# TEMPERATURE EFFECTS ON PLANT GAMETOPHYTE PERFORMANCE AND THEIR CONSEQUENCES FOR SEED REPRODUCTION IN WILD PLANTS

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## Declaration of manuscripts

This dissertation is a cumulative work based on the following papers:

**Chapter 2**

**Tushabe, D**., and Rosbakh, S. (2021). A compendium of *in vitro* germination media for pollen research. *Frontiers in Plant Science* 12, 709945. doi: 10.3389/fpls.2021.709945

**Chapter 3**

**Tushabe, D**., and Rosbakh, S. (2024). Patterns and drivers of pollen temperature tolerance. Under review in the Journal Plant, Cell, and Environment

**Chapter 4**

**Tushabe, D**., Altmann, F., Koehler, E., Woods, S., and Rosbakh, S. (2023). Negative effects of high-temperature stress on gametophyte performance and their consequences for seed reproduction in wild plants. *Environmental and Experimental Botany* 216, 105532. doi: 10.1016/j.envexpbot.2023.105532

**Chapter 5**

**Tushabe, D**., Altmann, F., Koehler, E., Woods, S., Kahl, S., and Rosbakh, S. (2024). Adaptation and acclimation of gametophytic traits to heat stress in a widely distributed wild plant along a steep climatic gradient. (In preparation)

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## Summary

Research is increasingly focusing on understanding the impacts of climate change on seed plant reproduction. Despite the ongoing efforts, gaps and biases still remain as most studies focus on cultivated crops and model species like *Arabidopsis thaliana*, with limited research on wild plants. Furthermore, methodological gaps such as the lack of standardized protocols for developing *in vitro* germination media for unstudied species or groups still exist.

The main aim of this thesis was to address these gaps by developing new methods for pollen research based on protocols in literature and investigate the impacts of temperature stress on plant sexual reproduction, with a particular emphasis on gametophytic performance in both wild and cultivated plants.

Chapter 1 introduces the research context of the thesis, highlighting existing gaps and biases in gametophytic studies. It provides a brief overview of the developmental stages of male (pollen) and female (ovule) gametophytes, the impacts of climate change-induced heat waves on gametophytic performance and the compensatory mechanisms they employ.

Chapter 2 is the methodological paper with a compendium of optimized *in vitro* pollen germination media (PGM) recipes from over 1800 articles published from 1926 to 2019. The compendium consists of 1572 PGM successfully used in 816 species from 412 genera and 114 families (monocots and dicots). Key components used include sucrose (89%), $H_3BO_3$ (77%), $Ca^{2+}$ (59%), $Mg^{2+}$ (44%), and $K^+$ (39%). Notably, concentrations of sucrose, calcium, and magnesium varied significantly across categories, highlighting the need for group-specific considerations in PGM composition. I therefore identified general rules for creating PGM tailored to different species groups, considering factors such as research focus (wild vs. cultivated species), phylogenetic relatedness, pollen physiology, biochemistry, and stigma properties. The compendium serves as a valuable data resource for PGM and facilitates future pollen research endeavors.

Chapter 3 examines the patterns and drivers of pollen temperature tolerance (cold and heat) across diverse plant populations and species globally. To achieve this, I compiled a dataset spanning 1933 to 2020, comprising cardinal temperatures (minimum, optimal, and maximum) for pollen germination and tube growth in 198 plant species. I investigated pollen temperature limits (PTLs) by examining their correlation with the thermotolerance of vegetative tissues and assessing variability at the intra- and interspecific levels across the species with contrasting phylogeny, cultivation history, biology, and ecology. The findings revealed positive correlations between PTLs and thermotolerance in vegetative tissues. At the species level, PTLs ranged from 6.1 to 39.5 ºC, displaying significant differences among growth forms and cultivation histories. While there were variations in pollen cold tolerance among species populations, optimum and maximum temperatures remained stable. Phylogenetic analysis indicated family-level conservation in pollen cold tolerance, contrasting with the evolutionary independence of heat tolerance. Climate emerged as a significant driver of PTLs, with species at higher elevations and latitudes exhibiting enhanced tolerance. Cultivated species displayed narrower temperature tolerances, highlighting potential vulnerabilities to global warming. Overall, this study sheds light on the complex relationships among pollen temperature limits, plant traits, and environmental factors, providing essential insights into the impact of climate change on plant reproduction.

In chapter 4, the impacts of chronic heat stress (CHS) at moderate (35/30 °C) and severe (40/35 °C) levels, on gametophyte performance and subsequent seed quantity, quality, and germination in four wild *Silene* species (*Silene coeli-rosa*, *Silene gallica*, *Silene laeta*, and *Silene noctiflora*) were investigated. I measured six traits related to the male (anther length, pollen production, and size) and female (ovary length, ovule production, and size) gametophyte performance, along with leaf chlorophyll fluorescence. Seeds from treated plants were used to measure seed mass and production, while seed germination was evaluated in terms of germination percentage, speed, and synchrony. The results revealed that both CHS treatments had a negative impact on overall plant performance.

All male gametophyte traits, ovary size, and ovule production were significantly reduced in CHS treatments, resulting in fewer seeds, but seed mass remained unaffected. Although the final germination percentage showed weak significant differences in the severe treatment, heat stress did not negatively impact seed germination. Treated plants exhibited significantly faster germination, with no effect on germination synchrony. Climate change-induced heat waves can adversely affect seed reproduction in wild plant populations, potentially impacting their long-term survival.

In chapter 5, the adaptive and acclimation potential of six male (anther length, pollen production, and size) and female (ovary length, ovule production, and size) gametophytic traits in eleven distinct populations of wild *Silene vulgaris* across a temperature gradient in Europe were examined. First, plants were cultivated in a common garden to reveal pre-adaptation of gametophytic traits to the local conditions. Next, flowering plants were subjected to chronic heat stress (CHS) treatments (moderate [35/30 °C] and severe [40/35 °C]) to assess the acclimation potential of gametophytic traits. Results from the common garden showed no intraspecific variation in gametophytic traits across the temperature gradient, suggesting limited influence of these traits on sexual adaptation to local habitats. Plants from colder climates produced more seeds with higher mass than those from warm climates. Under both CHS treatments, female gametophytes showed a higher ability to acclimate than males, with moderate CHS leading to increased ovary size and ovule numbers and severe CHS reducing ovule numbers but increasing their size. All pollen traits decreased under both CHS, with severe stress causing more significant reductions, thus translating to low seed quality and quantity. Acclimation potential did not vary among populations across the temperature gradient under both CHS treatments, except for pollen size under severe CHS, which was larger in warmer climates than in colder regions. Overall, the lack of adaptation and acclimation mechanisms in gametophytic traits suggests potential reliance on alternative strategies like shifting flowering time and phenotypic plasticity to cope with climate change-induced heat waves.

Chapter 6 provides a synthesis of the key findings from the four main chapters. The main conclusion drawn is that climate change-induced heat waves can have significant and complex impacts on the reproductive processes, particularly pollen performance, of both wild and cultivated plants. To obtain more precise estimates of the impact of heat stress on seed production, it is crucial to conduct further research that includes a broader array of genotypes or species. Additionally, the chapter discusses potential areas for exploration in future studies within this field.

# Chapter 1: General introduction

### *Impacts of climate change on plant reproduction*

Over the last two decades, there have been growing concerns on the impacts of climate change on the reproductive success of plants (Pachauri et al., 2014; Raza et al., 2019). Particularly, the increasing average temperatures and the rising frequency of extreme weather events, including droughts, floods, storms, and heatwaves, have negative effects on reproduction in both cultivated and wild plants (Rosbakh et al., 2017; Raza et al., 2019). For instance, drought limits water availability, negatively impacting plant reproduction processes such as pollen development, pollen tube growth, and fertilization (e.g., Fahad et al., 2017). Floods damage reproductive structures, wash away seeds, and disrupt water-dependent pollination (e.g., Fischer et al., 2021; Aslam et al., 2023). Strong storms and wind-gusts physically damage flowers, fruits, and seeds, affecting their viability (Gardiner et al., 2016). Heatwaves can cause flower, ovule, and pollen sterility by affecting enzymatic activity and metabolic processes required for optimal gametophyte development (Hasanuzzaman et al., 2013; Kumar et al., 2022). These climate change-induced impacts on reproductive processes ultimately result in reduced seed quantity and quality.

In cultivated crops, reduced crop yields are expected to affect global food security (Kumar, 2016; Ray et al., 2019). Projections indicate that there will be a 30% decline in crop yield by 2050 (Bapna et al., 2019). Within wild populations, a decrease in seed quantity and quality may result in reduced plant populations, increasing their vulnerability to extinction (e.g., Willis et al., 2008). Alterations in plant community composition have consequences on their ecological interactions, such as plant-pollinator relationships, with cascading effects throughout the ecosystem (Walck et al., 2011; Long et al., 2015; Bogdziewicz et al., 2016).

Over the past few decades, numerous studies have focused on understanding plant reproductive responses to extreme weather events (e.g., Gray and Brady, 2016; Harris et al., 2020). Attention is increasingly on the impact of heat stress on the gametophytic stage especially with the increasing frequency of heat waves, due to the high sensitivity of gametophytes to temperature fluctuations (Resentini et al., 2023; Tushabe et al., 2023). The physiological responses (e.g., adjustment in photosynthetic activity; dos Santos et al., 2022), biochemical (e.g., through enzymatic activity; El-Remaly, 2023), molecular (e.g., changes in gene expression patterns; Kan et al., 2023) and ecological (e.g., shifts in plant distribution and community interactions; Moran et al., 2022) have been investigated.

Progress has been made in developing various approaches to study these responses under controlled laboratory settings (*in vitro*), in greenhouses and other natural environments such as fields. For instance, experimental protocols and techniques have been developed for *in vitro* studies on gametophyte performance, where specific tissues or cells (e.g., pollen and ovules) are subjected to stress conditions (e.g., Rodriguez-Enriquez et al., 2013). The protocols include optimized growth media/ cultures for specific species that closely mimic natural conditions, enabling control over environmental factors like temperature, humidity, and nutrient levels (e.g., Brewbaker and Kwack, 1963). Additionally, recent advancements in imaging technology (e.g., scanning electron microscopy, fluorescence microscopy, and live cell imaging) have enabled a deeper understanding of molecular and cellular mechanisms (Lidke and Lidke, 2012; Bond et al., 2022). Such include signalling in pollen tube growth (Guan et al., 2013), male-female interactions from pollen germination to double fertilization (Higashiyama and Yang, 2017), and self-incompatibility (Bedinger et al., 2017). Plant breeders for instance, are applying these protocols and technologies to study how crops like rice, soybean, and cotton respond to extreme temperature shifts, particularly by examining *in vitro* pollen germination and pollen tube growth (Kakani et al., 2005; Salem et al.,

2007; Mesihovic et al., 2016). These investigations are used as breeding strategies for developing plant varieties with enhanced heat tolerance.

In the field and greenhouse experiments, studies on the plant reproductive responses to heat stress explore the ecological and physiological aspects. Such include plant-pollinator interactions (e.g., Dafni and Vereecken, 2016), shifts in flowering phenology (e.g., Scheepens and Stöcklin, 2013), range shifts (e.g., Flores-Rentería et al., 2018), and seed traits (e.g., Zhou et al., 2021; Amimi et al., 2023). Furthermore, scientists employ advanced remote sensing technologies like drones and satellites to monitor large-scale plant stress responses. These tools provide real-time temperature variation data, enabling studies across diverse geographic regions (Galieni et al., 2020; Smigaj et al., 2023). However, most studies and protocols developed are often biased towards model and cultivated species. This bias is primarily driven by the economic importance of the cultivated species (e.g., Zinn et al., 2010; Hedhly, 2011), with limited focus on wild species (e.g., Tushabe et al., 2023). Furthermore, there is a scarcity of studies in wild plants that specifically consider the effects on the gametophytic stage (pollen and ovule traits), which is highly temperature sensitive. Available evidence suggests that even slight increases in temperature beyond the optimal range (~10 to 30°C; Luo, 2011) during critical phases of gametophytic development can negatively impact plant reproduction (Hedhly, 2011; Arshad et al., 2017).

The ultimate goal of the thesis was to address these gaps by developing new methods for pollen research *in vitro* for wild plants and investigate how temperature stress affects plant sexual reproduction focusing on gametophyte performance in both wild and cultivated plants. To start with, the stages of male (pollen) and female (ovule) gametophyte development, the impact of temperature stress on gametophyte stages, and the compensatory mechanisms they employ are discussed.

### *Stages of male and female gametophyte development in angiosperms*

*Male gametophyte development*

The male gametophyte, pollen or microgametophyte, undergoes development within the anther of the stamen and consists of two sperm cells enclosed in a vegetative cell (McCormick, 1993). The process of pollen development has been extensively studied and documented in plant biology research (e.g., Borg et al., 2009; Gómez et al., 2015). It involves a series of distinct stages, each crucial for the formation of mature and functional pollen grains. These stages can be broadly categorized into microsporogenesis and microgametogenesis (McCormick, 1993; Gómez et al., 2015).

Microsporogenesis is the initial phase, taking place within the anther, in which the microsporocytes undergo meiosis to form a tetrad of haploid microspores (Twell et al., 2006, Figure 1). Each microspore and the tetrad are protected by a thick callose wall, isolating them from each other and the surrounding cells (McCormick, 1993). Microsporogenesis is supported by nutrients, carbohydrates, cell wall components, and enzymes in the locular fluid secreted by the tapetum (Liu et al., 2021). Enzymes such as calluses digest the callose walls of the tetrads, allowing the release of microspores into the second phase known as microgametogenesis (Gómez et al., 2015). In this phase, the microspore undergoes two mitotic divisions to produce bicellular or tricellular pollen grains. The vegetative cell decreases in size during pollen maturation, while the generative cell undergoes a second mitotic division to form two sperm cells (Borg et al., 2009). The pollen grain wall, consisting of the exine and intine, develops around the vegetative and generative cells. After maturation, the anther opens, releasing the mature pollen grains for pollination and fertilization by external agents such as wind or animal pollinators (Pacini, 2000).

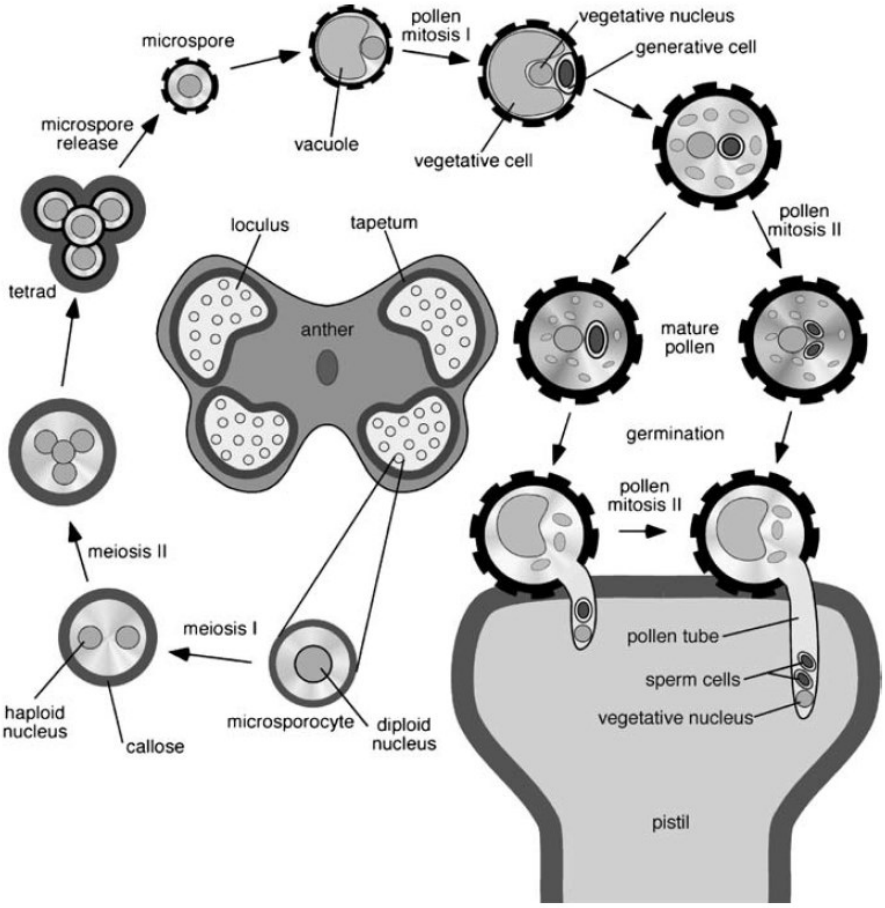


Figure 1: Morphological stages in pollen development (Source: Twell et al., 2006).

