective design of heat stress experiments.

2 | Key Considerations for Pollen Heat Stress Experimental Design

Pollen-focused experiments should be designed while taking specific pollen behavior into consideration. For example, the initial stress response can be rapid and transient, involving the induction of class A heat shock transcription factors (Swindell et al. 2007), while other stress effects occur later or build up over time. Additionally, mild heat can cause the abortion of developing tomato pollen; while 1 or 2 days of exposure may not show visible effects, 3–4 days of exposure significantly reduce mature pollen viability (Xu et al. 2021). Moreover, before designing temperature stress experiments aiming at the male gametophyte, it is important to consider adaptive differences between vegetative and reproductive tissues.

The heat shock response (HSR) is activated in response to the critical temperature (T_{crit}). In vegetative tissues, T_{crit} is generally 5°C–15°C above the optimal growth temperature (T_{opt}), but this range varies by species and genotypes (Staacke et al. 2025; Wahid et al. 2007). In contrast, pollen has a much narrower thermal window. In many crops, the temperature difference between T_{opt} and the maximum temperature (T_{max}) for pollen function is often less than 10°C (Stokes and Geitmann 2025). Consequently, the range between T_{crit} and T_{max}, the window for HSR activation before irreversible damage, is considerably smaller in pollen than in vegetative tissues (Hatfield and Prueger 2015; Wahid et al. 2007), highlighting pollen thermosensitivity. Temperature thresholds may also vary depending on the experimental setting. Pollen germinated in vitro (on a medium) may react differently from pollen germinating in vivo on a pistil (Eberle et al. 2012; Jiang et al. 2005; Zhang et al. 2025). Experimental factors such as the germination media, temperature increase, duration, and statistical models all influence the estimated thresholds (Tushabe and Rosbakh 2025).

Another key consideration lies in the different stress response strategies of vegetative versus reproductive tissues. Vegetative tissues typically cope with severe stress by slowing or halting growth to conserve resources (Rahmati Ishka et al. 2018). Conversely, many plants respond to abiotic stress by accelerating their transition to reproductive growth. This phenomenon, known as stress-induced flowering, is triggered by mild heat and acts as a pre-acclimation strategy that enables plants to rapidly complete their life cycle and set seeds (Riboni et al. 2014; Wada and Takeno 2010). Under stress, not only does flowering occur earlier, but subsequent reproductive processes, such as floral organ differentiation and pollen development, may also be accelerated (Takeno 2016; Zhu et al. 2021).

Therefore, when evaluating the effects of heat stress on specific stages of pollen development, precise staging is essential. Recent studies in maize emphasize the importance of staging. Transient heat stress during specific developmental stages shows differences in both susceptibility and response. The tetrad stage is most sensitive, causing the greatest impact on male sterility. In contrast, heat stress during the unicellular and bicellular stages accelerates pollen development, though this reduces pollen function (Begcy et al. 2019; Li et al. 2024). Precise staging can be achieved using cytological techniques like DAPI staining to identify nuclear configurations at different developmental stages (Begcy et al. 2019; Li et al. 2024) or through non-destructive staging methods of monitoring reproductive development progress (Table 1).

Although precise staging is important, several factors make this task highly challenging. First, growth and development of organs may be differentially affected by the stress, leading to asynchrony between pollen development and the proxy used for sampling. As shown in tomato, a slight increase in flower bud length after 4 days of heat treatment (31°/25° day/night) was observed when compared to control conditions (25°C/19°C day/night), while pollen developmental speed seemed to remain constant (Xu et al. 2021). Second, variation between genotypes was shown using established methods for pollen or anther developmental staging in wheat (Browne et al. 2018) and rice (Jagadish et al. 2013). Additionally, published morphological markers for cereal crops have wide phenotypic ranges and are therefore quite unlikely to be reliable indicators of meiosis in a

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TABLE 1 | Various phenotypic traits used for estimating pollen developmental stages in different crop species.

Plant species	Phenotypic traits used for pollen staging	References
Barley (*Hordeum vulgare*)	Nodes appearance, flag leaf emergence, and elongation	Gómez and Wilson (2012)
Tomato (*Solanum lycopersicum*)	Flower bud length	Jansma et al. (2022)
Tobacco (*Nicotiana tabacum*)	Flower bud length	Heidmann et al. (2016)
Maize (*Zea mays*)	Anther and tassel size	Begcy and Dresselhaus (2017); Chang and Neuffer (1989); Kelliher and Walbot (2012)
Wheat (*Triticum aestivum*)	Auricle distance	Browne et al. (2018)
Rice (*Oryza sativa*)	Auricle distance	Satake and Hayase (1974)
Sorghum (*Sorghum bicolor*)	Collar distance, anther length	Wood et al. (2006)

single genotype under a specific treatment (Masoomi-Aladizgeh et al. 2025). Genotype and genotype-environment interactions heavily influence these characteristics, and proxies should be recalibrated for individual experiments.

3 | Different Heat Stress Scenarios: Navigating the Confounding Variables

The study of pollen thermotolerance often focuses on endpoint indications for functionality such as viability, germination, and seed set (Mesihovic et al. 2016; Stokes and Geitmann 2025). However, transcript profiling of anthers at different developmental stages after heat stress revealed strikingly different stress responses (Jansma et al. 2022). Thus, the timing, developmental stage, and type of analysis greatly influence which part of the response will be revealed. Ideally, time series sampling should be used to recognize changes in the timing of responses; otherwise, acceleration and delays can be easily misinterpreted (Liu et al. 2020; Xu et al. 2021).

Additionally, the specific experimental design, including stress duration, day/night temperatures, ramp-up rate, and humidity, can greatly influence the measured outcomes. It is therefore essential to understand the different types of thermotolerance responses and how to test them.

Thermotolerance is the ability of an organism to withstand temperatures that differ from the optimal range for growth and development (Suzuki et al. 2008). It is typically divided into two categories: basal thermotolerance (BTT) and acquired thermotolerance (ATT) (Table 2).

BTT is the innate ability to survive a sudden and severe heat stress without prior conditioning. This is most often assessed by applying a Direct Acute Heat Stress (DAHS), which involves a rapid temperature increase from ambient conditions to a high, potentially damaging temperature (Figure 1).

For pollen, BTT can be evaluated at two key periods: (1) During development: Pre-anthesis flower buds are exposed to a heat stress of several hours, and pollen development is then monitored until maturity (Li et al. 2024; Ouonkap et al. 2024; Zhang et al. 2018) (Table 2), and (2) during the progamic phase (from pollination to fertilization). During this phase, DAHS is applied either in vivo (*in planta*) by exposing pollinated pistils to elevated temperatures for 30 min to several hours, or in vitro by treating germinating pollen on growth medium. Then, tube growth, fertilization success, or seed set are assessed. The temperatures used for DAHS typically range from 35°C to 45°C, with the specific temperature and duration depending on the plant species, genotype, and desired outcome (sub-lethal vs. lethal damage).