*Female gametophyte development*

The female gametophyte (i.e., embryo sac or megagametophyte), develops inside the ovule, situated in the ovary of the carpel (Drews et al., 1998). The development of the megagametophyte involves two interconnected phases: megasporogenesis and megagametogenesis (Figure 2). Megasporogenesis includes meiotic division and maturation, while megagametogenesis involves mitotic division, cellularization, and maturation (Huang and Russell, 1992). The female gametophyte in angiosperms typically consists of seven cells with four cell types; three antipodal cells, two synergid cells, an egg cell, and a central cell (Huang and Russell, 1992; Drews et al., 1998).

Ovule development starts with the initiation of a small bulge called the ovule primordium on the inner ovary wall (Gasser and Robinson-Beers, 1993). These primordia extend fingerlike projections, initiating one or two integuments. The inner integument forms from cells encircling the primordium, while the outer integument arises from epidermal and subepidermal layers. The integuments elongate covering the nucellus and leaving a micropyle for pollen tube entry (Drews and Yadegari, 2002; Hater et al., 2020). Within the nucellus, a single cell differentiates into a megasporocyte, which undergoes meiosis to produce four megaspores. Typically, only one megaspore survives while the others degenerate. The surviving megaspore develops into the megagametophyte or embryo sac (Yadegari and Drews, 2004; Drews and Koltunow, 2011).

Within the developing embryo sac, nuclei migrate and position themselves. Antipodal cells form at one end, polar nuclei fuse to form the secondary nucleus, and the egg cell and synergids form at the opposite end. Synergids guide the pollen tube during fertilization, while antipodal cells often degenerate (Drews et al., 1998; Hater et al., 2020). The embryo sac further develops, acquiring the organization and structure necessary for fertilization. The egg cell becomes the female gamete capable of fertilization by a sperm cell (Drews and Yadegari, 2002).

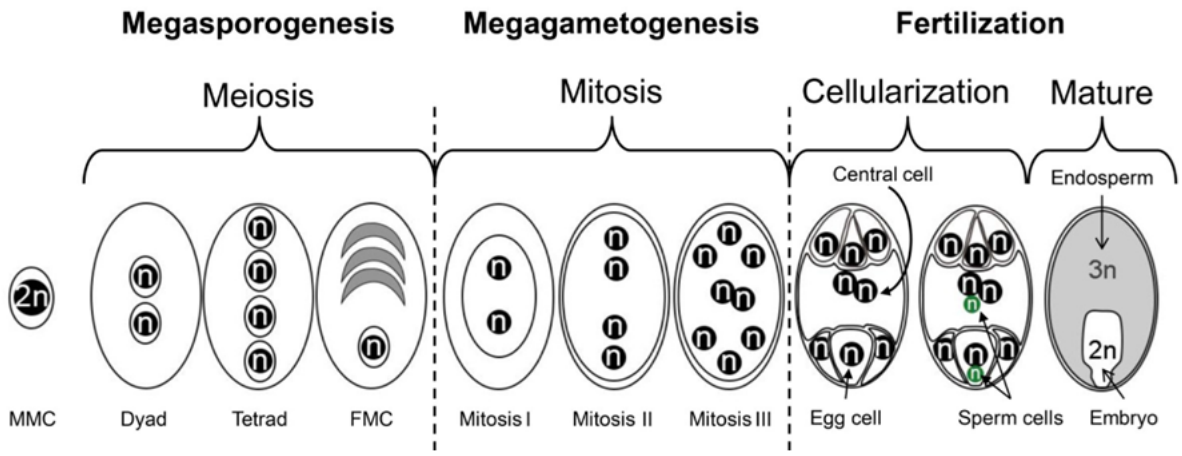


Figure 2: Morphological stages of megasporogenesis, megagametogenesis and fertilization in the female gametophyte. MMC (megaspore mother cell), FMC (functional megaspore cell), and n (number of chromosomes in a cell) (Source: Carballo et al., 2021).

***Effects of temperature stress on the various stages of male and female gametophytic development***

Within angiosperm gametophytic organs and tissues, ovules, which are protected by the pistil, exhibit greater tolerance to abiotic stress when compared to pollen grains and anthers (Zinn et al., 2010; Hedhly, 2011; Raja et al., 2019). This difference in sensitivity can be attributed to the smaller size, haploid chromosome set, lack of protective tissue, and direct exposure to the environment during anthesis that pollen experiences (Bedinger, 1992; Lohani et al., 2020). In general, stressful environmental conditions have negative effects on both the male and female gametophytes as well as on plant growth and flower production (De Storme and Geelen, 2014; Tushabe et al., 2023).

*Effects on pollen development*

The duration and exposure of plants to high temperature stress significantly impacts pollen development at all stages (Hedhly et al., 2020; Chaturvedi et al., 2021). High temperature alters essential enzymes, and hormones responsible for pollen growth (e.g., Rieu et al., 2017; Chaturvedi et al., 2021). This leads to morphological, structural, and metabolic changes in the male gametophytic

organs, disrupting cell division, impairing pollen grain development, and altering maturation processes (Hasanuzzaman et al., 2013; Iovane and Aronne, 2022).

The initial pollen developmental defects occur during meiosis, causing irregular chromosome behavior and abnormalities in meiotic divisions (De Storme and Geelen 2014). Abnormal tapetal cell development and degeneration, along with disrupted nutrient supply during microsporogenesis, contribute to a decrease in pollen viability (Sato et al. 2002; Endo et al. 2009). Furthermore, heat stress during microsporogenesis results in microspore abortion, making it the most heat-sensitive phase of pollen development (Iovane and Aronne, 2022).

During anther dehiscence, heat stress can also delay or inhibit pollen release leading to male sterility (De Storme and Geelen, 2014). Once released, mature pollen demonstrates higher tolerance to temperature fluctuations due to low plasma content, reduced metabolic activity, and protective structures (Hedhly, 2011). However, adverse weather conditions during pollen dispersal can still cause reduced pollen viability or cause male sterility (Kakani et al., 2005).

During fertilization, temperature stress can disrupt the adhesive properties of pollen grains, making it challenging for them to adhere to the stigma surface (e.g., Hedhly et al., 2005; Prasad et al., 2006). Germination of pollen on the stigma, formation of pollen tubes, and the fusion of male and female gametophytes are also susceptible to temperature fluctuations (Sakata et al., 2010; Çetinbaş-Genç et al., 2019; Liu et al., 2023), despite the protection provided by pistil tissue (Kakani et al., 2002). Consequently, any deviations in male gametophyte development can reduce the number and viability of pollen grains, leading to irregular and decreased seed yields (Zinn et al., 2010; Hedhly, 2011).

*Temperature effects on ovule development*

Temperature stress significantly affects various stages of ovule development such as megasporogenesis, megagametogenesis, fertilization, and embryo development (Choudhary et al.,

2022; Shi et al., 2022). During megasporogenesis, the most sensitive stage of the female gametophyte development, heat stress can disrupt the meiotic divisions and result in the production of abnormal or non-viable megaspores (Wang et al., 2021; Shi et al., 2022). Additionally, it can cause damage to the cellular structures within the megaspore mother cell and impair the formation of healthy megaspores resulting in reduced ovule fertility (Wang et al., 2021; Osorio et al., 2022). Temperature stress during megagametogenesis can alter cell differentiation, functioning, and the timing/duration of female gametophyte maturation, accelerating, delaying, or inhibiting these processes (Young et al., 2004; Płażek et al., 2019).

Heat stress also affects nuclear divisions in the developing embryo sac leading to abnormal ploidy levels and morphology (Shi et al., 2022; Yang and Pecinka, 2022). Abnormal embryo development, abortion, or reduced viability ultimately result in reduced seed and fruit production (Prasad and Djanaguiraman, 2014; Mácová et al., 2022).

***Compensatory mechanisms by the gametophytes to cope with heat stress during development***

Plants have evolved adaptive strategies to cope with the impacts of heat stress during gametophyte development (Hedhly et al., 2005). These include plasticity in reproductive responses encompassing genetic, molecular, physiological, or structural adaptations (Hasanuzzaman et al., 2013; Ahmad et al., 2021; Hoshikawa et al., 2021).

For example, gametophytes activate heat-responsive genes to produce protective molecules such as heat shock proteins, antioxidants, chaperones, and heat shock transcription factors (Hasanuzzaman et al., 2013; Kumar et al., 2022). Heat shock proteins and molecular chaperones prevent protein denaturation by facilitating proper folding under heat-stress conditions (Wahid et al., 2007). The antioxidant defence system produces enzymes such as superoxide dismutase, catalase, and peroxidases that counteract oxidative damage caused by reactive oxygen species produced during the stress (Gill and Tuteja, 2010; Kapoor et al., 2019). Epigenetic alterations, such as DNA methylation

or histone can also occur, influencing gene expression patterns that enhance heat tolerance and are heritable for transgenerational memory of heat stress responses (Boyko and Kovalchuk, 2011). Additionally, plants can adjust their reproductive cycle by altering the timing of flowering or pollen release to avoid extreme heat periods (e.g., Gugger et al., 2015; Rieu et al., 2017; Jagadish, 2020). This synchronization with more favourable temperature conditions increases the chances of successful pollination and fertilization (Kehrberger and Holzschuh, 2019). Plants can also adjust osmotically to reduce water loss and dehydration by accumulating compatible solutes such as sugars, proline, and betaine that maintain cellular water potential, preventing desiccation, and protecting gametophytes from heat-induced damage (Takahashi et al., 2020; Seleiman et al., 2021). These strategies can ensure the viability, germination, and successful fertilization of gametophytes under harsh heat conditions.

***Gaps and biases in gametophytic studies***

While significant progress has been made in understanding the general relationship between temperature variations and gametophytic performance, there are still knowledge gaps and biases including:

1) Methodological gap: particularly for *in vitro* studies, the use of optimized culture media is crucial for successful pollen and ovule germination. However, the applicability of existing protocols in establishing general rules for developing germination media/ culture remains uncertain, particularly when studying or experimenting with unstudied species where relevant information is lacking.
2) Taxonomic bias: the widely accepted sensitivity of gametophytes to temperature stress is primarily based on experiments with cultivated plants (e.g., rice, wheat, cotton; Lohani et al., 2020) and a few model species such as *Arabidopsis thaliana* (Bac-Molenaar et al., 2015).

However, research on wild species and their response to temperature variations is scarce, making it unclear how temperature affects seed production in natural populations.

3) Developmental stage bias: studies often focus on specific stages such as microgametogenesis (e.g., Elsahookie et al., 2021) or fertilization (e.g., Snider and Oosterhuis, 2012). However, the cascading effects of temperature throughout the life cycle of gametophytes to regeneration by seed is critical for a comprehensive understanding as impacts on one stage may affect other stages (e.g., Prasad and Djanaguiraman, 2014; Mácová et al., 2022).
4) Aspect bias: most studies favor molecular/physiological approaches over ecological/evolutionary aspects of gametophyte performance. Ecological aspects are crucial for understanding how the negative effects translate and impact species characteristics, distribution limits, and seed crop quality (e.g., Rosbakh and Poschlod, 2016). Ecological aspects of gametophytic performance especially in wild species, from sporogenesis to gamete fusion remain under studied.
5) Climate change context bias: the effects of temperature on gametophytic performance in the context of climate change-associated heat waves are not well studied. Most studies focus on average temperature trends rather than the challenges posed by heat wave events. More research is needed to understand the responses and resilience of gametophytes during episodes of extreme temperature stress, especially in wild plants.

***Thesis outline and research questions***

The primary objective of this thesis was to investigate the impacts of high-temperature stress on gametophyte performance at interspecific and intraspecific levels, with a focus on evaluating their potential importance for the success of plant seed regeneration in wild and cultivated species. I supplemented this with a methodological study of *in vitro* pollen germination media, which plays a crucial role in facilitating pollen research.

Specifically, I aimed to:

1) develop new methods for pollen research in wild and cultivated plants by compiling a compendium of *in vitro* pollen germination media recipes available in published literature,

2) examine the patterns and drivers of pollen cold and heat tolerance across multiple populations and species of wild and cultivated plants occurring worldwide,

3) assess how exposure of wild herbaceous plants at the reproductive stage to heat-stress affects their gametophyte performance at various stages and how these effects are translated into seed quantity and quality at both species and population level, and

4) assess the adaptive and acclimation potential of gametophytic traits to heat stress in eleven populations of wild *Silene vulgaris*, occurring along a steep climatic gradient of temperature in Europe.

Below is the overview of the subsequent chapters:

Chapter 2 addresses objective one, aimed at compiling a compendium of *in vitro* pollen germination media scattered in literature, and developing generalizable germination media for studying species for which such information is not available. The compendium serves as a valuable resource for future pollen research, facilitating experimental design and pollen germination studies.

In chapter 3, the primary goal was to investigate the patterns and drivers of pollen temperature tolerance among diverse plant populations and species on a global scale. The research explores the variability of pollen temperature limits (PTLs) across different plant species, considering factors such as phylogeny, cultivation history, biology, and ecology. I correlated PTLs with vegetative tissue thermotolerance, assessed intra- and interspecific variability, and examined the impact of climate on these temperature limits. Generally, this study emphasizes the complex relationships between pollen

temperature limits, plant characteristics, and environmental factors in the context of climate change impacts on plant reproduction.

Chapter 4 investigates the impacts of chronic heat stress (CHS) on plant regeneration through seeds, with a specific focus on the gametophyte stage. I assessed the effects of moderate (35/30 ºC) and severe (40/35 ºC) CHS treatments on the performance of male and female gametophytes in four wild *Silene* species. I aimed to understand how these effects translate into seed quantity, quality, and germination. The findings highlight the sensitivity of gametophytes to high-temperature stress and suggest that climate change-associated heat waves could significantly affect seed reproduction in wild plants, potentially influencing the long-term survival of plant populations and the performance of granivores.

Chapter 5 examines the adaptive and acclimation potential of male and female gametophytic traits in wild *Silene vulgaris* populations across a temperature gradient in Europe. Specifically, this involved cultivating plants under common garden conditions to reveal whether there is evidence of local adaptation in gametophytic traits and assess how these traits acclimate to moderate and severe chronic heat stress (CHS) treatments. The results suggest limited influence of the gametophytic traits on sexual adaptation to local habitats. Overall, there was a lack of adaptation and acclimation potential in the gametophytic traits, resulting in low seed quality and quantity.

Chapter 6 concludes with the consequences and practical applications derived from the preceding chapters. The focus is on the significance of these findings in conservation and ecosystem assessments, with a specific emphasis on tackling the challenges posed by climate change-induced environmental changes.

# Chapter 2: A compendium of *in vitro* germination media for pollen research


## Abstract

The correct choice of *in vitro* pollen germination media (PGM) is crucial in basic and applied pollen research. However, the methodological gaps (e.g., strong focus of current research on model species and cultivated plants along with the lack of general rules for developing a PGM) makes experimenting with pollen difficult.

We closed these gaps by compiling a compendium of optimized *in vitro* PGM recipes from more than 1800 articles published in English, German, and Russian from 1926 to 2019.

The compendium includes 1572 PGM recipes successfully used to germinate pollen grains or produce pollen tubes in 816 species representing 412 genera and 114 families (both monocots and dicots). Among the 110 components recorded from the different PGM recipes, sucrose (89% of species), $H_3BO_3$ (77%), $Ca^{2+}$ (59%), $Mg^{2+}$ (44%), and $K^+$ (39%) were the most commonly used PGM components. PGM pH was reported in 35% of all studies reviewed. Also, we identified some general rules for creating PGM for various groups of species differing in area of research (wild and cultivated species), phylogenetic relatedness (angiosperms vs. gymnosperms, dicots vs. monocots), pollen physiology (bi- and tri-cellular), biochemistry (starchy vs. starchless pollen grains), and stigma properties (dry vs. wet), and compared the component requirements. Sucrose, calcium, and magnesium concentrations were significantly different across most categories indicating that pollen sensitivity to sugar and mineral requirements in PGM is highly group-specific and should be accounted for when composing new PGM. This compendium is an important data resource on PGM and can facilitate future pollen research.


Keywords: cookbook, experiment, medium, *in vitro*, pollen

## Introduction

Pollen, the male gametophyte, is an evolutionary development in higher plants that ensures successful genetic exchange, establishment, and survival of the species (Ashman et al., 2004; Pacini and Dolferus, 2016). Because of their crucial role in successful seed development (Shivanna and Rangaswamy, 1992; Dafni and Firmage, 2000; Rosbakh et al., 2018), pollen germination (PG), and pollen tube growth (PTG) have been in the focus of many studies ranging from research on physiological and biochemical aspects of these processes (Taylor and Hepler, 1997; Wang et al., 2010; Williams and Reese, 2019) to large-scale screenings of pollen abiotic stress-tolerance (Kakani et al., 2005; Rosbakh et al., 2018). Additionally, pollen is an excellent model system for studying a number of basic processes including plant cell growth, cell wall synthesis, intracellular transport, and cell-cell interaction (Johnson-Brousseau and McCormick, 2004; Boavida and McCormick, 2007). That is why pollen remains one of the most attractive objects in plant research.