ATT is the ability to survive a lethal high temperature after an initial exposure to a sub-lethal heat stress, a process known as priming or acclimation. This initial priming phase, or Prior Induction of Heat Stress (PIHS), typically lasts 30 min to 3 h, followed by a recovery period at ambient temperatures (Figure 1) (Mesihovic et al. 2016). While PIHS typically does not cause substantial cellular damage, it triggers the HSR, which allows the plant tissues to acclimate to subsequent, more severe stress conditions. Another priming method that induces ATT is Gradually Increasing Heat Stress (GIHS). A gradual temperature increase over several hours, which mimics natural diurnal warming, is potentially a more physiologically relevant priming protocol. Similarly, any diurnal HS treatment that lasts for several days eventually induces acclimation, such as short-term HS (STHS) and Mild/Moderate Chronic Heat Stress (MCHS; Figure 1). STHS is a diurnal heat treatment lasting several days (2–7 days) that affects specific stages of pollen development, while MCHS, a longer period of mild or moderate heat (over 2 weeks), covers the entire reproductive development phase. This regime, with a daily temperature increase averaging between 5°C and 10°C above ambient conditions (Mesihovic et al. 2016), ensures that all pollen developmental stages are similarly exposed to stress. MCHS experiments are typically carried out

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TABLE 2 | Heat stress regimes applied to whole plants and their effects on pollen performance and reproduction.

Species	Stress conditions	Pollen stage	Pollen and reproductive readout	References
DAHS: Direct acute heat stress				
Arabidopsis	22°C (16/8 h) → 37°C 8H → 22 C; GC	Late bolting stage (with two to three siliques)	Seed setting, pollen viability, in pistil pollen tube length and ovule targeting rate, pollen germination in vitro	Ci et al. (2025); DOI: https://doi.org/10.1111/jipb.13846
Rice	30°C/24°C (12/12 h) → 40°C for 2 h with or w/o auxin spraying; GC	Anthesis/fertilization (Spikelets in the middle of the panicle)	Seed setting, in pistil pollen tube growth and ROS level	Zhang et al. (2018); DOI: https://doi.org/10.1186/s12284-018-0206-5
Tomato	43°C–45°C, 2 h, control 26°C/22°C (14/10 h)	A-7 to A-3; post meiotic to vacuolated pollen	Pollen germination and viability, transcriptomics	Frank et al. (2009); DOI: https://doi.org/10.1093/jxb/erp234
Tomato (cv MicroTom)	50°C, 2 h → 26/22°C (14/10 h) day/night	A-4 to A-1 (unicellular to early bicellular pollen)	Pollen viability and germination, proteomics	Jegadeesan et al. (2018); DOI: https://doi.org/10.3389/fpls.2018.01558
MTHS: Moderate transient heat stress				
Barley	HS at 30°C/25°C 2 days (meiosis) or 5 days (mitosis), then back to constant 17°C	Anthers at meiosis (PMC) and post-meiotic stages (mitosis I and II)	Anthers and carpels morphology, spikelet set, seed set, cytokinesis defects, pollen starch level, sterility rate	Callens et al. (2023); DOI: https://doi.org/10.3389/fpls.2022.918730
Arabidopsis	Flowering Arabidopsis plants exposed to 12, 24, 36, 48 h at 30°C–32°C	Flower buds at male meiosis (tetrad)	Tetrad-stage male meiocytes were microscopically analyzed immediately after heat treatment—male meiotic cell division products (Dyads/triads/polyads); meiotic restitution; altered crossovers	De Storme and Geelen (2020); DOI: https://doi.org/10.1038/s42003-020-0897-1
Maize	48 h at 35°C/25°C, optimal—25°C/21°C; GC	Tetrad stage (microsporogenesis)	Germination defects; seed setting; morphology, pollen enzymatic activity/viability, metabolomics, transcriptomics; pollen germination	Begcy et al. 2019 DOI: https://doi.org/10.1104/pp.19.00707
DAHS/MTHS				
Wheat	30°C or 35°C for 20 h, control: 20°C under continuous light: CER	Booting at premeiotic, early and late meiotic PMCs stages	Meiosis progression, number of grains per spike	Draeger and Moore (2017); DOI: https://doi.org/10.1007/s00122-017-2925-1

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TABLE 2 | (Continued)

Species	Stress conditions	Pollen stage	Pollen and reproductive readout	References
PIHS: Prior-induction of heat stress				
Tobacco	PRS: 30°C, 1 h → 25°C, 3 h → 35°C, 2 h → 25°C; 1 h compared with P or PR: 30°C, 1 h → 25°C 1 h or 5 h; S: 35°C, 2 h → 25°C, and; C: 25°C, 2 h	Germinating pollen in vitro[a]	Pollen viability, germination, and tube length; distribution of Actin filaments, sugars and ATP levels	Mareri et al. (2021); DOI: https://doi.org/10.3390/ijms22168535
GIHS: Gradual increase in heat stress				
Rice	Ramping from 29°C to 38°C over 2.5 h → 38°C for 6 h; GC	Anthesis	12 days after stress—Spikelet fertility, anther dehiscence characteristics, anther length and width, pollen number, pollen germination, proteomics of anthers	Jagadish et al. (2010); DOI: https://doi.org/10.1093/jxb/erp289
STHS: Short-term heat stress				
Wheat	3–5 days heat episodes (~34°C–37°C/28°C–27°C) at −8 to −6 DAA and −2 to 0 DAA	Meiosis and anthesis	Pollen viability and germination	Bheemanahalli et al. (2019); DOI: https://doi.org/10.2135/cropsci2018.05.0292
Rice	Optimal—26°C, HS—35°C/26°C (12/12 h) for 7 days	Flowering	Pollen viability, in vitro germination, seed setting	Ci et al. (2025); DOI: https://doi.org/10.1111/jipb.13846
Barley	HS: 30°C/25°C for 2 days (PMC) or 5 days (Mitosis) C: 17°C/17°C (16/8 h)	PMC meiosis or pollen mitosis I and II	Seed set, tiller sterility	Callens et al. (2023); DOI: https://doi.org/10.3389/fpls.2022.918730
MCHS: Moderate chronic heat stress				
Canola	Diurnal ramping: 23°C → 35°C, over 14 days	10–24 days after flowering	Floral set, seed set, pollen morphology, Pollen viability	Ibrahim et al. (2024); DOI: https://doi.org/10.1016/j.jplph.2024.154302
Soybean	High daytime temperature 36°C–39°C/28°C (day/night) compared with optimal (29°C–32°C); Field exp.	Gametogenesis and full bloom	In vitro germination abnormal anatomy; pod set	Djanaguiraman et al. (2019); DOI: https://doi.org/10.1111/pce.13421
Sorghum	Diurnal HS 36°C/26°C or 39°C/29°C compared with optimal 30°C/20°C; GC	From booting (before panicle emergence) to seed set	Cross fertilization comparing male vs. female sensitivity, determined by seed set	Djanaguiraman et al. (2018); DOI: https://doi.org/10.1111/pce.13089
Tomato	HS—32°C/26°C and 31°C/25°C, control—24°C/14°C day/night; GH and GC	From 2-month-old plants onwards	Seed set, fruit set, pollen number, pollen viability, and germination rate	Firon et al. (2012); DOI: https://doi.org/10.1093/aobpla/pls024

Note: This table provides several representative examples for various species, specifically highlighting the conditions of the stress treatments, the developmental stage during which the stress was applied, and the phenotypic analyses performed on reproductive traits.

Abbreviations: C: control; CER: controlled environment room; DAP: days after pollination; GC: growth chamber; GH: greenhouse; P: priming; PMC: pollen mother cell; PRS: priming followed by recovery and then heat stress; ROS: reactive oxygen species; S: stress.

[a]Indicates the only example of HS conducted in vitro.

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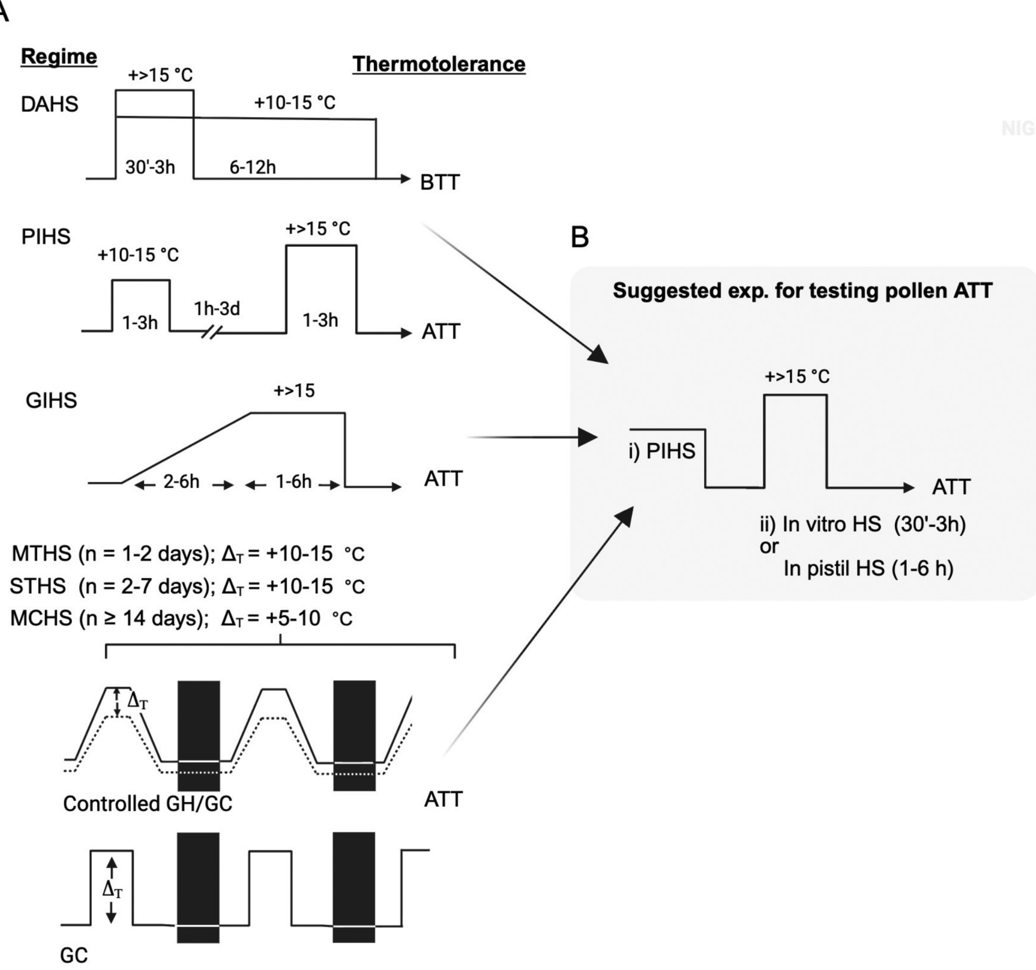

FIGURE 1 | Heat stress (HS) regimes proposed for performing pollen thermotolerance analysis in plants. (A) Major regimes for testing pollen thermotolerance: Direct applied HS (DAHS) used to determine basal thermotolerance (BTT), pre-induction HS (PIHS) for acquired thermotolerance (ATT), and gradually increasing HS (GIHS) for assessing ATT by mimicking natural conditions. HS regimes of longer duration that also induce ATT in pollen over time are moderate transient heat stress (MTHS), short-term HS (STHS), and moderate chronic HS (MCHS), designed to target a specific stage, several stages, and all stages of pollen development, respectively. Shown are the proposed time periods of HS application and the severity of the stress. HS can be applied at various stages of pollen development, and the outcome can be evaluated at different time points depending on the output trait of interest. (B) A proposed experiment for directly testing ATT and thermomemory in pollen. This figure is an adaptation with modifications of fig. 1 presented in Mesihovic et al. (2016).

in a temperature-controlled greenhouse or a climate chamber, starting at the beginning of flowering until fruit maturation, and are particularly useful for comparing the thermotolerance of different plant genotypes (Bashary et al. 2024; Firon et al. 2006; Rutley et al. 2021; Sato et al. 2002).

When the goal is to target a specific stage of pollen development, a Moderate Transient Heat Stress (MTHS) regime, lasting 1–2 days, is most effective (Begcy et al. 2019; Callens et al. 2023; De Storme and Geelen 2020). Following the stress, plants are put back to an optimal temperature to allow pollen to mature, after which fertility is assessed (Table 2).

While GIHS, MTHS, STHS, and MCHS all induce ATT, they do not test it in the same way as PIHS. The PIHS protocol has been a traditional method for evaluating heat acclimation in vegetative tissues (Balazadeh 2022; Larkindale and Vierling 2008). Priming involves creating a stress memory through physiological, biochemical, and epigenetic changes that enable a quicker and more robust response to a subsequent stress. Using PIHS for studying pollen ATT in vitro may be impractical because pollen may not have sufficient time to develop an effective acclimation response between the priming and acute stress. A recent study on PIHS-treated tobacco pollen showed no significant improvement in thermotolerance compared to DAHS-treated pollen (Mareri et al. 2021) (Table 2).

The ability of pollen to develop thermomemory has not yet been thoroughly tested. A practical experiment would be to collect mature pollen from plants that have undergone any in vivo heat stress regime (e.g., MCHS) and expose it to an intense heat stress (Figure 1b). Pollen vigor could be recorded to quantify the level of thermotolerance gained. While such experiments would evaluate ATT in pollen, it remains unknown whether pollen can independently undergo acclimation, and whether this would be of developmental significance, considering the short-term relevance of pollen and the high dependency of pollen on the well-being of the plant.

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Acclimation and priming are considered systemic processes involving cell signaling and metabolic changes at the whole-plant level (SAA, Systemic Acquired Acclimation) (Devireddy et al. 2021; Mittler and Blumwald 2015). This suggests that ATT triggered during pollen development likely relies on interactions with surrounding tissues like the tapetum and may also depend on systemic signals from floral or vegetative organs. However, since in vitro germinating pollen exposed to sub-lethal temperatures can activate HSR transcriptionally (Poidevin et al. 2021), it is plausible that stress memory and acclimation can be induced and maintained to some degree independently of the sporophytic tissue.

4 | Methods and Considerations for Pollen Harvesting

The successful evaluation of pollen thermotolerance starts with careful pollen harvesting. It is a crucial factor that can greatly affect the purity, viability, and uniformity of the harvested pollen, thereby impacting all downstream analyses. As HS decreases the amount of pollen produced and may influence its availability to be extracted, it is important to use the most effective extraction method to maximize pollen yield.