In basic and applied research, pollen functioning has been studied with the help of two approaches, *in vivo* and *in vitro*. *In vivo* studies are carried out directly at the stigmatic surface in the natural state, while *in vitro* approaches rely on a culture medium that simulates conditions of the style-stigma (Rodriguez-Enriquez et al., 2013). The advantage of the *in vivo* methods is that they consider all natural conditions pollen grains experience on stigma (Dawkins and Owens, 1993; Albert et al., 2018). However, such methods have sometimes proved difficult (Shivanna and Rangaswamy, 1992). Partly, this is due to the involvement of the pistillate tissue that interacts with the growing pollen tubes thereby affecting physiological and biochemical investigations (Shivanna and Rangaswamy, 1992; Zheng et al., 2019; Xu et al., 2020). Moreover, the complex and labour-intensive nature of the *in vivo* approach (e.g., maintenance of pistil tissue viability and post-experimental sample processing) limits its applicability in large-scale research, such as breeding programs (Kakani et al., 2005) or multispecies ecological screenings (Rosbakh et al., 2018). The comparatively technically

simple *in vitro* approach, which is based on the ability of pollen to germinate and grow without the pistillate tissue, solves this problem making it possible to conduct comprehensive pollen research (Taylor and Hepler, 1997). Although being sometimes criticized for inaccurate replication of the biological context (Rodriguez-Enriquez et al., 2013), the *in vitro* approach is generally preferred because it provides results comparable to *in vivo* studies (Sulusoglu and Cavusoglu, 2014; Jayaprakash, 2018; Luo et al., 2020). It is also easier to detect alterations in PG or PTG performance using *in vitro* approaches (Procissi et al., 2003; Steinebrunner et al., 2003; Cole et al., 2005; Hashida et al., 2007) since the parameters can easily be tested for defects which is difficult to perform *in vivo*. Notably, *in vitro* PG rates are considered the best estimate of pollen viability *in vivo* (Shivanna et al., 1991; Stone et al., 1995).

Typically, an *in vitro* PG protocol includes cultivating of fresh or stored pollen grains in/on a germination media contained within a hanging drop/well or on a membrane support (Conner, 2011; Soares et al., 2013; Jayaprakash, 2018). In all such protocols, the correct choice of pollen germination media (PGM) remains the most important part, as pollen is highly sensitive to the PGM composition (Dafni et al., 2005). To begin with, pollen of several species requires either liquid (Hoffmann et al., 1990; Golan-Goldhirsh et al., 1991; Montaner et al., 2003) or solid (e.g., with addition of agar) medium (Shivanna and Sawhney, 1995; Jayaprakash, 2018) to germinate, while others can germinate in/on both solid and liquid media (Bilderback, 1981; Boavida and McCormick, 2007). Additionally, most pollen grains need a carbohydrate source to germinate successfully, and sucrose solution is generally used (Montaner et al., 2003; Silva et al., 2016; Lagera et al., 2017). In some cases, other sugars, and sugar derivatives such as lactose, maltose, raffinose, and fructose among others, are also used (Shaoling et al., 2005; Hirsche et al., 2017; Lagera et al., 2017; Impe et al., 2019). Furthermore, several inorganic compounds affect *in vitro* PG with boron being one of the most important element for most species (Brewbaker and Kwack, 1963; Wang et al., 2003; Yao and Zhao, 2004; Fang et al., 2016). Besides boron, minerals such as calcium, magnesium, potassium are

also known to have stimulatory effects on PG (Brewbaker and Kwack, 1963; Čapková-Balatková et al., 1980; Song et al., 2009; Biswas and Mondal, 2014; Jayaprakash, 2018). The different compounds in the medium affect the pH that must therefore be adjusted to species-specific values, to allow for optimal conditions for pollen to germinate (Tupý and Řhová, 1984; Fricker et al., 1997; Fan, 2001). Trisaminomethane (Tris) and 2-ethanesulfonic acid (MES) belong to the buffers that have been often used to maintain a constant pH in the medium (Tupý and Řhová, 1984; Holdaway-Clarke et al., 2003). Further, several other substances such as hormones, vitamins, buffers, proteins, lipids, antibiotics, enzymes, plant, and animal extracts are sometimes added to increase the percentage of PG and to accelerate the rate of PTG (Vasil, 1960; de Bruyn, 1966; Boavida and McCormick, 2007; Jayaprakash, 2018). Finally, different plant groups are also suggested to have different PGM requirements (e.g., plants with binucleate vs. trinucleate pollen grains (Hoekstra, 1979; Bergamini and Mulcahy, 1988; Zhang et al., 1997; Gibernau et al., 2003); those with dry vs. wet stigmas (Boavida and McCormick, 2007; Rodriguez-Enriquez et al., 2013); angiosperms vs. gymnosperms (Paoletti and Bellani, 1990); monocots vs. dicots (Jayaprakash, 2018); and in plants with starchy vs. starchless pollen grains (Bellani et al., 1985; Franchi et al., 2007).

Despite the fact that several PGM are widely available (Brewbaker and Kwack, 1963; Hong-Qi and Croes, 1982; Roberts et al., 1983; Rodriguez-Enriquez et al., 2013), experimentation on PG *in vitro* is still challenged by several problems. Firstly, information on PGM requirements for a species in question is usually extremely scattered in works published in very different journals and/or years. Secondly, although numerous protocols describe methods to induce PG, they are not applicable to all species as they are strongly biased either to model species, such as *Arabidopsis thaliana* (Boavida and McCormick, 2007; Rodriguez-Enriquez et al., 2013) or domesticated plant species, their cultivars and wild relatives (Roberts et al., 1983; Cheng and Mcomb, 1992; Jayaprakash et al., 2018). In contrast, PG studies for any given wild species are limited (Mortenson et al., 1964; Fernando et al., 2001; Sorkheh et al., 2011). Thirdly, it is not clear whether the available protocols can be

generalized to create some general rules for developing a PGM for a single species or various species groups especially those that have not yet been studied.

Here, we close these gaps by compiling a compendium of *in vitro* PGM recipes available in the published literature. Specifically, we first provide a list of optimized media successfully used to germinate pollen grains and/or produce pollen tubes in different species. In addition, we identify the key PGM components required for various groups of species differing in area of research (wild and cultivated species), phylogenetic relatedness (angiosperms vs. gymnosperms, dicots vs. monocots), pollen physiology (bi- and tri-cellular), biochemistry (starchy vs. starchless pollen grains), and stigma properties (dry vs. wet) that will help to create and/or optimize PGM recipes for the species, for which such information is not available.

**Materials and methods**

To extract available information on PGM composition, we first reviewed all studies published from 1926 to 2019 that included keywords ‘‘pollen’’, ‘‘germination,’’ and ‘‘media’’ either in the title or abstract. The literature was searched using the Web of Science database with the searched databases including, “Web of Science Core Collection,” “KCI-Korean journal database,” “Medline,” “Russian science citation index,” and “SciELO Citation.” Only publications in English, Russian, and German were considered. The search resulted in 1800 studies.

In the second step, we sorted out the publications that were accessible in a digital form and/or contained information about PGM composition with an estimate of its efficacy to stimulate PG or PTG in the abstract or full text. From each of the 675 studies that fulfilled the selection criteria, we extracted the author’s name(s), year of publication, full literature reference and whether the publication was available in digital form. For each species/variety studied in the selected publications, we further extracted information on the PGM composition including the ingredients used and their concentrations. When several PGMs were used in a publication, only PGM reported to

be most effective, i.e., resulting in maximum PG, longest pollen tubes, or minimum pollen bursting obtained, were extracted. If component concentrations of effective PGMs were given as a range, average values of such ranges were considered.

Additionally, we included unpublished data on PGM composition for 104 Central European plant species collected during PG studies at the University of Regensburg from 2014 to 2020 (S. Rosbakh unpublished).

*Data analysis*

For the statistical analysis, all entries in the data set were standardized by recalculating sucrose and agar concentrations to percentages and the rest of the ingredient concentrations to millimolar (mM). The species taxonomy was standardized against the "Plant List" (2013).

In order to infer group-specific concentrations of PGM components, we classified all species present in the data set into several categories: (1) wild vs. cultivated species, (2) angiosperms vs. gymnosperms, (3) dicots vs. monocots plants, (4) bi- vs. tri-nucleate pollen (Brewbaker, 1967), (5) starchy vs. starchless pollen (Baker and Baker, 1979), and (6) dry vs. wet stigmas (Heslop-Harrison and Shivanna, 1977). This analysis was carried out only with entries that included information on the most frequent PGM components (agar, sucrose, $H_3BO_3$, $Ca^{2+}$, $Mg^{2+}$, $K^+$, and pH). The variation in the PGM ingredients in the species groups were visualized with the help of ggplot2 package (Wickham, 2016) in the R software version 4.0.0 (R Core Team, 2020). A Kruskal–Wallis non-parametric test at a 95% confidence interval was performed to test for significant differences among PGM requirements in the different plant groups.

## Results and Discussion

*The compendium of PGM for pollen research*

The final version of the PGM compendium (Tushabe and Rosbakh, 2021) is composed of 1572 recipes successfully used to germinate pollen grains and/or produce pollen tubes in 816 species representing 412 genera and 114 families (both monocots and dicots). All together, we recorded 110 components from the different *in vitro* PGM, used under varying conditions and concentrations. Out of 816 species, 51% (420) and 32% (260) germinated in liquid or solidified (mainly agar with concentrations 0.5–1.5%) media, respectively, while 17% (136) germinated in both solid and in liquid media. The liquid media is preferred when pollen needs to reach a certain turgescence level to germinate (Martin and Brewbaker, 1971; Montaner et al., 2003), but also because water serves other hydrolytic and synthetic reactions (Brink, 1924). However, in some species, cultivation in liquid media leads to pollen bursting, due to quick hydration thus solid (e.g., agarified) media is required (Burke, 2002; Sun et al., 2008; Jayaprakash, 2018). In such media, agar also enables incorporation of sucrose or other stimulants, helps to maintain relative humidity at constant levels and provides appropriate aerobic conditions for adequate PG (Linskens and Stanley, 1974).

Among other components, sucrose (89% of species), $H_3BO_3$ (77%), $Ca^{2+}$ (59%), $Mg^{2+}$ (44%), and $K^+$ (39%) were the most frequently used while enzymes, vitamins, and amino acids were less common (1, 6, and 1%, respectively); PGM pH values were reported in 35% of all studies reviewed. The high frequency of the former five components in the extracted PGM correspond to other studies on PGM (e.g., Imani, 2012; Jayaprakash, 2018; Wani et al., 2020) and might reflect the wide application of the classic Brewbaker and Kwack PGM (Brewbaker and Kwack, 1963) in pollen research. As for the roles of the most frequent PGM components, sucrose serves as an effective energy source and an osmoticum for PG *in vitro* (Heslop-Harrison and Heslop-Harrison, 1992; Rodriguez-Enriquez et al., 2013; Selinski and Scheibe, 2014; Reinders, 2016). Pollen bursting and failure to germinate *in vitro*

is often associated with inadequate sucrose concentrations (Baloch et al., 2001). The mineral elements boron and calcium have also been found to play several critical regulatory and structural functions in PG. For instance, boron is required for the pollen wall structure, absorption, and metabolism of sugars by forming a sugar–borate complex, and increases oxygen uptake for metabolism (Vasil, 1960; Sidhu and Malik, 1986; Yang and Li, 1999; Wang et al., 2003). Boron deficiency in PGM, often leads to pollen tube bursting or failed PTG (Cheng and Rerkasem, 1993; Fang et al., 2019).

Similarly, calcium is a central regulator, providing various governing roles in the initiation and regulation of PG through ionic balance and cell signaling (Brewbaker and Kwack, 1963; Steinhorst and Kudla, 2013). Calcium in *in vitro* PGM has proved essential in pollen tip growth (Steinhorst and Kudla, 2013) with its deficiency leading to morphological abnormalities such as coiling and tip swelling (Shivanna and Rangaswamy, 1992; Taylor and Hepler, 1997). Other combined roles of boron and calcium (e.g., in sugar synthesis and accumulation) have been emphasized in different studies (Shu-juan, 2010; Muengkaew et al., 2017). Elements such as magnesium and potassium were also frequently used ingredients in PGM because of their role in cellular physiological processes, such as osmotic balance and membrane potential (Čapková-Balatková et al., 1980; Taylor and Hepler, 1997; Song et al., 2009; Wu et al., 2011). They also improve the germination and elongation of pollen tubes by enhancing the calcium effect (Brewbaker and Kwack, 1963). The pH of the *in vitro* germination medium is an important factor controlling PG and pollen tube development in different plant species (Bellani et al., 1997; Munzuroglu et al., 2003; Burke et al., 2004; Zaman, 2011; Fragallah et al., 2019), because it affects physiological processes through enzyme activation or inhibition (Fan, 2001; Bisswanger, 2014). Finally, the comparatively low percentages of PGM components including enzymes, vitamins, and amino acids can be explained by the fact that pollen grains are rich in these components and therefore do not generally require exogenous supply of such substances in the *in vitro* PGM (Campos et al., 2008; Komosinska-Vassev et al., 2015).

*PGM requirements in different plant groups*

The median concentration requirements for the frequently used ingredients varied among the plant groups with different degrees of magnitude. Because data distribution in the majority of the plant groups was skewed, we preferred median values over mean values when discussing the results.

To begin with, successful PG and PTG was observed mainly in a liquid PGM (agar = 0%, Table 1) regardless of the characteristics of the tested species, except for a few cases such as in Poaceae where agar was required for some special needs (see below). The strong dominance of liquid PGM recipes in our compendium could be also explained by high popularity of the "hanging drop" cultivation approach in pollen research (Stanley and Linskens, 1974). Furthermore, pollen of the plant families strongly represented in the compendium (e.g., Solanaceae 10% and Rosaceae 9%), prefer to germinate in liquid medium. Finally, researchers might prefer liquid PGMs over the solid ones because they are slightly more practical (i.e., no need to add agar every time you want to germinate pollen).

Table 1: The median (M), mean (μ) concentrations, standard error (SE) and p-values of the most frequently used ingredients in *in vitro* pollen germination media across the categories.

| Group | n | Agar (%) | | | | Sucrose (%) | | | | $H_3BO_3$ (mM) | | | | $Ca^{2+}$ (mM) | | | | $Mg^{2+}$ (mM) | | | | $K^+$ (mM) | | | | pH | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M | μ | SE± | p | M | μ | SE± | p | M | μ | SE± | p | M | μ | SE± | p | M | μ | SE± | p | M | μ | SE± | p | M | μ | SE± | p |
| **Cultivated** | 449 | 0 | 0.3 | 0.02 | **<0.001** | 10 | 13 | 0.27 | 0.94 | 1.6 | 1.6 | 0.05 | **<0.001** | 1.3 | 1.7 | 0.06 | **<0.001** | 0.8 | 1 | 0.03 | **<0.001** | 1 | 1 | 0.05 | **<0.001** | 6.1 | 6.3 | 0.06 | **<0.001** |
| **Wild** | 689 | 0 | 0.2 | 0.02 | | 12 | 12 | 0.29 | | 1.3 | 1.2 | 0.03 | | 1.8 | 2.7 | 0.09 | | 0.8 | 1 | 0.02 | | 3 | 2.3 | 0.09 | | 5.5 | 6.1 | 0.07 | |
| **Angiosperm** | 1092 | 0 | 0.3 | 0.01 | 0.27 | 12 | 12 | 0.21 | **<0.001** | 1.6 | 1.3 | 0.03 | 0.43 | 1.7 | 2.4 | 0.07 | **0.013** | 0.8 | 1 | 0.02 | **0.005** | 1 | 1.9 | 0.07 | 0.16 | 5.9 | 6.2 | 0.05 | 0.42 |
| **Gymnosperm** | 46 | 0 | 0.3 | 0.07 | | 10 | 8 | 0.71 | | 1.6 | 1.3 | 0.08 | | 1.3 | 1.3 | 0.08 | | 0.8 | 0.8 | 0.1 | | 1 | 1.3 | 0.22 | | 5.8 | 5.9 | 0.15 | |
| **Dicotyledon** | 818 | 0 | 0.2 | 0.01 | 0.27 | 14 | 13 | 0.25 | **<0.001** | 1.6 | 1.4 | 0.04 | 0.43 | 1.8 | 2.5 | 0.08 | **0.01** | 0.8 | 1 | 0.02 | **0.005** | 1.3 | 1.9 | 0.08 | 0.16 | 5.8 | 6.2 | 0.07 | 0.42 |
| **Monocotyledon** | 274 | 0 | 0.4 | 0.03 | | 10 | 12 | 0.38 | | 1.6 | 1.3 | 0.04 | | 1.3 | 2 | 0.11 | | 0.8 | 1 | 0.03 | | 1 | 1.8 | 0.17 | | 6.3 | 6.1 | 0.08 | |
| **Binucleate** | 913 | 0 | 0.3 | 0.01 | 0.16 | 10 | 12 | 0.22 | **<0.001** | 1.6 | 1.4 | 0.03 | 0.23 | 1.7 | 2.4 | 0.07 | **0.01** | 0.8 | 1 | 0.02 | **0.004** | 2.2 | 1.9 | 0.08 | 0.08 | 5.9 | 6.1 | 0.06 | 0.59 |
| **Trinucleate** | 173 | 0 | 0.3 | 0.04 | | 15 | 15 | 0.59 | | 1.6 | 1.3 | 0.05 | | 1.7 | 2.3 | 0.14 | | 1 | 1 | 0.03 | | 1 | 1.7 | 0.21 | | 5.8 | 6.5 | 0.14 | |
| **Dry** | 604 | 0 | 0.3 | 0.02 | 0.2 | 15 | 12 | 0.3 | **<0.001** | 1.6 | 1.3 | 0.04 | 0.43 | 1.8 | 2.6 | 0.09 | **0.004** | 0.8 | 1 | 0.02 | **0.001** | 3 | 2.2 | 0.1 | 0.06 | 5.8 | 6.3 | 0.08 | 0.58 |
| **Wet** | 475 | 0 | 0.3 | 0.02 | | 10 | 12 | 0.29 | | 1.6 | 1.4 | 0.05 | | 1.3 | 2.1 | 0.09 | | 0.8 | 1 | 0.02 | | 1 | 1.6 | 0.1 | | 6.0 | 6.1 | 0.06 | |
| **Starchless** | 268 | 0 | 0.3 | 0.03 | 0.47 | 10 | 12 | 0.41 | 0.19 | 1.6 | 1.3 | 0.05 | 0.99 | 1.3 | 2.2 | 0.13 | 0.92 | 0.8 | 1 | 0.03 | 0.39 | 2.6 | 2 | 0.16 | **0.02** | 6.2 | 6.1 | 0.09 | 0.55 |
| **Starchy** | 86 | 0 | 0.1 | 0.04 | | 10 | 11 | 0.79 | | 1.6 | 1.4 | 0.11 | | 1.8 | 3.1 | 0.25 | | 1 | 1.1 | 0.05 | | 3 | 2.5 | 0.2 | | 5.5 | 6.0 | 0.21 | |

Among other components considered, pollen requirements for $H_3BO_3$ (1.6 mM) and pH (range 5.5–6.3) in PGM were similar across all plant groups, except for species with different cultivation status (CW; Table 1). The former being similar could be attributed to the broad usage of Brewbaker–Kwack protocol in our dataset, whose boric acid concentration has proved to be right in many studies (Chauhan et al., 1987; Kumari et al., 2015; Souza et al., 2017). Correspondingly, boric acid is the least variable component in PGMs, markedly affecting PG and PTG (Stanley, 1971) with small deviations making it either inadequate (Cheng and Rerkasem, 1993; Fang et al., 2019) or toxic (Fang et al., 2016). Similarly, slight, or drastic changes in the pH media can affect the pollen cytoplasmic pH resulting in slow pollen growth or total growth inhibition (Tupý and Řhová, 1984; Fricker et al., 1997; Fan, 2001). This suggests that boron has equal importance in PG and PTG regardless of the pollen anatomy, morphology, and physiology.

As for the remaining most frequently used PGM components, $Ca^{2+}$ and $Mg^{2+}$ were significantly different in all categories except for species with starch vs. starchless pollen (SS). Sucrose concentration was also significantly different in all groups compared, except for SS and CW (Table 1). Notably, although $Mg^{2+}$ was statistically significant in all categories (except SS), the differences in both median and mean values were marginal ($Mg^{2+}$ median concentration range: 0.8–1 mM; Table 1). This can be explained by low data variability in our dataset (Tushabe and Rosbakh, 2021), affected by wide usage of PGM based on the Brewbaker and Kwack PG protocol (Brewbaker and Kwack, 1963). The group-specific difference and possible underlying reasons are discussed in the following paragraphs.

In cultivated plants, the median concentration of $H_3BO_3$ (1.6 mM) and pH (6.1) were significantly higher than in wild plants ($H_3BO_3$ = 1.3 mM and pH = 5.5). In contrast, wild species had significantly higher concentrations of $Ca^{2+}$ (1.8 mM) and $K^+$ (3.0 mM) than the cultivated ones ($Ca^{2+}$ = 1.3 mM, $K^+$ = 1.0 mM; Table 1 and Figure 3). Some studies indicate that pollen of wild species

have lower PGM requirements than the cultivated ones (Jayaprakash, 2018) as was observed in the case of boric acid. However, we have no a plausible explanation for the pH differences or why the mineral concentrations were higher in the wild plants than the cultivated. The differences could be attributed to domestication and artificial selection, which may cause cultivars to change their physiological and morphological attributes thus differing from the wild counterparts (Chaudhary, 2013; Gepts, 2014; Liu et al., 2019). Finally, the experimental design and selection of various conditions for domesticated and wild species by different researchers could as well have contributed to the observed differences.

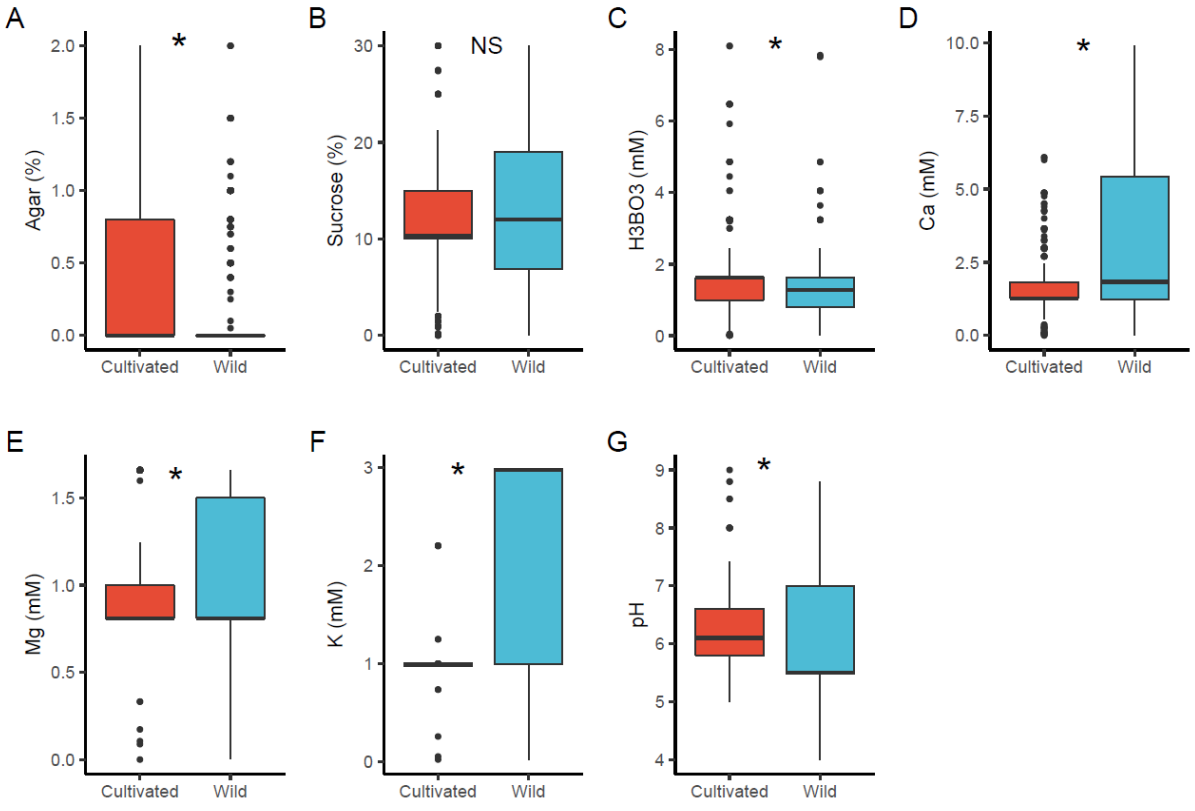


Figure 3: Pollen germination media requirements for cultivated (n = 449) and wild species (n = 689): agar (A), sucrose (B), boric acid (C), calcium (D), magnesium (E), potassium (F), and pH (G). *, significant ($p<0.05$); NS, not significant.

In angiosperms, the median concentrations of sucrose (12%) and $Ca^{2+}$ (1.7 mM) were significantly higher than in gymnosperms (sucrose = 10%, $Ca^{2+}$ = 1.3 mM; Table 1 and Figure 4). These results are in line with the study by Brewbaker and Kwack (1963) that shows that gymnosperm pollen generally require lower concentration of these substances (Brewbaker and Kwack, 1963). The lower calcium requirements can be attributed to the generally comparatively slow growth of germinating gymnosperm pollen (Brewbaker and Kwack, 1963; Williams, 2012). The dissimilarity in the cytology and wall structure between angiosperms and gymnosperms pollen (e.g., Pacini et al., 1999; Fernando et al., 2005) could probably also contribute to the observed differences. Further, pollen cytology plays a significant role in the metabolic processes (Bergamini and Mulcahy, 1988). For instance, pollen grains of most gymnosperms are wind pollinated (Faegri and van der Pijl, 1979) and hence, released with a low moisture content to reduce weight (Fernando et al., 2005). In order for gymnosperm pollen to germinate, rehydration takes place quickly in the liquid medium/pollination drop within the micropylar (Nepi et al., 2005; Firon et al., 2012). This suggests that a higher osmotic potential (lower salt content) is required probably explaining the lower concentrations of sucrose and $Ca^{2+}$ observed in the PGM for gymnosperms. Contrary, angiosperm pollen is released with higher moisture content with rehydration being slower, and grows hurriedly, utilizing the available ingredients quickly (Dawkins and Owens, 1993; Pacini et al., 1999; Nepi et al., 2005; Firon et al., 2012). This probably explains the higher concentration of sucrose and $Ca^{2+}$ in the *in vitro* media.

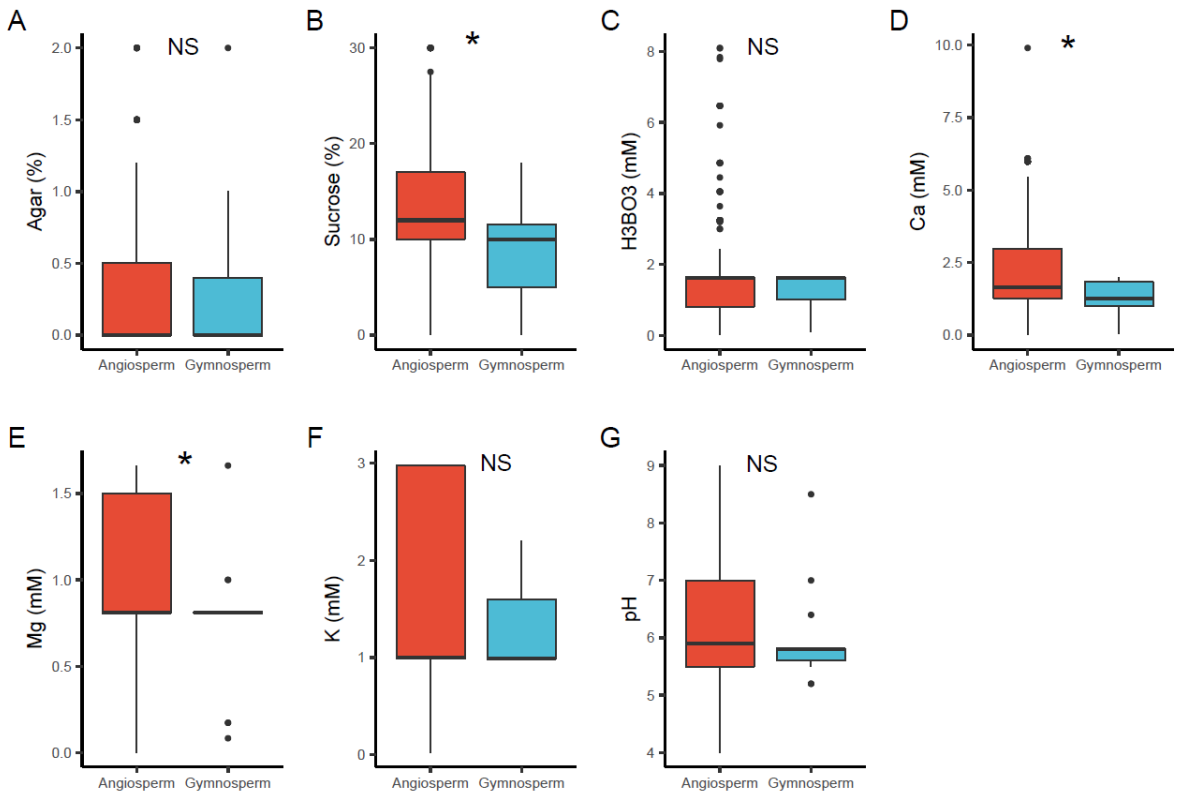


Figure 4: Pollen germination media requirements for angiosperms (n = 1092) and gymnosperms (n = 46): agar (A), sucrose (B), boric acid (C), calcium (D), magnesium (E), potassium (F), and pH (G). *, significant ($p<0.05$); NS, not significant.

In dicots, the median concentrations of sucrose (14%) and $Ca^{2+}$ (1.8 mM) were significantly higher, than in monocots (sucrose = 10%, $Ca^{2+}$ = 1.3 mM; Table 1 and Figure 5). These results are in accordance with previous research showing that monocot pollen require lower mineral content than dicots (Jayaprakash, 2018). A possible explanation is that monocot pollen is usually recalcitrant (i.e., have high moisture content of 30–40%) as compared to orthodox pollen of dicots (1–5%; Franchi et al., 2011; Jayaprakash, 2018). This suggests therefore, that monocot pollen requires lower ion concentrations in a PGM to reach the turgence level needed to germinate. Additionally, pollen of several monocots such as *Typha latifolia* L., are released with high concentration of sugars in it (mainly sucrose) and other protective molecules to confer stability after release, an adaptation against

desiccation (Wolkers et al., 2001; Pacini et al., 2006). They therefore require low concentrations of these in the media. Contrastingly, the largely orthodox dicot pollen are desiccation tolerant and are released with low moisture and mineral content (Wolkers et al., 2001; Pacini et al., 2006; Franchi et al., 2011), hence requiring a higher supply of these substances in *in vitro* media.

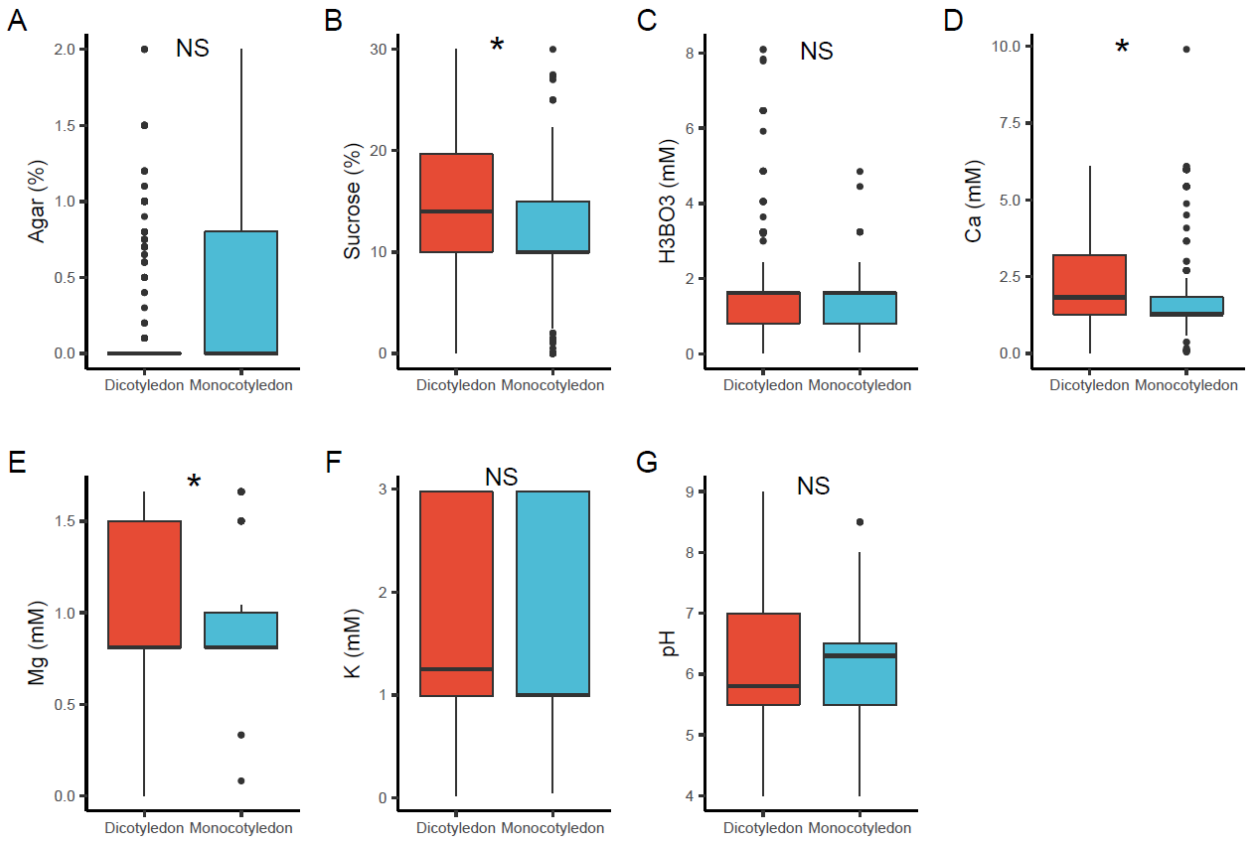


Figure 5: Pollen germination media requirements for dicotyledons (n = 818) and monocotyledons (n = 274): agar (A), sucrose (B), boric acid (C), calcium (D), magnesium (E), potassium (F), and pH (G). *, significant ($p<0.05$); NS, not significant.