There are various pollen extraction methods, with their suitability depending on species characteristics and the intended downstream application. Floral organs show great diversity in size, shape, and developmental patterns among angiosperm species; thus, extraction methods should be optimized accordingly. The amount of pollen and handling ease are influenced by the shape, size, and structure of the flower and anthers (Reddi and Reddi 1986). Whether a plant has orthodox (i.e., desiccation-tolerant) or recalcitrant (i.e., desiccation-sensitive) pollen can also impact extraction ease. Since orthodox pollen (e.g., of the Brassicaceae, Solanaceae, Fabaceae, and Rosaceae plant families) loses water and prepares for dispersal, it is drier than recalcitrant pollen and may be easier to extract. Another factor that might affect extraction efficiency is the pollination strategy of the plant species. For example, pollen grains of Cacao (*Theobroma cacao* L.), which are naturally pollinated by insects, are highly sticky, making extraction more difficult (Weinstein et al. 2024).

Pollen harvesting methods can be broadly categorized into: *collecting shed pollen* or *extracting pollen from anthers*. The easiest and quickest way to collect mature dry pollen is by releasing it from anthers through sonication or vibration and collecting the shed pollen or by using vacuum harvesting onto a filter mesh (Johnson-Brousseau and McCormick 2004). This method works well for species that produce abundant, easily shed pollen and have an open flower structure; mainly those that are naturally insect- or wind-pollinated (Vallejo-Marin and Russell 2024). The timing of this type of pollen extraction relative to anthesis can be crucial, as extracting it too early may lead to suboptimal shedding, while extracting it too late could result in a significant amount of pollen already being lost. Yet sometimes, even at the right timing, depending on the shape of the anthers or if dehiscence is partial, dry collection can be relatively poor. To prevent this, anther dehiscence and pollen shedding can be aided by additional mechanical force by using a needle (Impe et al. 2020). While quick, this approach may not provide a representative sample of all pollen produced and is limited to collecting only mature, dehisced grains.

Wet harvesting methods involve releasing pollen by vortexing open flowers in a liquid solution, often pollen germination media (PGM), followed by filtering through a mesh to collect pollen grains. This approach typically empties the entire locular space, resulting in a representative sample of pollen. However, much care and haste are needed when handling the sample for downstream application, as the wet pollen imbibes and may start germinating in the PGM within a couple of hours.

In cases where pollen grains are not easily released from the anthers, such as when they are recalcitrant or partially dehisced, alternative methods for collecting pollen from closed anthers suitable for analyzing earlier pollen developmental stages can be used. One option is to cut the anthers (or whole flower buds) into small pieces (Xu et al. 2017), followed by vortex-mixing in a buffer. This can be combined with sample crushing (Bokshi et al. 2019), sonication (Kakui et al. 2020), or squeezing (Heidmann et al. 2016) to obtain free-flowing pollen. In this way, most of the pollen is extracted, which is important for determining total pollen count and assessing developmental defects. A key consideration is the risk of anther tissue contamination and physical damage to pollen grains, which may affect viability and downstream analyses. Another option, suitable for follow-up microscopy-based assays, is to isolate anthers and carefully open them using a syringe needle or fine tweezers under a microscope, as described for *Arabidopsis thaliana* (Hedhly et al. 2018). This method provides better control over sampling when the target species has very small flowers. Although it is laborious and time-consuming, the yield is likely to be more representative.

Regardless of the harvesting method used for downstream analyses, it is important to be consistent with it throughout all subsequent analyses to preserve the connection between the physiological state of the pollen and cellular or gene expression activities, and to prevent artifacts.

5 | Methods and Considerations for Estimating Pollen Performance

Various methods are used to assess pollen quality and the impact of stresses like high temperature on pollen development and quality at maturity (thoroughly reviewed recently by Stokes and Geitmann 2025).

"Pollen viability" and "pollen vigor" are two distinct aspects of pollen fitness used to evaluate its performance. Pollen viability is a binary (yes/no) measure indicating whether a pollen grain is metabolically active. Pollen vigor, on the other hand, is a ranking system that assesses the rate at which it performs. It is usually measured based on the time it takes for pollen to reach a specific milestone, such as tube length, morphology, or reaching the ovule (Stokes and Geitmann 2025).

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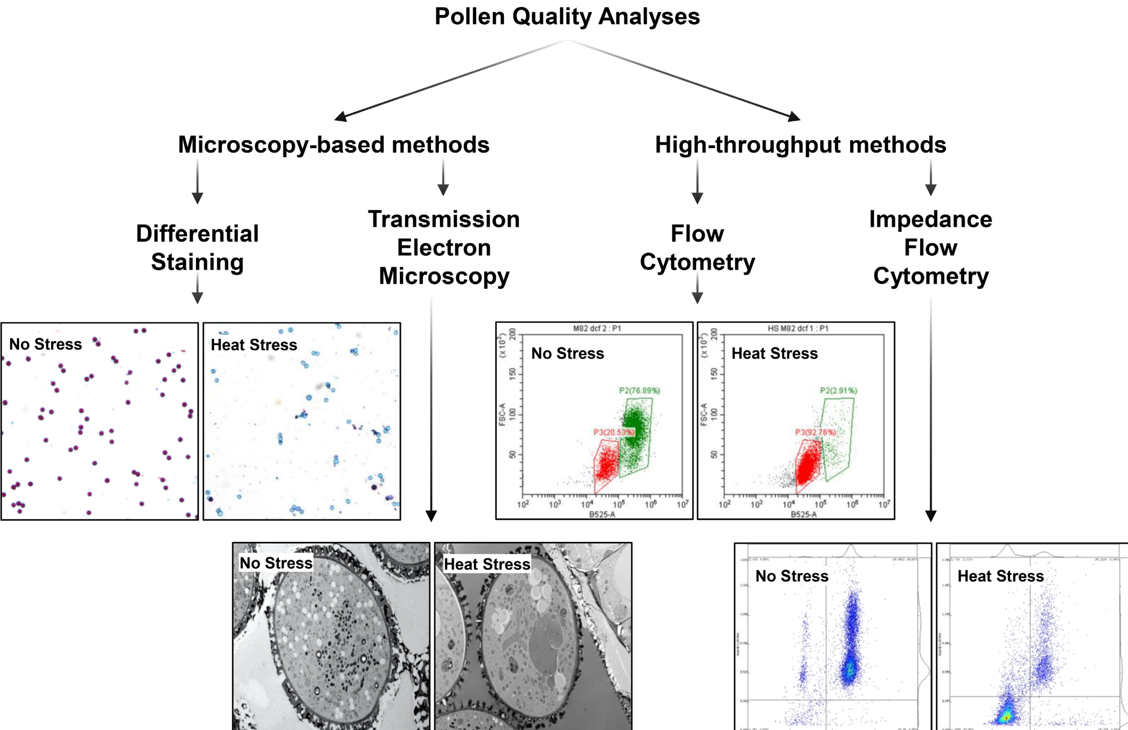

FIGURE 2 | Schematic summary of example methodologies for assessing pollen quality, including a representation of the heat stress effect. Differential staining (DS): Mature pollen grains extracted from no stress (~26°C/20°C day/night) and heat-stressed tomato plants (32°C/26°C day/night) stained with Alexander dye. Viable pollen is stained purple, while non-viable pollen appears blue. Transmission Electron Microscopy (TEM) (20.000×) of a representative pollen grain from *Arabidopsis thaliana*. Flowering plants were exposed to either no stress (21°C) or stress (32°C) conditions for 24 h before pollen isolation. Pollen from heat treated plants show a reduced complexity of vesicular structures and accumulate multivesicular bodies. Flow cytometry (FC): Mature pollen grains extracted from no stress (~26°C/20°C day/night) and heat-stressed tomato plants (32/26°C day/night) stained with H_2DCFDA were plotted according to the forward scatter count (FCA), and florescence intensity (B525 laser). The fraction of high florescence (i.e., high viability) pollen is marked in green, while the low florescence (low viability) pollen is marked in red, showing a decrease in the high-viability sub-population under heat stress conditions. Impedance flow cytometry (IFC) plot of mature tomato pollen collected from plants grown at optimal (~26°C/20°C day/night) and in a high temperature (32°C/26°C day/night) regime for 14 days. Pollen at the right top quadrants is considered viable, while those in the left top and bottom quadrants represent dead pollen, presenting a decrease in viable pollen under heat stress conditions.