In tri-nucleated pollen, requirements for sucrose in PGM were significantly higher than in binucleated pollen (15 vs. 10%, respectively; Table 1 and Figure 6). The component requirements *in vitro* highly depend on the existing endogenous pollen reserves, whether these are sustainable autotrophically and are readily available for metabolism (Read et al., 1993; Stephenson et al., 2003;

Carrizo García et al., 2012). Trinucleated pollen is found to be highly dependent on exogeneous supply of substances important for PG and PTG (Mulcahy and Mulcahy, 1988). This can be associated with tricellular pollen germinating faster, a heterochronic evolutionary shift from bicellular pollen (Brewbaker, 1967; Mulcahy and Mulcahy, 1988), accounting for the higher sucrose requirements observed. In contrast, binucleated pollen grains initially rely on their own reserves when germinating *in vitro*, hence the lower sucrose requirements (Mulcahy and Mulcahy, 1988). Pollen requirements for $Ca^{2+}$ in PGM were also statistically different between tri- vs. bi-nucleated pollen but the median values were similar (1.7 mM; Table 1) as was the case for $Mg^{2+}$.

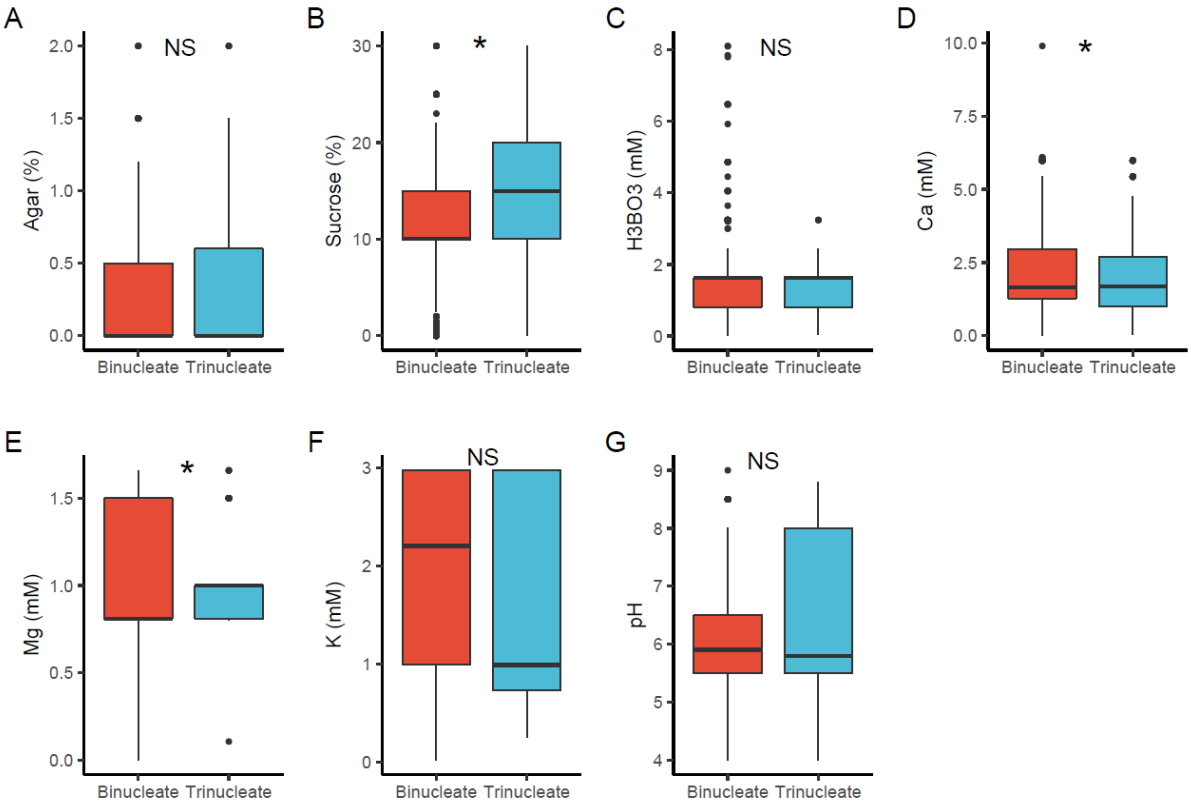


Figure 6: Pollen germination media requirements for binucleate (n = 913) and trinucleate pollen (n = 713): agar (A), sucrose (B), boric acid (C), calcium (D), magnesium (E), potassium (F), and pH (G). *, significant ($p<0.05$); NS, not significant.

In plant with dry stigmas, sucrose (15%) and $Ca^{2+}$ (1.8 mM) requirements were significantly higher than in wet stigma plants (sucrose = 10%, $Ca^{2+}$ = 1.3 mM; Table 1 and Figure 7). Under natural conditions, wet stigmatic plants produce exudates rich in minerals and sugars (Labarca et al., 1970; Hawker et al., 1983; Kandasamy and Vivekanandan, 1983; Lau et al., 2017) in which pollen germinates (Park and Lord, 2003; Allen and Hiscock, 2010; Rejón et al., 2014). This would suggest that pollen in wet stigmatic plants should as well require a higher exogenous supply of these substances *in vitro*. However, this was contrary with our results. A possible explanation is that most dry stigmatic plants tend to have trinucleate pollen with high requirements for exogenous minerals (see also above), whereas wet stigmas are associated with binucleated pollen (lower supply; Brewbaker, 1967; Montaner et al., 2003).

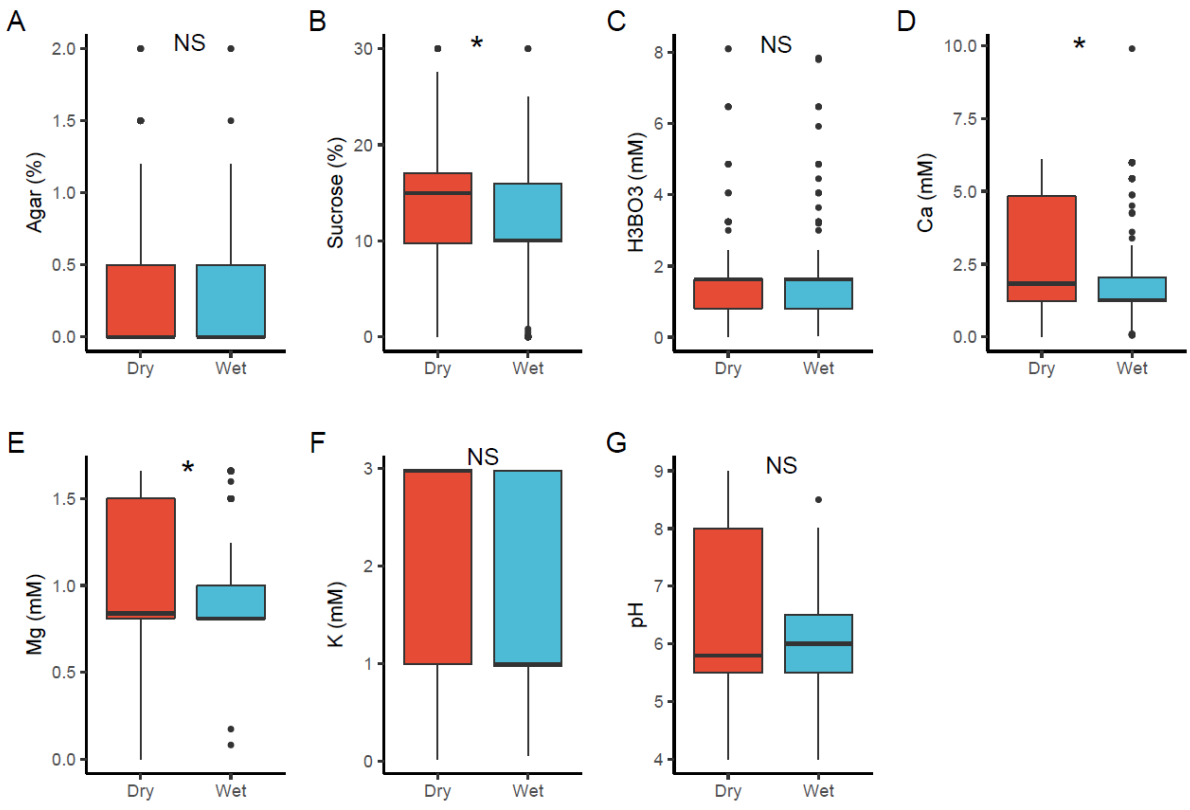


Figure 7: Pollen germination media requirements for dry stigma (n = 604) and wet stigma plants (n = 475): agar (A), sucrose (B), boric acid (C), calcium (D), magnesium (E), potassium (F), and pH (G). *, significant ($p<0.05$); NS, not significant.

In plants with starchy vs. starchless pollen, we detected statistically significant differences for $K^+$ requirements only (Table 1 and Figure 8). The lower requirements of starchless pollen (median 2.6 mM) than in starchy (3.0 mM) can be attributed to the fact that the former are usually associated to binucleated pollen which require lower mineral input than in starchy/trinucleate (Lora et al., 2012). Notably, both pollen types can occur in the same anther (Baker and Baker, 1979; Lora et al., 2012) and yet may need different requirements to germinate *in vitro* (Franchi et al., 2007).

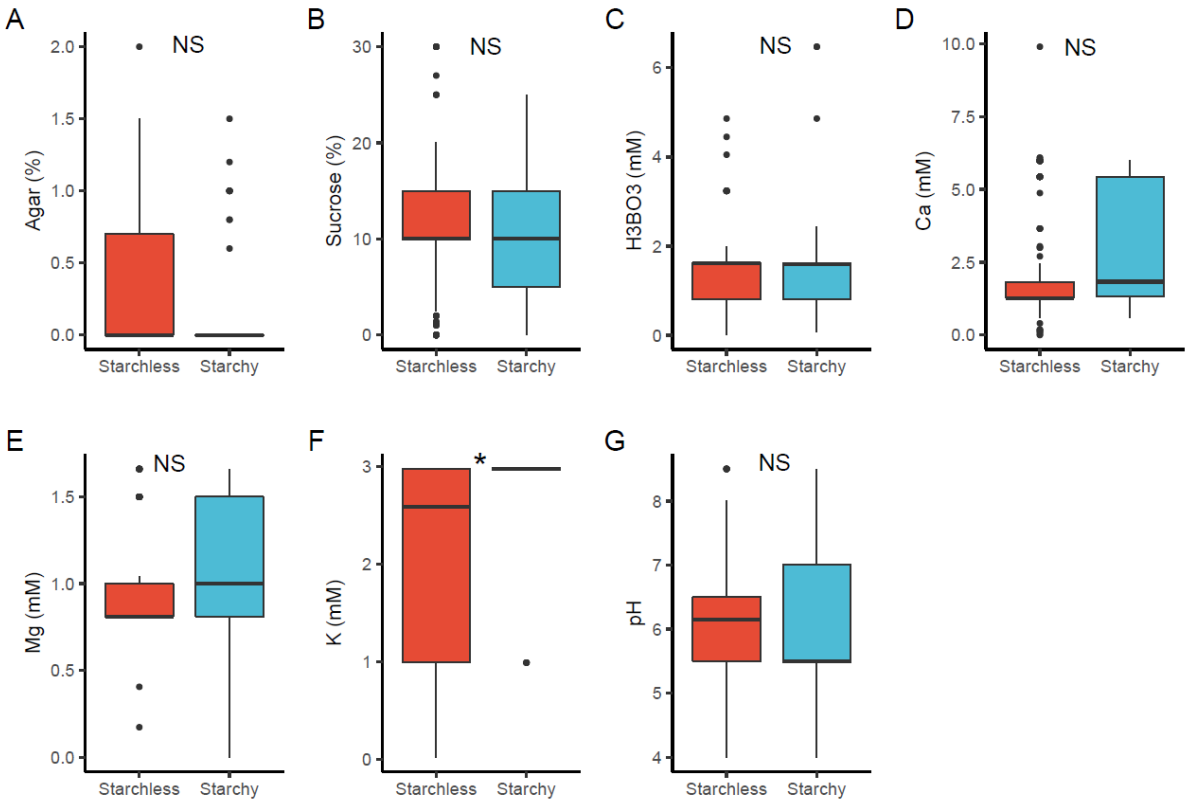


Figure 8: Pollen germination media requirements for starchy (n = 86) and starchless pollen (n = 268): agar (A), sucrose (B), boric acid (C), calcium (D), magnesium (E), potassium (F), and pH (G). *, significant ($p<0.05$); NS, not significant.

As for the groupings by taxonomy, plant families Solanaceae (10%), Rosaceae (9%), Fabaceae (8%), Poaceae and Pinaceae at 5% had the strongest representation in the compendium accounting all

together for 586 PGM recipes for 285 (35%) species. The high contribution from these five families can be explained by the fact that many species from these families are cultivated plants (e.g., Solanaceae: tomato, potato; Rosaceae: almond, apple, pear, cherry; Fabaceae: peas and beans; Poaceae: rice, wheat, barely; Pinaceae: pines). *Nicotiana tabacum* L. (27 recipes), *Zea mays* L. (25), and *Lilium longiflorum* Thunb. (22), which are also economically (*Z. mays*) and scientifically (*N. tabacum* and *L. longiflorum*) important plants, were the most studied species. *N. tabacum* L., the tobacco plant, has also been widely used as a model plant in pollen research, as it produces long-living pollen in large quantities (Cheung et al., 1995; Conze et al., 2017). The medium requirements for the most frequently cultivated species as well as model species such as *A. thaliana* (L.) Heynh. are summarized in the Table 2.

Table 2: The median (M), mean (μ) concentrations, and standard error of the most frequently used ingredients in *in vitro* pollen germination media for the most studied species.

| Species | n | Agar (%) | | | Sucrose (%) | | | $H_3BO_3$ (mM) | | | $Ca^{2+}$ (mM) | | | $Mg^{2+}$ (mM) | | | $K^+$ (mM) | | | pH | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M | μ | SE± | M | μ | SE± | M | μ | SE± | M | μ | SE± | M | μ | SE± | M | μ | SE± | M | μ | SE± |
| ***Arabidopsis thaliana* (L.) Heynh.** | 6 | 0.6 | 0.6 | 0.16 | 18 | 16 | 2.72 | 1.6 | 1.5 | 0.16 | 4 | 2.9 | 0.93 | 1 | 0.7 | 0.3 | 0.6 | 0.6 | 0.37 | 7 | 6.9 | 0.25 |
| ***Lilium longiflorum* Thunb.** | 19 | 0 | 0.1 | 0.07 | 10 | 9 | 1.05 | 0.2 | 0.8 | 0.16 | 1.3 | 1.7 | 0.64 | 0.9 | 0.9 | 0.09 | 1 | 1 | 0 | 5.6 | 5.5 | 0.11 |
| ***Lotus corniculatus* L.** | 11 | 0 | 0.1 | 0.09 | 0 | 6 | 2.6 | 0.8 | 1 | 0.11 | 5.4 | 4.8 | 0.53 | 1.2 | 1.2 | 0.12 | 3 | 2.6 | 0.37 | 5.5 | 5.9 | 0.44 |
| ***Lycopersicon esculentum* Mill.** | 12 | 0 | 0.5 | 0.19 | 12 | 12 | 1.78 | 1 | 1.2 | 0.18 | 1.3 | 1.6 | 0.35 | 0.8 | 1 | 0.21 | - | - | - | 6 | 6.1 | 0.37 |
| ***Nicotiana tabacum* L.** | 23 | 0 | 0 | 0.03 | 10 | 10 | 0.9 | 1.6 | 1.6 | 0.13 | 1.3 | 1.6 | 0.25 | 0.8 | 1 | 0.08 | 1 | 1 | 0 | 6 | 6.3 | 0.14 |
| ***Olea europaea* L.** | 8 | 0.5 | 0.4 | 0.12 | 10 | 12 | 1.38 | 1.6 | 2.5 | 0.71 | 1.5 | 1.5 | 0.28 | 1.2 | 1.2 | 0.43 | 1 | 1 | - | 5.3 | 5.4 | 0.23 |
| ***Petunia hybrida* Vilm.** | 13 | 0 | 0.1 | 0.09 | 10 | 11 | 1.15 | 1.6 | 1.6 | 0.17 | 1.3 | 1.7 | 0.69 | 0.8 | 1.1 | 0.28 | 1 | 1 | - | 6 | 6.0 | 0.07 |
| ***Prunus dulcis* (Mill.) D.A.Webb** | 5 | 1 | 0.6 | 0.23 | 15 | 13 | 1.22 | 1.6 | 1.3 | 0.31 | 1.5 | 1.5 | 0.28 | 0.8 | 0.8 | - | 1 | 1 | - | 5.8 | 5.8 | - |
| ***Zea mays* L.** | 16 | 0.6 | 0.6 | 0.11 | 15 | 15 | 0.51 | 1.6 | 1.3 | 0.15 | 1.8 | 1.8 | 0.14 | - | - | - | - | - | - | 6 | 6.1 | 0.29 |

The PGM requirements for the most used components also tend to be different for the families. Among the frequent families, only Poaceae pollen tends to germinate better and produce pollen tubes on solid medium (0.6% agar; Table 3) with the rest of the families preferring liquid medium. The tendency of Poaceae pollen to germinate in PGM with higher agar concentration can be explained by the dry stigmata in this family. Germinating pollen of plants with dry stigmas has often proved challenging as it is more difficult to imitate the complex interactions on dry stigmatic surfaces (Allen and Hiscock, 2010; Rodriguez-Enriquez et al., 2013). Therefore, the higher agar concentration in PGM of pollen from dry stigmatic plants helps in incorporating the ingredients, maintaining humidity, and adequate aerobic conditions for PG (Linskens and Stanley, 1974). In contrast, the rest of frequent families mostly have wet stigmas with exudates (Allen and Hiscock, 2010) which are easier to imitate *in vitro* and germinate the pollen in liquid media.

Table 3: The median (M), mean (μ) concentrations, and standard error of the most frequently used ingredients in *in vitro* pollen germination media for the most represented families.

| Family | n | Agar (%) | | | Sucrose (%) | | | $H_3BO_3$ (mM) | | | $Ca^{2+}$ (mM) | | | $Mg^{2+}$ (mM) | | | $K^+$ (mM) | | | pH | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M | μ | SE± | M | μ | SE± | M | μ | SE± | M | μ | SE± | M | μ | SE± | M | μ | SE± | M | μ | SE± |
| **Fabaceae** | 90 | 0 | 0.1 | 0.04 | 10 | 12 | 0.94 | 1.6 | 1.3 | 0.07 | 1.8 | 3.1 | 0.23 | 0.8 | 1.1 | 0.05 | 2.6 | 2 | 0.19 | 5.5 | 5.91 | 0.18 |
| **Liliaceae*** | 58 | 0 | 0.2 | 0.05 | 10 | 9 | 0.63 | 1.6 | 1.1 | 0.09 | 1.8 | 2.7 | 0.33 | 1 | 1.2 | 0.09 | 2 | 1.9 | 0.43 | 5.7 | 5.98 | 0.20 |
| **Pinaceae** | 38 | 0 | 0.3 | 0.07 | 9 | 8 | 0.78 | 1.6 | 1.3 | 0.09 | 1.3 | 1.3 | 0.09 | 0.8 | 0.7 | 0.12 | 1 | 1.4 | 0.26 | 5.8 | 5.72 | 0.06 |
| **Poaceae** | 59 | 0.6 | 0.6 | 0.07 | 15 | 16 | 0.99 | 1.6 | 1.3 | 0.1 | 1.5 | 1.8 | 0.17 | 0.9 | 1.1 | 0.17 | 0.7 | 0.7 | 0 | 5.8 | 5.88 | 0.08 |
| **Rosaceae** | 87 | 0 | 0.4 | 0.05 | 10 | 12 | 0.52 | 1.6 | 1.5 | 0.16 | 1.3 | 1.7 | 0.17 | 0.8 | 1 | 0.05 | 1 | 1.6 | 0.37 | 6 | 6.12 | 0.14 |
| **Solanaceae** | 139 | 0 | 0.2 | 0.03 | 15 | 14 | 0.54 | 1 | 1.4 | 0.09 | 1.3 | 1.5 | 0.14 | 0.8 | 1 | 0.06 | 1 | 1.1 | 0.08 | 6 | 6.03 | 0.08 |

*Includes species from other families such as Amaryllidaceae

Furthermore, Poaceae and Solanaceae tend to have comparatively higher sucrose requirements than the other frequent families (15 vs. 9–10%; Table 3). Notably, some Poaceae species such as *Triticum aestivum* L. even require higher concentrations of other sugars such as maltose (18–30%; Tushabe and Rosbakh, 2021). This can be explained by Poaceae having trinucleated pollen that mostly rely on the exogenous mineral supply (also see above). Solanaceae having high sucrose requirements can be attributed to the high data variability in our dataset for this family (sucrose concentration range 10–20%). Generally, pollen anatomy, morphology, and physiology can vary considerably among species (Baker and Baker, 1979; Pacini, 1996; Speranza et al., 1997) contributing to observed differences in the media requirements among families (Table 3).

*Developing PGM recipes*

The detected (dis)similarities in pollen requirements for the most frequently used PGM components can be used for creating a new PGM where no ready recipe is available in our data set. Below, we suggest a number of "base" recipes for various plant groups:

- Angiosperms: liquid media, 12% sucrose, 1.6 mM $H_3BO_3$, 1.7 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 1.0 mM $K^+$, and pH 5.9
- Gymnosperms: liquid media, 10% sucrose, 1.6 mM $H_3BO_3$, 1.3 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 1.0 mM $K^+$, and pH 5.8
- Dicots: liquid media, 14% sucrose, 1.6 mM $H_3BO_3$, 1.8 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 1.3 mM $K^+$, and pH 5.8
- Monocots: liquid media, 10% sucrose, 1.6 mM $H_3BO_3$, 1.3 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 1.0 mM $K^+$, and pH 6.3
- Binucleate pollen: liquid media, 10% sucrose, 1.6 mM $H_3BO_3$, 1.7 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 2.2 mM $K^+$, and pH 5.9

• Trinucleate pollen: liquid media, 15% sucrose, 1.6 mM $H_3BO_3$, 1.7 mM $Ca^{2+}$, 1.0 mM $Mg^{2+}$, 1.0 mM $K^+$, and pH 5.8

• Dry stigmatic plants: liquid media, 15% sucrose, 1.6 mM $H_3BO_3$, 1.8 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 3.0 mM $K^+$, and pH 5.8

• Wet stigmatic plants: liquid media, 10% sucrose, 1.6 mM $H_3BO_3$, 1.3 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 1.0 mM $K^+$, and pH 6.0

• Starchless pollen: liquid media, 10% sucrose, 1.6 mM $H_3BO_3$, 1.3 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 2.6 mM $K^+$, and pH 6.2

• Starchy pollen: liquid media, 10% sucrose, 1.6 mM $H_3BO_3$, 1.8 mM $Ca^{2+}$, 1.0 mM $Mg^{2+}$, 3.0 mM $K^+$, and pH 5.5.

For some frequent families, the following from our data set can be used as a guide in creating new PGM:

• Fabaceae: liquid media, 10% sucrose, 1.6 mM $H_3BO_3$, 1.8 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 2.6 mM $K^+$, and pH 5.5

• Pinaceae: liquid media, 9% sucrose, 1.6 mM $H_3BO_3$, 1.3 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 1.0 mM $K^+$, and pH 5.8

• Poaceae: 0.6% agar, 15% sucrose, 1.6 mM $H_3BO_3$, 1.5 mM $Ca^{2+}$, 0.9 mM $Mg^{2+}$, 0.7 mM $K^+$, and pH 5.8

• Rosaceae: liquid media, 10% sucrose, 1.6 mM $H_3BO_3$, 1.3 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 1.0 mM $K^+$, and pH 6

• Solanaceae: liquid media, 15% sucrose, 1.0 mM $H_3BO_3$, 1.3 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$, 1.0 mM $K^+$, and pH 6.

# Chapter 3: Patterns and drivers of pollen temperature tolerance

## What drives pollen tolerance to low and high temperatures?

**Abstract**

Pollen, a pivotal stage in the plant reproductive cycle, is highly sensitive to temperature fluctuations, impacting seed quality and quantity. While the importance of understanding pollen temperature limits (*Tmin*, *Topt*, *Tmax* – collectively PTLs) is recognized, a comprehensive synthesis of underlying drivers is lacking.

Here, we examined PTLs, correlating them with vegetative tissue thermotolerance and assessing variability at the intra- and interspecific levels across 198 species with contrasting phylogeny, cultivation history, biology, and ecology.

At the species level, the PTLs range from 6.1 to 39.5 ºC, with considerable differences among growth forms and cultivation histories. Positive correlations were found between PTLs and leaf/stem temperature tolerances. Notably, pollen cold tolerances varied significantly across species populations, while *Topt* and *Tmax* values remained stable. Phylogenetic analysis revealed family-level conservation in pollen cold tolerance, contrasting with heat tolerance's independence from evolutionary history.

Climate emerged as a significant PTL driver, with species at higher elevations and latitudes exhibiting enhanced cold and heat tolerance. Cultivated species displayed narrower temperature tolerances (10.3-39.9 °C) than their wild counterparts (5.2-42.3 °C), highlighting potential crop vulnerabilities to global warming. Herbaceous plants exhibited superior tolerance to both low and high temperatures compared to shrubs and trees, reflecting divergent thermal conditions during anthesis.

This study illuminates complex relationships between pollen temperature limits, plant characteristics, and environmental factors, providing crucial insights into climate change impacts on plant reproduction.

Keywords: climate change, cold, crop, heat, limit, pollen, temperature, tolerance, reproduction, seed

## Introduction

Globally, there is a growing concern in the scientific community about the adverse impacts of climate change on the reproduction processes of plants (Hedhly et al., 2009; Fahad et al., 2017; Piao et al., 2019). Climate change-driven extremes in temperature, such as cold spells and heatwaves occurring during critical plant developmental stages related to seed production, consistently lead to diminished seed quality and quantity (Hatfield and Prueger, 2015; Raza et al., 2019; Yadav et al., 2022). Consequently, the altered rates of viable seed production are anticipated to exert profound ecological impacts on plant population dynamics, with potential implications for species demography and long-term survival. For instance, in cases where population growth hinges on seed availability, persistently low seed production may lead to a decline in species abundance and even the eventual extinction of certain plant species (Turnbull et al., 2000; Willis et al., 2008). Moreover, fluctuations in fruit and seed production in wild plants can ripple through ecosystems, impacting various trophic levels and engaging in intricate interactions with numerous animal species. Reduced plant reproduction can affect birds and insects that rely on seeds and fruits as food sources, with downstream consequences for mammals and the prevalence of human pathogens (Lewis et al., 2014; Bogdziewicz et al., 2016). Lastly, climate-induced alterations in the reproductive performance of cultivated plants may have detrimental implications for food security, potentially resulting in significant reductions in crop yields in the coming years (Ray et al., 2019; Caparas et al., 2021).

While temperature exerts control over all aspects of seed production (Slafer et al., 2015), pollen, the male gametophyte, emerges as the most temperature-sensitive within the plant reproductive cycle when compared to other tissues and developmental stages (Sharkey and Schrader, 2006; Zinn et al., 2010; Hedhly, 2011; Prasad et al., 2017). The enhanced pollen sensitivity, as opposed to ovules, is attributed to several factors, including its comparatively small size, haploid set of chromosomes, lack of protective tissue, and direct exposure to the environment (Bedinger, 1992; Pacini and Dolferus, 2016). Consequently, an array of experimental studies has demonstrated that even mild temperature stress applied at various stages of pollen development (anther wall development, microsporogenesis, microgametogenesis, pollen germination [PG] and pollen tube growth [PTG]) results in a substantial decline in pollen performance, often yielding irreversible effects (Kakani et al., 2002; Sato et al., 2002; Raja et al., 2019; Tushabe et al., 2023). These findings collectively underscore that pollen sensitivity to both low and high temperatures (‘cold and heat tolerance’) is a pivotal limiting factor in seed productivity and is particularly susceptible to the effects of global climate change (Hedhly et al., 2009; Eckert et al., 2010; Hassan et al., 2021).

Understanding pollen temperature tolerance is a subject of considerable interest among plant scientists spanning various disciplines (Kakani et al., 2005; Mesihovic et al., 2016; Rosbakh et al., 2018; Djanaguiraman et al., 2019). This interest is particularly pronounced in the context of ongoing climate change, as it holds the potential to shape the future of both wild plant conservation and agricultural productivity. However, despite the pivotal role of this trait in plant reproductive processes, our knowledge regarding the critical temperature thresholds of pollen remains relatively limited. Equally elusive is our understanding of the extent of variability in these thresholds and the underlying factors contributing to such variability. The existing body of literature on pollen thermal limits predominantly concentrates on either a select few cultivars of a single crop species (e.g., Kakani et al., 2002; Coast et al., 2016; Paupière et al., 2017) or a limited assortment of wild species

confined to specific environments (e.g., Rosbakh and Poschlod, 2016; Wagner et al., 2016). This focused approach, although valuable in its own regard, presents challenges for researchers seeking to draw any general conclusions on the adaptability and susceptibility of pollen temperature tolerances to changing environmental conditions, both in space and time.

Here we bridge this knowledge gap by examining patterns and drivers of pollen cold and heat tolerance across multiple populations and species of wild and cultivated plants occurring worldwide. To accomplish this, we harness a distinctive dataset (Appendix 1, Table 1) encompassing crucial temperature parameters for pollen germination (PG) and pollen tube growth (PGT) – including minimum, optimal, and maximum temperatures – for 198 species measured in 412 populations and/or cultivars with >500 000 georeferenced species occurrences covering all world's biomes. Our first objective is to delve into the comprehensive temperature limits governing pollen performance and explore their potential correlations with the thermotolerance observed in vegetative tissues *(aim 1).* While it has been often suggested that all stages of plant sexual regeneration function within a narrower range compared to leaves, stems, and roots (Luo, 2011; Nievola et al., 2017), this assumption has not been empirically examined until now.

Next, we explore the presence and extent of intraspecific variability in pollen temperature tolerance *(aim 2)*. Previous research has shown that plants tend to adapt their pollen performance to the local growing conditions, with example of populations from colder regions exhibiting better pollen cold tolerance as opposed to their counterparts from warmer habitats (Morrison et al., 2016; Ranasinghe et al., 2018; Zebro et al., 2023). However, these investigations have typically centred on individual species or focused solely on specific temperature thresholds, such as minimum or maximum temperatures. Consequently, we are still lacking a comprehensive understanding of the adaptability and plasticity of pollen performance in response to changing thermal conditions, both temporally and spatially.

In the next part of the analysis, we tackled drivers of pollen thermal limits at the species (interspecific) level. First, we tested for presence of phylogenetic signal in the pollen temperature tolerance data *(aim 3)*. The rationale for this analysis stems from the well-established understanding that closely related taxa often retain ecological traits and environmental preferences of their ancestors (Crisp et al., 2009; Burns and Strauss, 2011; Kamilar and Cooper, 2013; Liu et al., 2015). Accordingly, we anticipated that pollen thermal limits would exhibit a certain degree of phylogenetic conservation. Subsequently, we assessed whether interspecific variation in pollen tolerance to both low and high temperatures could be attributed to specific temperature extremes encountered in the locations where these plants grow *(aim 4)*. Building upon insights from prior research (Rosbakh and Poschlod, 2016; Zhu et al., 2018; Lancaster and Humphreys, 2020; Sentinella et al., 2020), we posited that pollen, inhabiting climates characterized by substantial temperature fluctuations – such as high-latitude regions and areas with continental climates – might have evolved enhanced temperature tolerance mechanisms. These adaptations would enable them to cope with short- and long-term temperature shifts, including frost events, heatwaves, and seasonal temperature variations. We conducted these assessments separately for wild and cultivated plants, as the adaptability to local climate conditions can differ due to natural selection pressures for wild plants and selective breeding in cultivated varieties (Lippmann et al., 2019).