5.1 | Pollen Viability Measurements

Quantifying pollen performance is essential for determining the effects of stress during pollen development and the progamic phase. While traditional methods rely on manual microscopy, the need for high-throughput and precise analysis has led to the development of automated techniques. Broadly, viability assays can be categorized into staining-based methods and label-free, biophysical methods (Figure 2).

Staining-based assays are a cornerstone of pollen viability analysis. Some stains, such as FDA (Fluorescein Diacetate) and TTC (2,3,5-triphenyltetrazolium chloride), assess cell vitality based on enzymatic activity, when a positive result indicates an active metabolism. When applied to pollen, FDA non-fluorescent fatty acids esters of fluorescein enter the vegetative cell, where they are hydrolyzed by esterases to release fluorescein, which produces a fluorescent signal. If the plasma membrane is not intact, fluorescein cannot accumulate in the cell and be hydrolyzed, thus no signal is detected. TTC is a redox indicator, used to detect cellular respiration. TTC is colorless, and it turns dark red upon reduction by oxidative and dehydrogenase enzymes in viable cells (Stokes and Geitmann 2025). In contrast, other stains like Alexander's stain or acetocarmine differentiate viable and non-viable cells by detecting specific cellular components, such as a full cytoplasm or intact cell wall, respectively (Stokes and Geitmann 2025). Traditionally, pollen counts were done manually using a hemocytometer under a microscope. This approach is laborious, time-consuming, and prone to user-subjectivity, which can affect the accuracy of viability estimates.

To overcome the limitations of manual counting and precise quantification of pollen grains in high quantities, automated high-throughput methods were developed: (1) *Flow cytometry* (*FC*): The combination of differential staining with optical FC allows for rapid and precise quantification of large pollen populations. Zhang et al. (1992) used FDA and propidium iodide with fluorescence-activated cell sorting (FACS) to assess maize pollen viability (Zhang et al. 1992). This method is particularly powerful as it is non-destructive, enabling the sorted, viable pollen to be used for subsequent functional assays like germination tests (Luria et al. 2019). The use of fluorescent probes allows for the isolation of specific subpopulations, such as those with high or low ROS levels determined by the reactive oxygen species probe (i.e., H_2DCFDA) (Luria et al. 2019), offering new avenues for studying heat stress mechanisms. (2) *Automated image*

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analysis: An alternative route to high-throughput phenotyping is the use of image analysis software to process stained samples. Tools like PollenCounter (Ayenan et al. 2020; Tello et al. 2018) and CellProfiler (Ascari et al. 2020; Dao et al. 2016) automatically classify and count pollen grains from images. Recent advancements in Artificial Intelligence (AI) have further improved this approach, allowing for automated viability classification in a variety of crops, including rice, tomato, and cotton, across different staining methods (Phumeechanya et al. 2024; Tan et al. 2022). This approach reduces manual labor and subjectivity, improving reproducibility and speed. (3) *Impedance flow cytometry* (*IFC*): This label-free high-throughput method uses the inherent dielectric properties of pollen grains, including cell size, membrane integrity, and cytoplasmic conductivity, to distinguish viable from non-viable cells visualized in a multidimensional plot (Heidmann and Di Berardino 2017). IFC can rapidly screen thousands of samples, making it ideal for large-scale experiments. The method has been successfully validated against traditional staining assays and applied to assess pollen viability across numerous species and evaluate the effects of heat stress in crops such as chickpea, pepper, and tomato (Jansen et al. 2024; Lin et al. 2022; Pattison et al. 2021). These days, IFC is implemented within portable instruments that enable high-throughput pollen viability measurements in the field.

Importantly, the data obtained from staining and FC measurements do not necessarily represent in vivo pollen performance in absolute terms. Staining-based assays, for instance, are often presented as if they show a clear phenotype—stained or unstained—without discussing the subjective thresholds used during microscopy or the potential to analyze other phenotypes that may have biological significance. However, with the advent of automated image analysis, which measures color and intensity as continuous traits, and the continuous distributions observed in FC plots, researchers can pay closer attention to the impact of thresholds and reconsider whether a binary classification is the most effective way to describe pollen quality. Furthermore, analyzing a continuous phenotype allows for the dissection of pollen into distinct sub-populations (Luria et al. 2019; Rutley et al. 2021).

5.2 | Pollen Germination and Tube Growth Assays

Pollen germination ability is also used to understand the effects of heat stress on pollen development or the progamic phase. Germination tests include in vitro (on media), semi in vivo (pollen growth through excised stigma/style), and in vivo (pollen growth within intact pistils) assays (Figure 3).

In vitro and semi in vivo assays have been used across various species, and detailed protocols are available for Arabidopsis and tomato (Desnoyer and Grossniklaus 2023; Dickinson et al. 2018; Flores-Tornero et al. 2025). In vitro and semi in vivo assays can be performed using liquid or solid germination media, with specific formulations depending on the species (Tushabe and Rosbakh 2021). Both methods allow for pollen tube phenotyping by observing tube length, morphology, and abnormalities, such as swelling at the tip or impaired tube integrity (tube rupture). The in vitro method is useful for evaluating pollen grain germination ability, counting how many pollen grains germinate within a set time, or measuring pollen tube length after a specific growth period. Microscopy is usually employed for direct scoring and tube length measurement, but alternatives with higher throughput include an ELISA-based method (Harris et al. 1987) or semi-quantitative fluorescence assays that use Congo Red staining of growing pollen tubes as a proxy for extension in microtiter plates (Hartman et al. 2014). Recently, a high-throughput in vitro pollen germination assay was developed for wheat using a 96-well plate incubated in a PCR machine under a temperature gradient, enabling the concurrent assessment of pollen morphology, germination, and tube growth across a wide range of temperatures through microscopy and imaging analysis (Zhao et al. 2024). However, a major limitation of this approach is that it overlooks the role of female tissues. As a result, pollen germination and tube growth may differ substantially from in vivo conditions. Recent research showed that heat stress-induced ROS production in maize stigmatic tissue (silk) causes pollen tube growth arrest (Gong et al. 2024), showing that female tissues can influence pollen tube growth under stress conditions.

Semi in vivo germination assays overcome some limitations of the in vitro methods by incorporating the influence of female tissues. This approach allows for the quantification of pollen grains that germinate and grow through the stigma and style, as well as measuring the number and average length of pollen tubes emerging from the style and reaching the ovaries. Similarly to in vitro assays, it also enables testing of various temperatures using different temperature-controlled chambers. An additional benefit is that ovules can be placed near the cut end of the style, permitting the assessment of ovule targeting and fertilization potential (Higashiyama et al. 1998; Palanivelu and Preuss 2006). However, semi in vivo assays are more labor-intensive.