We further considered differences in pollen thermal limits response to latitudinal gradients in plants with different growth forms, as relatively short herbs tend to experience warmer thermal environments compared to tall trees. This difference arises from the ground-level radiative heating experienced by herbs in the upper air layer, in contrast to tall trees, which have a thermal coupling with the ambient atmosphere (Geiger et al., 2009; Treml et al., 2019). Finally, we examined the impact of elevation on pollen temperature tolerance, recognizing that plants thriving in uplands may

have developed heightened cold and high thermotolerance strategies to thrive in the unique conditions at these elevations (Rosbakh and Poschlod, 2016; Körner, 2022).

## Materials and methods

### *Data collection*

In this study, we define pollen tolerance to low and high temperatures as temperatures, falling below or exceeding, respectively, which causes stress that affects pollen morphological, physiological, biochemical, and molecular properties and ultimately its performance (Wahid et al., 2007; Bewley and Black, 2013; Hasanuzzaman et al., 2013; Liu et al., 2023). Moreover, we investigate the optimal temperature range, wherein pollen exhibits its peak performance. This ideal range corresponds to the conditions fostering the highest proportion of germinated pollen grains and the fastest pollen tube growth. Pollen temperature tolerances are frequently assessed through three key temperatures, which are *Tmin* (the minimum), *Topt* (the optimum), and *Tmax* (the maximum). These parameters are essential for understanding pollen germination (PG) and pollen tube growth (PTG), as highlighted in studies such as Kakani et al. (2005) and Rosbakh and Poschlod (2016). *Tmin* and *Tmax* represent the temperature extremes at which neither pollen grains can germinate, nor pollen tubes can grow. In contrast, *Topt* is the temperature range wherein a species' pollen grains exhibit their highest germination rates, and pollen tube growth is maximized in terms of length (Figure 9).

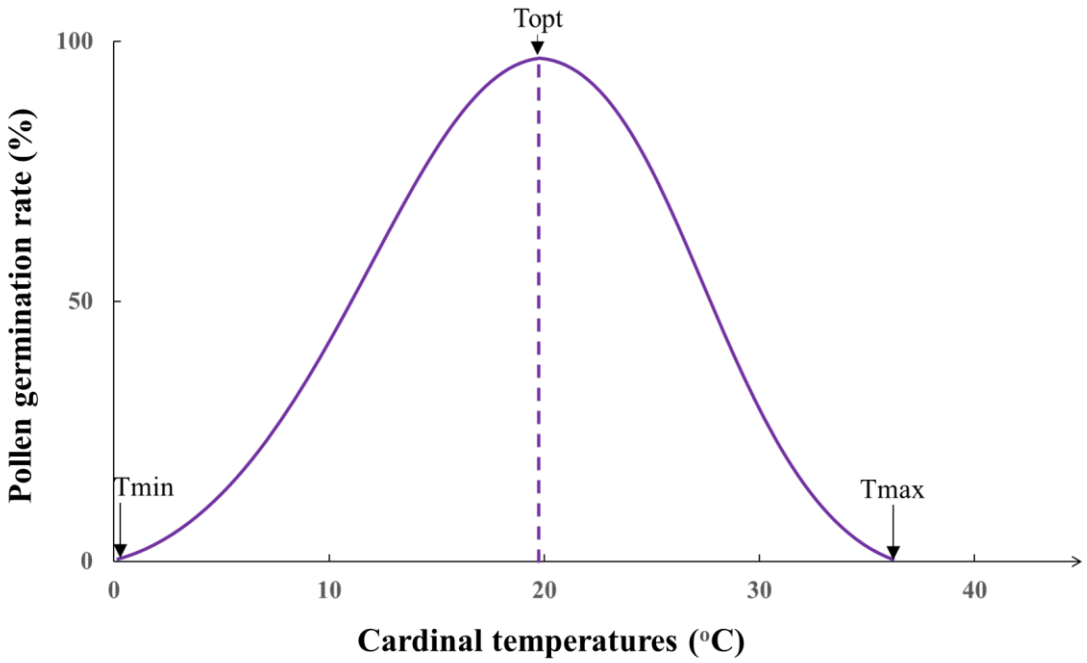


Figure 9: Cardinal temperatures for pollen germination; *Tmin* (minimum), *Topt* (optimum), and *Tmax* (maximum).

To compile the dataset on pollen thermotolerance (Appendix 1, Table 1), we first extracted available information on cardinal temperatures for PG and PTG by reviewing all studies published from 1933 to 2020 via search in the Web of Science database with the keywords 'pollen', 'germination', and 'temperature'. The search resulted in a total of 1268 studies, out of which 98 contained information on the PG and PTG cardinal temperatures that were subsequently extracted. From each selected publication, we extracted data on study species and their cultivars (if applicable), the cardinal temperatures for PG and/or PTG, whether pollen cultivation was conducted *in vivo* or *in vitro*, the range of test temperatures (e.g., 0-40 or 20-35 °C) and the statistical modelling technique employed to derive temperature threshold estimates (such as linear, bilinear, or quadratic models). In cases where the temperatures were given as a range, the average values of these ranges were used.

To enhance the dataset, we integrated additional experimental data concerning the cardinal temperatures for pollen tube growth (PTG) from a set of 91 Central European plant species collected at the University of Regensburg, Germany, in 2014-2020 (S. Rosbakh, unpublished); the detailed information on pollen cultivation is given in Rosbakh and Poschlod (2016). The species taxonomy was harmonized against the Plant List (The Plant List, 2013) with the R package *taxonstand* (Cayuela et al., 2021). In total, the consolidated dataset encompasses 641 entries for 198 species from 128 genera and 56 distinct families. Due to the strong and statistically significant correlations observed between the cardinal temperatures for pollen germination (PG) and pollen tube growth (PTG) at both the intra- and interspecific levels, we adopted a pragmatic approach in our subsequent analysis. Specifically, we utilized the arithmetic means of the respective cardinal temperatures – *Tmin*, *Topt*, and *Tmax* – to represent overall pollen performance. In instances where cardinal temperatures were available for either PG or PTG but not both, we relied on the available values to define pollen thermal limits.

Each species was then characterized in terms of its distribution (in terms of latitude, longitude, and elevation), cultivation status (wild or cultivated) and growth form (herbaceous vs. woody). To characterize species position along the gradients of latitude, longitude, and elevation, we extracted geographic coordinates from the Global Biodiversity Information Facility (GBIF) using the package *rgbif* (Chamberlain et al., 2023) in R software v.4.3.0 (R Core Team, 2023). Only species with georeferenced locations obtained from known herbarium vouchers were considered in the analysis. Elevation data were extracted data from the WorldClim database (Fick and Hijmans, 2017).

To accomplish our first objective of comparing temperature tolerance between pollen grains and vegetative organs, we conducted an additional search to identify studies reporting the lethal minimal and maximal thermal limits for leaves, stems, and whole plants of species for which we had available pollen temperature tolerance data. Optimal thermal limits of vegetative tissues have been rarely

studied and thus were omitted from the analysis. This targeted search was executed in the Web of Science database, utilizing keywords ‘vegetative’, ‘thermotolerance’ and the corresponding species name. This search yielded a dataset consisting of 13 species with minimal thermal limits and 17 species with maximal thermal limits for vegetative organs (almost exclusively leaves), all of which were concurrently represented in our pollen temperature tolerance dataset.

*Data analysis*

All statistical analyses were performed using R software version 4.3.0 (R Core Team, 2023).

*Relationship between the pollen and vegetative organs’ temperature tolerance (aim 1)*

To examine the relationship between temperature tolerance in pollen and vegetative organs, we fitted a linear mixed-effect model with family included as a random effect (to account for potential phylogenetic autocorrelation).

*Intraspecific pollen temperature tolerance variability (aim 2)*

To explore intraspecific variability in pollen temperature tolerance, we focused on eleven species from our dataset that had data available for more than five distinct populations. Importantly, all these species were cultivated (Table 4; most of the wild species in the dataset were represented by only a single study population), and these populations effectively correspond to cultivars. To visualize the pollen thermal limits variability within the selected species, we used boxplots integrated into violin plot. Additionally, to estimate the degree of intraspecific variation in pollen *Tmin*, *Topt* and *Tmax*, we calculated coefficient of variation (CV) for each temperature for each of the species. A linear model in combination with post-hoc Tukey test was used to test for the differences in CVs among the pollen temperature tolerance. The CVs values were log-transformed, to improve the normality of residuals; all model requirements were met.

*Phylogenetic signal in pollen temperature tolerance (aim 3)*

Pollen temperature tolerance data were then plotted on the species' phylogeny using the package *phytools* (Revell, 2012). The phylogenetic tree for the study species was compiled using the package *V.PhyloMaker* (Jin and Qian, 2019).

To test, whether the pollen thermal limits were phylogenetically constrained, we first calculated Blomberg's K-statistics, Brownian motion-based metric of the strength of phylogenetic signal (Blomberg et al., 2003), using the phylosignal function in the *picante* library (Kembel et al., 2010). $K = 1$ indicates that closely related species have trait values that are similar to those expected given Brownian motion; $K < 1$ indicates that closely related species have trait values that are less similar than expected given a Brownian model of evolution. Additionally, we run Moran's I test for *Tmin* (the only trait with significant phylogenetic signal, see below), an alternative estimate of phylogenetic signal indicating how phylogenetic signature changes across the phylogeny (Gittleman and Kot, 1990). The resulting values of this analysis do not offer any quantitative interpretation of the phylogenetic signal, because the expected value of the statistic under the assumed model is unknown *a priori*. However, stronger deviations from zero indicate stronger relationships between trait values and the phylogeny (Münkemüller et al., 2012). The phylogenetic autocorrelation in the data was estimated at three taxonomic levels: family, class, and order.

*Interspecific trait variation (aim 4)*

To estimate the variability in pollen low and high temperature tolerance variability at the species level, we fitted three linear mixed effects with one of temperatures (*Tmin*, *Topt* or *Tmax*) being the response variable in the corresponding models. The model predictors (i.e., fixed effects) were latitude, longitude (used as an absolute value), elevation, cultivation status and growth form. To account for phylogenetic signal (see above), family was included in all models as a random factor.

The models were fitted using the packages *lme4* (Bates et al., 2015), *lmertest* (Kuznetsova et al., 2017), and *mumin* (Bartoń, 2023). Differences in pollen temperature tolerances among plant groups with different characteristics (e.g., cultivation status and pollination mode) were estimated with the help of the post hoc Tukey test ($p<0.05$), implemented in the packages *emmeans* and *multcomp* (Hothorn et al., 2008; Lenth, 2023). All numeric variables were scaled prior to the analysis. The model assumptions were met in all the cases. The model outputs were visualized using the package *interactions* (Long, 2019).

**Results**

*Temperature limits of pollen temperature tolerance*

Of the 641 entries in the dataset, representing 198 species from 128 genera and 56 families, the most common family was Fabaceae (21%) and *Glycine max* (L.) Merr. the most studied species (14%). In general, studies on cultivated species (78%) were more frequent than those on wild species (22%).

The pollen cold tolerance (*Tmin*) ranged from -5 ºC in early-flowering, dwarf shrub *Polygala chamaebuxus* occurring on calcareous soils in temperate climate to 18.2 ºC in *Prunus arabica*, a tree cultivated in Mediterranean climate, with an average of 6.1 ºC. The 5$^{th}$ and 95$^{th}$ percentiles of the *Tmin* values distribution ranged from 0 to 15.1 ºC (Figure 10).

On average, the optimal temperature of pollen performance (*Topt*) was 24.8 ºC, ranging from 11.3 ºC in *Prunus armeniaca* (apricot; a fruit tree cultivated in continental climates) to 35.0 ºC in *Theobroma cacao* (cacao tree) grown mainly in (sub)tropical climates. The 5$^{th}$ and 95$^{th}$ percentiles of the *Topt* values distribution ranged from 17.1 to 32 ºC (Figure 10).

The pollen heat performance ranged from 30 ºC in *Betula pendula*, a wild tree common in temperate and boreal climates with early flowering phenology, to 50 ºC in *Campanula patula* and *Fragaria vesca*, two typical grassland species occurring across a wide range of climates. The 5$^{th}$ and 95$^{th}$

percentiles of the *Tmax* values distribution ranged from 33.6 to 45.4 ºC (Figure 10). Averaged over all species, *Tmax* value was 39.5 ºC.

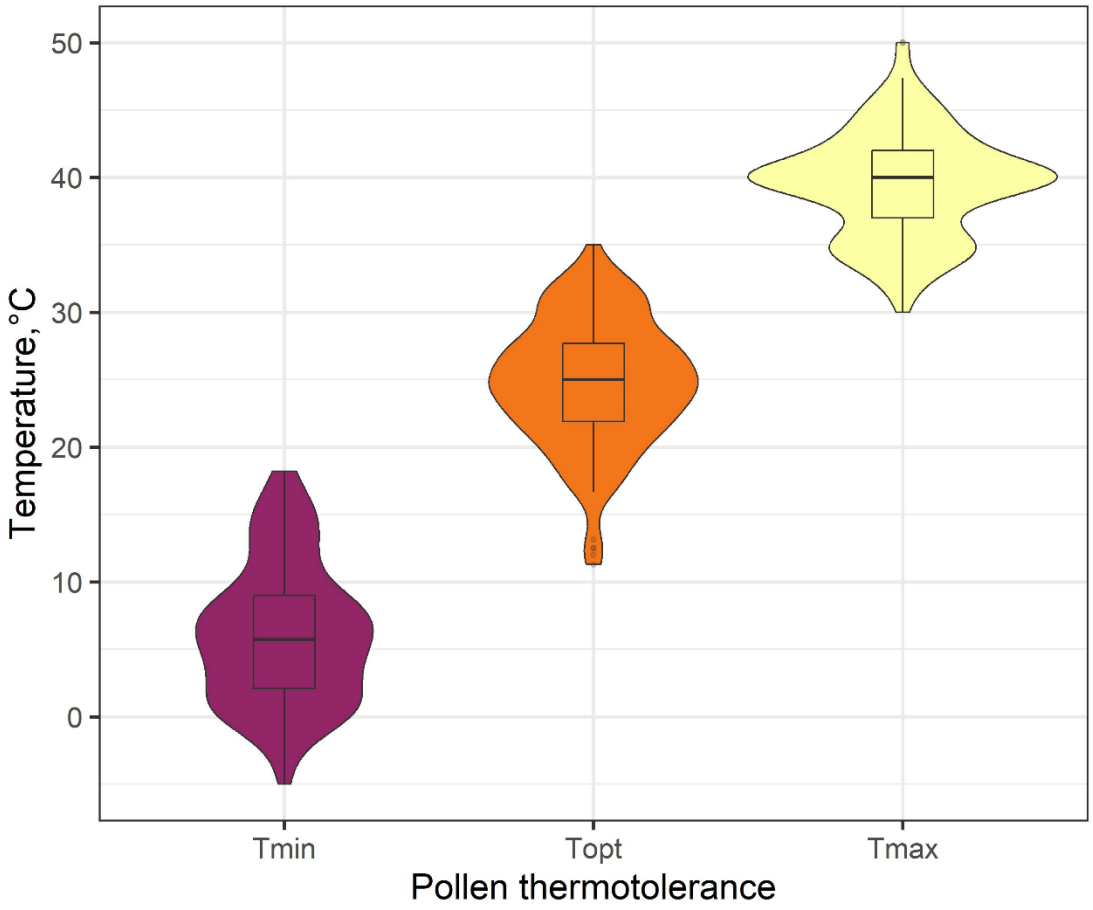


Figure 10: Average pollen thermal limits for 198 species in the dataset. *Tmin*, *Topt* and *Tmax* are minimal, optimal and maximal temperatures of pollen performance.

*Relationship between the pollen and vegetative organs' temperature tolerance (aim 1)*

The linear models revealed that pollen and vegetative organ tolerance to low temperatures were significantly, strongly, and positively correlated with each other (Figure 11A, $r^2 = 0.55$, $p = 0.003$, $n = 13$). A similar pattern was detected for high temperature tolerances, although this relationship was weaker (Figure 11B, $r^2 = 0.25$, $p = 0.03$, $n = 17$).