In vivo assays provide the most direct assessment of reproductive success. In this approach, pollen is deposited onto intact pistils, and pollen tube performance is evaluated within the natural tissue environment. Aniline blue staining allows visualization of pollen tube number, position, and length throughout the style and ovary, and fertilization efficiency can be quantified by subsequent fruit and seed set.

This method has been applied to tomato to assess thermotolerance across genotypes (Ouonkap et al. 2024). In addition, some systems allow the use of GUS-based "blue-dot assays," in which pistils pollinated with LAT52::GUS pollen are dissected and stained so that each fertilized or ovule-targeted embryo sac appears as a discrete blue dot, providing a rapid, ovule-level measure of fertilization success (Fíla et al. 2020; Hafidh et al. 2016). Yet, true in vivo assays are time-consuming and space-intensive: they require controlled growth chambers for heat treatments, and—depending on the species—full fruit ripening for seed-set measurements may take weeks or months.

Semi–in vivo assays, by contrast, remove the pistil after pollination and allow pollen tubes to continue growing out of the cut style onto a defined medium. This preserves the early in vivo interaction between pollen and pistil tissues, while enabling more rapid imaging, manipulation, and quantification of tube growth under controlled conditions. These systems capture key pistil-mediated effects on germination and early tube growth,

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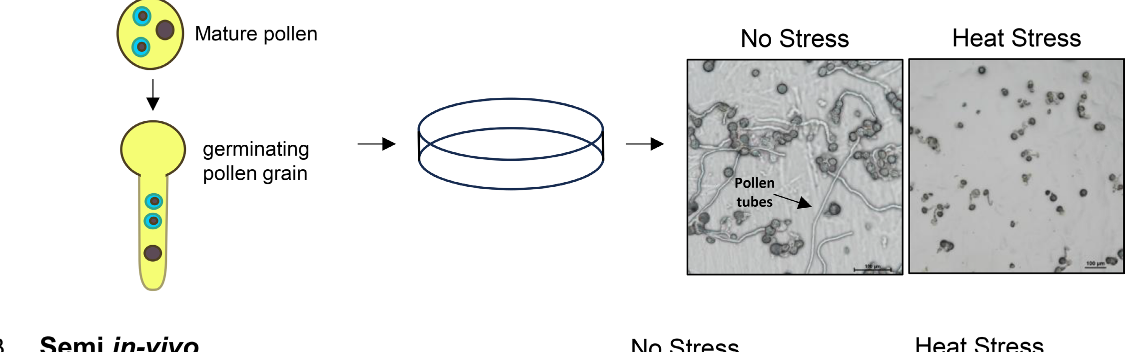

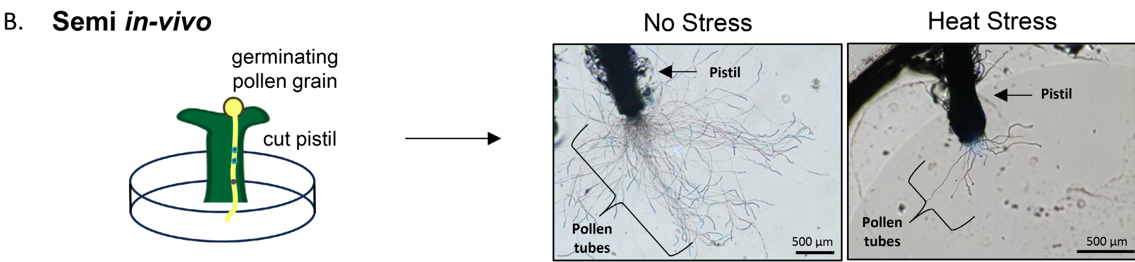

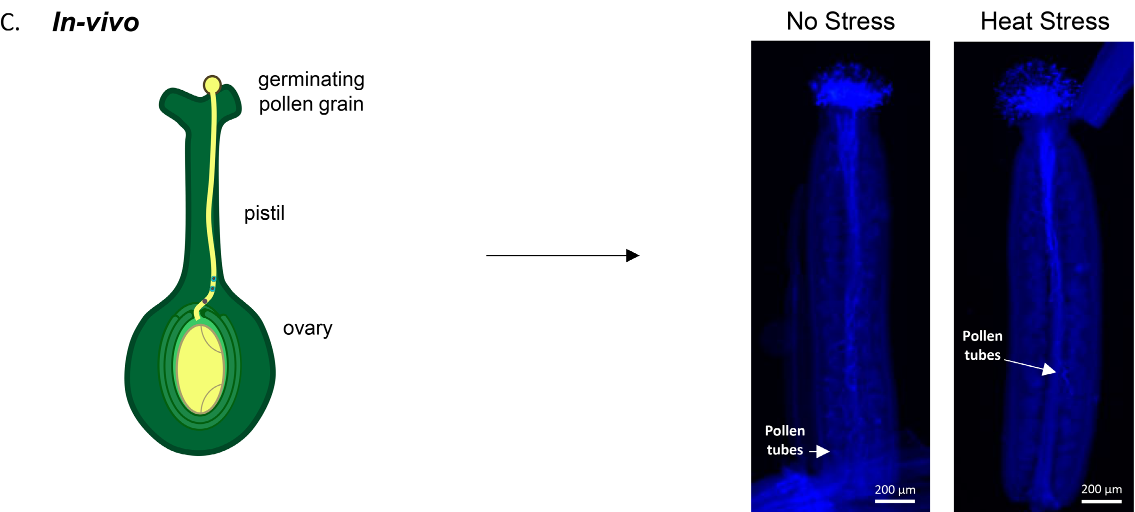

FIGURE 3 | Schematic summary of example methodologies for assessing pollen germination, including a representation of the heat stress effect. (A) An in vitro assay. Mature pollen grains were extracted from no stress (~26°C/20°C day/night) and heat-stressed tomato plants (32°C/26°C day/night), and incubated in petri dishes with germination media. Under heat stress conditions, pollen fail to germinate and pollen tubes are shorter. (B) A semi in vivo assay. Emasculated pistils were pollinated with freshly harvested mature pollen and incubated at 25°C in a humid chamber for 1 h to allow pollen hydration and germination. Pistils were then excised and placed in pollen germination media in a humid chamber for 17 h under each either no-stress (25°C) or heat stress (30°C) conditions. (C) An in vivo assay. Emasculated *Arabidopsis thaliana* pistils were pollinated and incubated for 5 h at 22°C (no stress) or 34°C (heat stress) conditions during the progamic phase. Pistils were then collected, fixed and stained with aniline blue to visualize pollen tube growth under UV light microscopy. As indicated by arrows at 22°C, pollen tubes reached the ovary base; at 34°C, growth was restricted with pollen tubes not reaching the bottom ovules.

but avoid the long timeframes associated with tracking fertilization and fruit development.

5.3 | Cytological and Structural Analysis of Pollen Quality

Pollen size is often employed as a biological indicator to estimate the ploidy and viability of mature pollen grains. Volume-based particle size techniques that use impedance variance (e.g., Coulter principle) possess strong discriminative ability to detect differences in pollen size, enabling reliable estimates of pollen viability (De Storme et al. 2013). A more detailed microscopy approach involves examining semi-thin sections of paraffin-embedded samples under light microscopy. This method is less popular as the procedure is time-consuming and has lower throughput compared to newer pollen viability assays. However, *ultrathin sections* of both anther somatic cells and pollen grains have been utilized to study the impact of heat stress on sub-cellular structures through electron microscopy (De Storme

et al. 2013; Feng et al. 2018; Porch and Jahn 2001), offering new insights that are inaccessible with high-throughput pollen viability methods. Likewise, *detecting callose deposition*. A unique feature of male meiosis in flowering plants is the deposition of callose between the primary cell wall and the plasma membrane, which acts as a mold for pollen exine development. Additionally, a peripheral callose layer surrounds the meiocyte cell unit, maintaining the emerging microspore tetrad in a compact shape. This callose basket is later dissolved by callases secreted from the tapetum to release individual spores (Rhee and Somerville 1998; Worrall et al. 1992). Callose, composed of ß-1,3-glucan, is selectively stained with aniline blue, producing a distinct green fluorescence under a fluorescence microscope (Regan and Moffatt 1990). The deposition of the microspore surrounding callose wall is significantly impaired under heat stress conditions, although the callose interstitial walls still form (Schindfessel et al. 2021). The callose between cells appears not to dissolve well under heat stress, resulting in sticky microspores that retain their tetrad structure at the mature pollen stage, similar to the quartet phenotype, which lacks poly-galacturonase activity (De Storme and Geelen 2020).

5.4 | Scanning and Transmission Electron Microscopy for Pollen Wall Ultrastructure Analysis

The pollen wall is a highly complex structure made up of an inner wall, the intine, and an outer layer—the exine. The intine is made of cellulose and pectin, while the exine is composed of sporopollenin, a highly cross-linked polymer mainly composed of long-chain fatty acids, phenylpropanoids, and phenolics. This makes it mechanically very resistant and helps preserve the shape and function of the pollen. *Scanning electron microscopy* (SEM) and *transmission electron microscopy* (TEM) are commonly used techniques for imaging the outer structure and the internal details of pollen, respectively. The tough cell wall, small size of pollen, and low water content allow for excellent dehydration and chemical fixation. Both methods are also used to examine live and fossil pollen grains (Ulrich and Grímsson 2020). A detailed overview of the terminology used to describe pollen structure and the pollen cell wall is provided by Halbritter et al. (2018).

SEM helps to identify the outer wall shape and ornaments of pollen. Although SEM analysis of pollen has shown grain collapse and changes in exine patterning caused by heat stress (Ermolaev et al. 2024; Jiang et al. 2015; Kim et al. 2001; Kumar et al. 2015; Liu et al. 2021), these effects appear when the damage is already severe; thus, earlier effects may not be detected. SEM samples are prepared by removing water and coating the sample with a thin layer of conductive material, usually gold, to dissipate excess electrons. A common dehydration technique uses critical point drying with liquid carbon dioxide, which prevents surface tension and structural damage (Boyde 1980). However, critical point drying can cause shrinkage and potentially lead to inaccurate measurements of pollen size (Ulrich and Grímsson 2020). An alternative, less costly method involves hexamethyldisilazane (HMDS) (Chissoe et al. 1994; Ermolaev et al. 2024).

TEM is often used to assess the impact of heat stress on the internal structure and organization of pollen (Lohani et al. 2025) (Figure 2). A common effect of high temperature on pollen is increased thickness and structural changes in the cell wall, observed in both the intine and exine (Hinojosa et al. 2019; Jiang et al. 2015; Porch and Jahn 2001). The traditional approach involves vacuum-infiltration with a fixative such as glutaraldehyde, paraformaldehyde, or a combination of both (Jia et al. 2017). Samples are then dehydrated and embedded in a resin like Spurr. Ultrathin sections can also be stained with uranyl acetate and lead citrate to enhance contrast. Cryofixation followed by freeze-substitution or chemical fixation improves preservation of both ultrastructure and antigenicity. The best preservation of ultrastructural features, such as vacuoles in pollen, is achieved through high-pressure freezing and freeze-substitution, enabling whole-cell electron tomography (Liang et al. 2024).

Complementary methods for analyzing 3D structural information of pollen *include x-ray tomography* (Li et al. 2016), optical diffraction tomography (Kim et al. 2018), and the pollen surface composition using Fourier transform infrared spectroscopy (FTIR) and FT-Raman spectroscopy (Kenđel and Zimmermann 2020).

5.5 | Biochemical and Molecular Approaches

5.5.1 | Assessing Oxidative Stress and Antioxidant Dynamics in Pollen

Reactive oxygen species (ROS) play key signaling roles during pollen development, germination, tube growth, and fertilization (Zhang et al. 2023; Zhou and Dresselhaus 2023). Maintaining ROS homeostasis is vital, as moderate ROS levels support reproduction, but excessive levels, such as during intense stress, can be toxic. Plants constantly regulate ROS through ROS-scavenging enzymes and nonenzymatic antioxidants like flavonoids (Czarnocka and Karpiński 2018). In tomato, recent research identified elevated ROS levels as a key factor in promoting pollen tube growth under heat stress (Ouonkap et al. 2024).

Several methods are available to detect ROS production along with enzymatic and non-enzymatic ROS scavengers (Akter et al. 2021). *Fluorescent ROS sensors* are typically used and imaged with a microscope for individual pollen grains and growing pollen tubes. For example, the ROS sensor 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), which converts into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation by ROS (Hempel et al. 1999), has been employed to visualize and quantify ROS in tomato, kiwi, and tobacco pollen grains and tubes (Muhlemann et al. 2018; Ouonkap et al. 2024; Potocký et al. 2007; Rutley et al. 2021; Speranza et al. 2012). The hydrogen peroxide-specific probe Peroxy Orange has also been used to visualize and quantify ROS in tomato pollen grains and tubes (Muhlemann et al. 2018). Likewise, the colorless NBT dye has also been used to detect ROS and assess cell function, turning a deep blue/black (formazan) where ROS are present, as was shown in Arabidopsis and *Nicotiana tabacum* pollen tubes (Lassig et al. 2014; Potocký et al. 2007). Unlike the fluorescent ROS sensors described earlier, NBT is cytotoxic, thus, this method provides a snapshot of superoxide production but does not allow for in vivo monitoring.

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5.5.2 | Omics-Based Methodologies

(Multi-)omics techniques offer a powerful framework to better understand the mechanisms behind heat-stress responses in pollen. Combining insights from transcriptomics, proteomics, and metabolomics of heat-stressed pollen and anthers helps identify potential signaling pathways and physiological and metabolic processes affected. *Transcriptomics* studies the RNA transcripts produced in a system, serving as a proxy for the system's "status" at a specific moment and enabling the identification of differentially expressed genes (DEG) between various conditions or cell types (Browne et al. 2021; Frank et al. 2009; Jansma et al. 2022; Liu et al. 2020; Müller et al. 2016; Zhang et al. 2022). Many studies have explored the transcriptional response to heat stress in anthers and pollen across diverse plant species, including wheat (Browne et al. 2021), cotton (Zhang et al. 2022), and tomato (Frank et al. 2009; Müller et al. 2016). Most reveal upregulation of heat-shock protein genes, highlighting the crucial role of these proteins in the response to heat stress. It is interesting to note that heat stress-responsive genes could be highly expressed in early stages of pollen development without the occurrence of stress conditions (Swindell et al. 2007), therefore, it is important to consider the basal level of expression. *Proteomics* examines the abundance of all proteins. Likewise, analyzing the proteomic response to heat stress shows how the expression of heat shock proteins supports protein stability and oxygen scavenging (Jegadeesan et al. 2018). *Metabolomics* studies analyze numerous metabolites simultaneously, revealing heat-induced changes such as an accumulation of flavonoids in microspores of tomato in response to heat stress, which appears to be an acclimation response (Paupière et al. 2017). *Lipidomics* enables qualitative and quantitative analysis of various lipid classes and fatty acid compositions. Research on heat stress-induced lipidomic changes in pollen grains and pollen tubes is limited. Nonetheless, a clear effect of high temperature on lipid composition was observed in wheat (Narayanan et al. 2018) and tobacco (Krawczyk et al. 2022). Lipidomic methodologies include a gas chromatography–mass spectrometry (GC–MS)-based approach (Bashir et al. 2013) or a multi-step method combining solid-phase extraction, thin-layer chromatography (TLC), and gas chromatography (GC) (Hernández et al. 2020). In addition, an electrospray ionization-tandem mass spectrometry approach can be used (Narayanan et al. 2018). Given the crucial role of lipids in maintaining membrane structural integrity and fluidity under heat conditions, further lipidomic studies are needed to better understand the lipid adjustments that contribute to enhanced pollen thermotolerance.

In stress response studies, it is important to understand that the observed changes or responses are a combination of: (1) the damage pathway that causes the stress-induced defects (e.g., male sterility), (2) adaptive pathways through which the plant adjusts to the stress, and (3) inconsequential "side effects." While research often aims to identify the first two types of responses, omics studies alone cannot distinguish between them; this often requires specific follow-up experiments that independently validate new hypotheses, such as studying knockout or overexpression mutants (Rieu et al. 2017; Swindell et al. 2007). Nonetheless, omics techniques have transformed plant biology by providing unprecedented insights into the full range of cellular, tissue, or organ responses to stress, and by helping to develop increasingly specific and high-quality datasets.

5.5.3 | Single-Cell Sequencing to Study Stage-Specific Heat Stress Responses of Developing Pollen

An important factor in omics methodologies is the diversity of the sample. Most studies depend on bulk tissue analysis, such as whole anthers or flower buds (Browne et al. 2021; Müller and Rieu 2016; Zhang et al. 2022). Such samples include high biological variation, like different cell types and developmental stages, since even within a single anther, pollen development is not synchronized (Carrizo García et al. 2017). Thus, signals and mechanisms behind heat stress can only be examined on a broad, global scale, which may hide cell-type-specific responses that operate at finer spatial and temporal levels.

Single-cell RNA sequencing (scRNA-seq) provides a powerful tool to examine the effects of heat stress on pollen and anthers at a higher resolution. Using scRNA-seq, cells or nuclei from a tissue or organ are separated and analyzed individually (Grones et al. 2024). The most challenging step is obtaining a high-quality single-cell or nuclei suspension (Wang et al. 2023). It is important to utilize a protocol that minimizes processing time to preserve the transcriptome snapshot of the stress. In this regard, using a protocol in which samples are either flash frozen or fixed before creating the single-cell suspension is recommended (Grones et al. 2024).

Isolation and purification protocols also require optimization. Established procedures for protoplast purification (Denyer et al. 2019; Jean-Baptiste et al. 2019; Zhang et al. 2019) or nuclei extraction (Farmer et al. 2021; Thibivilliers et al. 2020) from *Arabidopsis thaliana* roots may not work for more resilient tissues like anthers and pollen. Another key issue is obtaining single-cell or nuclei samples free of debris. When cleaning samples with FACS (Fluorescent Activated Cell/Nuclei Sorting), cells or nuclei are stained with DNA-binding fluorescent dyes (such as DAPI or PI) and sorted from debris based on fluorescence (Ichino et al. 2022). An alternative is density gradient centrifugation (Wang et al. 2023). Ultimately, there is always a trade-off between the amount of debris in a sample, the time required, and the stress applied to the single-cell or nuclei suspension before reaching sufficient quality for the sequencing step.

The high resolution provided by scRNA-seq enables differentiation of transcriptional responses across different cell types and developmental stages (Denyer et al. 2019; Farmer et al. 2021; Grones et al. 2024). This can be of immense interest in pollen studies, as scRNA-seq enables the comparison between vegetative and generative nuclei, for example.

High-resolution data allows the identification and characterization of intermediate cell states. By capturing the transcriptome of individual cells, scRNA-seq facilitates the comparison between heat-stressed and control pollen of the same developmental stage, revealing affected molecular mechanisms and specific developmental stages where these defects occur. The use of scRNA-seq is rapidly increasing in plant research (Grones et al. 2024; Ichino et al. 2022; Lee et al. 2025). Future

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advancements aim to study the transcriptome with spatial information (spatial transcriptomics) and to explore other molecular layers, such as single-cell proteomics.

6 | Conclusion

Pollen development in flowering plants is vital for reproductive success and crop yields, but is highly vulnerable to heat stress, which is intensified by ongoing climate change. This review highlights key methodologies used to evaluate pollen function under heat stress conditions and provides practical recommendations for experimental design. Methods for assessing pollen performance include viability and vigor assays, microscopy, and advanced phenotyping using flow cytometry and automated image analysis, and a range of germination assays. Biochemical and molecular analyses highlight the complexity of the heat stress response, underlying the challenge in generating standardized protocols and comparable data. Effective experimental design must therefore account for variability in plant species, genotype, developmental stage, and the type of heat stress regime applied. Evaluating pollen thermotolerance is further complicated by the difficulty in synchronizing pollen developmental stages and accounting for differences between experimental systems. Although anther and pollen development may initially appear to be straightforward processes, careful planning and the choice of assays are crucial for obtaining meaningful data.

The next phase of research should focus on enhancing the mechanistic understanding of heat tolerance and filling current methodological gaps. The roles of lipidomic changes, antioxidant dynamics, and systemic signals in pollen thermotolerance remain underexplored, as does the molecular response during the progamic phase. For instance, comprehensive in vivo analyses of how pollen tubes respond to heat stress while growing through the pistil are still lacking. Future studies must carefully consider the maternal environment, since the pistil-pollen interaction is a crucial yet often overlooked factor in modulating thermotolerance. Additionally, combining integrated-omics approaches (e.g., transcriptomics and proteomics) with detailed phenotyping will be vital to link physiological responses with key regulatory genes and protective pathways, ultimately revealing the biological mechanisms behind ATT and stress memory in pollen. High-throughput, multi-parametric phenotyping driven by AI-based data analysis will allow objective measurement of complex traits like pollen tube growth kinetics and subtle morphological changes. Importantly, these technological advances need to be supported by validation frameworks that accurately link laboratory stress responses to yield stability and field performance. By establishing rigorous, validated screening pipelines, we can unlock the full potential of genetic tools such as GWAS and QTL mapping, greatly accelerating the development of new genetic and management approaches to tackle plant male fertility issues in agriculture amid climate change.

Author Contributions

All authors contributed to the structure and content and wrote the original manuscript. Additionally, G.M. generated Figure 1 and table 2, and revised the manuscript. M.L.-L. coordinated the writing, generated Figures 2 and 3 and table 1, and revised the manuscript. D.G. contributed the T.E.M. image in Figure 2. H.S. contributed the semi in vivo and in vivo images in Figure 3.

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Disclosure

ChatGPT was used for grammar check and text editing.

Data Availability Statement

The authors have nothing to report.

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