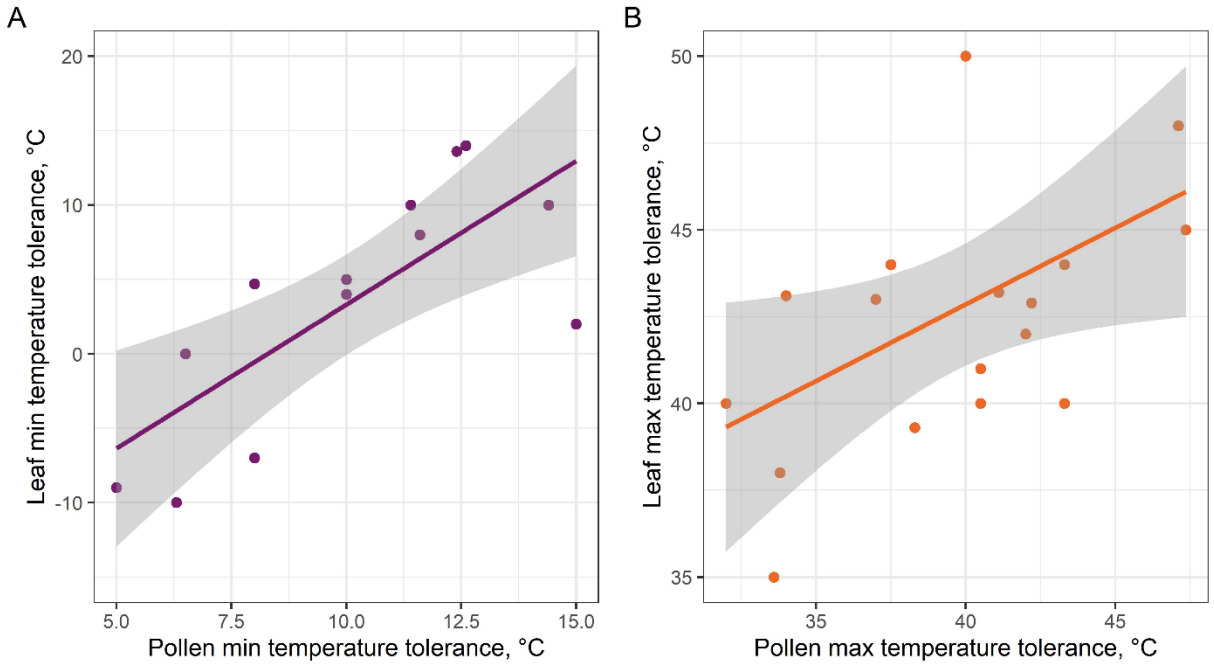


Figure 11: Correlation between (A) minimal and (B) maximal pollen and vegetative organ temperature tolerance.

*Intraspecific variability in pollen temperature tolerance (aim 2)*

Eleven species with data for more than five cultivars were tested for intraspecific variability in pollen thermal limits (Table 4; Figure 12). Averaged over all species and temperatures, coefficient of variation (CV) ranged from 0.01 (*Tmax* in *Brassica napus* and *Capsicum annuum*) to 0.47 (*Tmin* in *Sorghum bicolor*). The linear model revealed that degree of intraspecific variability, as deduced from the CVs, was significantly larger ($p<0.001$) in *Tmin* (0.22) compared to *Topt* (0.06) and *Tmax* (0.04).

Table 4: Intraspecific trait variation in pollen temperature limits in eleven species. *Tmin*, *Topt* and *Tmax* are minimal, optimal and maximal pollen tolerance temperatures; CV – coefficient of variation.

| **Species** | **Characteristic** | **n** | ***Tmin*** | | ***Topt*** | | ***Tmax*** | |
|---|---|---|---|---|---|---|---|---|
| | | | Mean | CV | Mean | CV | Mean | CV |
| *Arachis hypogaea* (Groundnut) | A legume cultivated in (sub)tropical climates | 22 | 14.4 | 0.10 | 32.2 | 0.06 | 43.3 | 0.06 |
| *Brassica napus* (Rapeseed or canola) | An oil seed crop mainly grown in temperate climates | 12 | 6.5 | 0.23 | 24.3 | 0.03 | 33.1 | 0.01 |
| *Capsicum annuum* (Bell or chili pepper) | A vegetable typically grown in warm and temperate regions | 14 | 12.4 | 0.10 | 28.1 | 0.07 | 41.1 | 0.01 |
| *Cocos nucifera* (Coconut) | A fruit commonly cultivated in tropical regions | 19 | 13.8 | 0.17 | 27.4 | 0.05 | 41.3 | 0.05 |
| *Glycine max* (Soybean) | A legume grown in diverse climates | 92 | 10.7 | 0.23 | 30.4 | 0.10 | 47.1 | 0.05 |
| *Gossypium hirsutum* (Upland cotton) | A fiber crop primarily grown in (sub)tropical climates | 26 | 12.6 | 0.10 | 28.6 | 0.06 | 43.2 | 0.03 |
| *Juglans regia* (Walnut) | A nut that thrives best in temperate regions | 10 | 13.7 | 0.08 | 30.0 | 0.06 | 40.5 | 0.03 |
| *Pennisetum glaucum* (Pearl millet) | A cereal well-adapted to hot and dry climates | 28 | 9.4 | 0.24 | 28.3 | 0.07 | 47.3 | 0.05 |
| *Pistacia vera* (Pistachio) | A nut primarily cultivated in mediterranean climate | 5 | 6.1 | 0.25 | 23.9 | 0.07 | 40.8 | 0.03 |
| *Saintpaulia ionantha* (African violet) | An ornamental plant cultivated in tropical climates | 30 | 4.6 | 0.43 | 25.4 | 0.04 | 46.5 | 0.07 |
| *Sorghum bicolor* (Sorghum) | A cereal cultivated in (sub)tropical regions | 15 | 11.4 | 0.47 | 28.8 | 0.04 | 42.9 | 0.04 |
| | | **Mean** | | 0.22 | | 0.06 | | 0.04 |

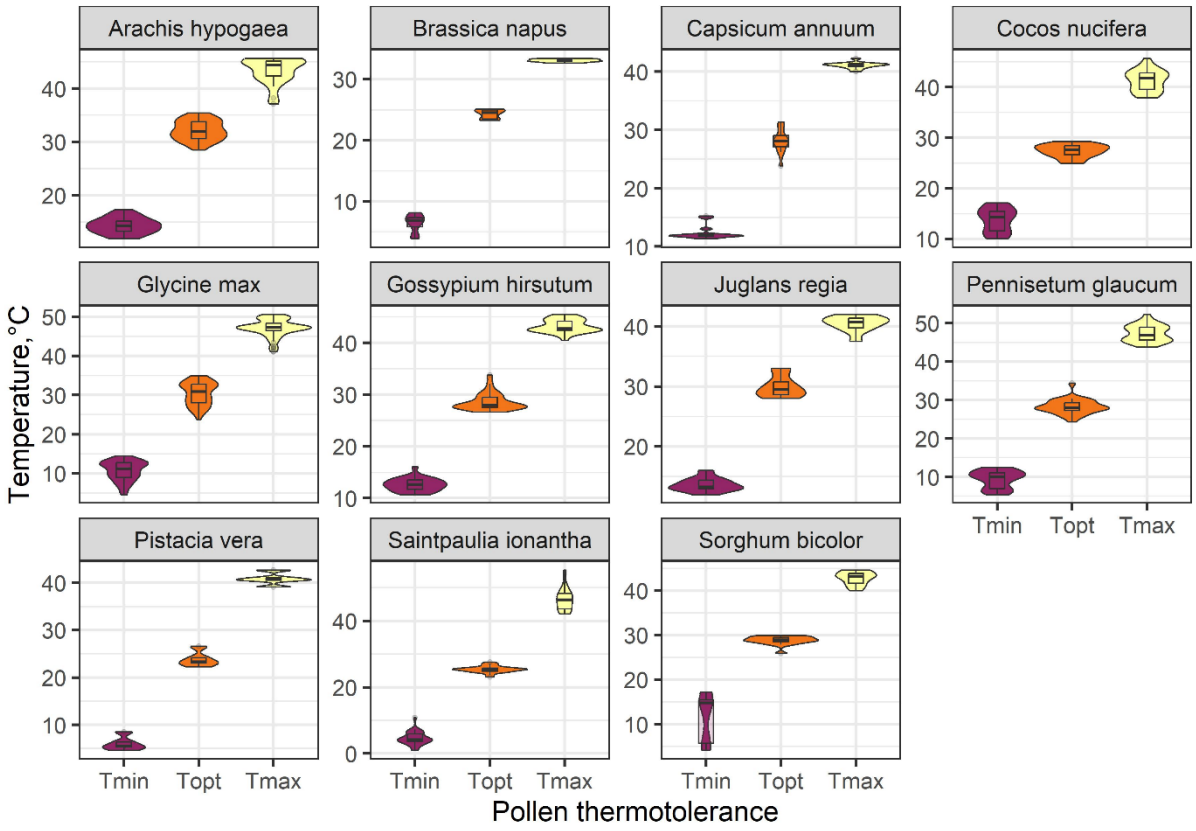


Figure 12: Intraspecific variation in pollen tolerance to low and high temperatures in eleven species.

*Phylogenetic signal in pollen temperature tolerance (aim 3)*

The distribution of pollen thermal limits across the phylogenetic tree is shown in Figure 13. Blomberg's K indicated a weak but significant phylogenetic signal in low-temperature pollen tolerance (K = 0.07, p = 0.03, n = 143). The Moran's I test revealed that the plants from the same family tended to share similar low-temperature tolerance (family: I = 0.29, p<0.001: order: I = -0.02, p = 0.89; class: I = -0.02, p = 0.42).

In contrast, the *Topt* and *Tmax* were randomly distributed across the phylogenetic tree as indicated by non-significant results of the K-statistics (*Topt*: K = 0.07, p = 0.18, n = 172; *Tmax*: K = 0.07, p = 0.16, n = 136).

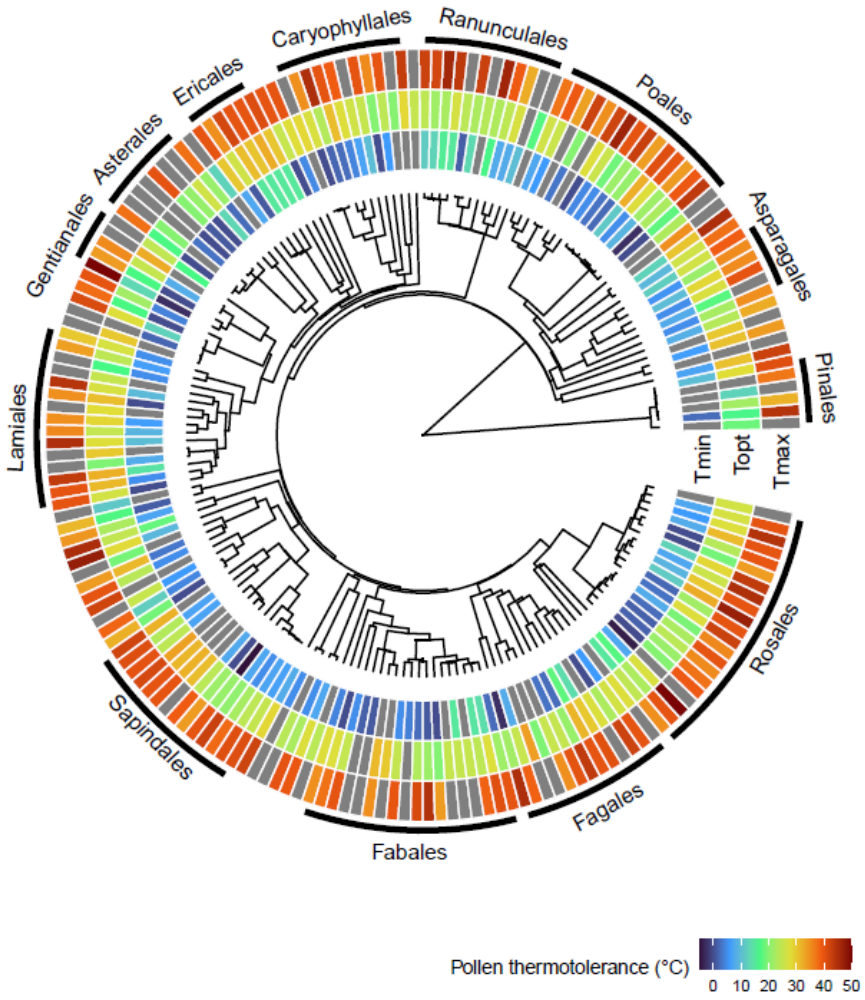


Figure 13: Distribution of minimal (the inner ring), optimal (the middle ring) and maximal (the outer ring) temperatures of pollen temperature tolerances across the species phylogeny. Grey cells are species with unavailable data.

*Interspecific trait variability (aim 4)*

The linear mixed effects models revealed that all predictors had statistically significant effects on minimal, optimal and maximal temperatures of pollen performance (Table 5; Figures 14-16).

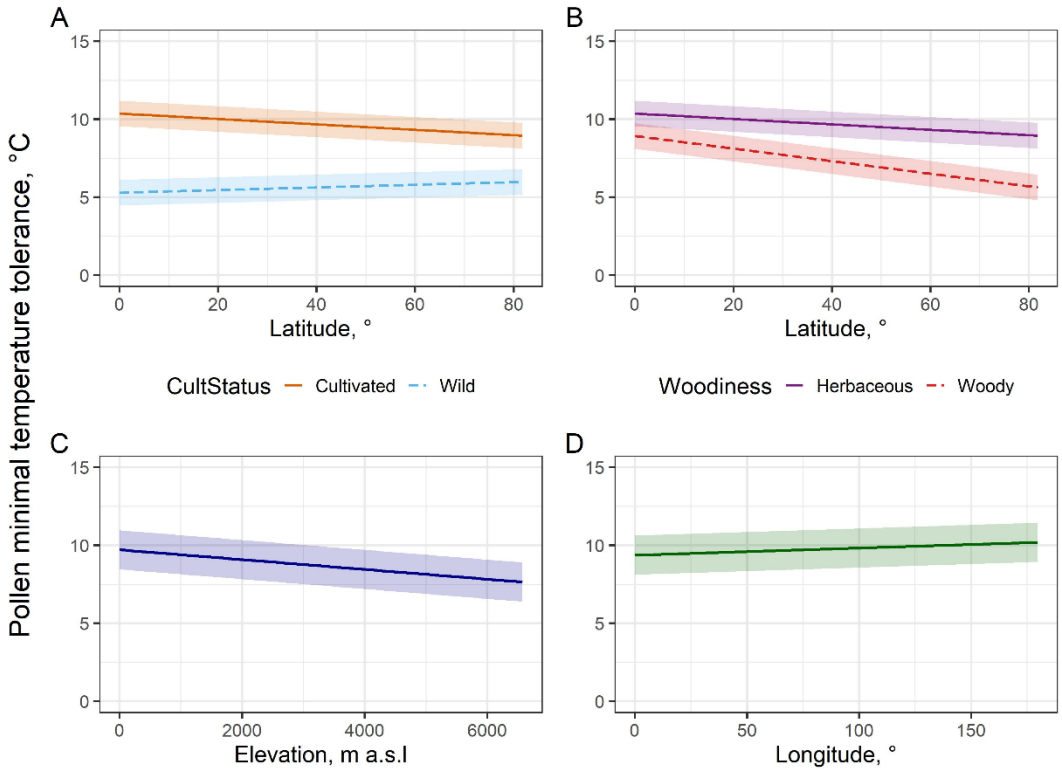


Figure 14: Variation of pollen cold tolerance (*Tmin*) along gradients of latitude (A, B) longitude (D) and elevation (C). The panels A and B show variation of *Tmin* values along latitudinal gradient among wild and cultivated plants (A) representing herbaceous and woody growth forms (B). Shaded areas denote 95% confidence interval.

The variation in pollen cold tolerance (*Tmin*) was mainly driven by the difference in plant cultivation status and growth form. Cultivated species had significantly large *Tmin* values as compared to their wild counterparts (10.3 ± 0.6 vs. 5.2 ± 0.6 ºC, respectively), whereas pollen of woody species had a significantly better tolerance to low temperatures than that of the herbaceous species (10.3 ± 0.6 vs. 8.9 ± 0.6 ºC, respectively). Across all species, *Tmin* values decreased towards the pole at the rate 0.02 ºC per degree of latitude, $p<0.001$; Figure 14), whereas species differing in their cultivation status and growth forms differed in their response to the latitudinal gradient. Specifically, the slopes were steeper for cultivated (-0.03 vs. -0.003 in wild; Figure 14A) and woody species (-0.03 vs. -

0.004 in herbs, Figure 14B). Elevation has a similar significant effect on *Tmin* variation, both in terms of effect sizes and nature of the relationship (Table 5; Figure 14C), with species occurring at high elevations having significantly lower *Tmin* values (decrease of 0.03 ºC per 100 m of elevation, $p<0.001$; Figure 14C). We also detected a significant longitudinal trend in pollen minimal temperature tolerance with species occurring in high longitudes having larger *Tmin* values (0.005 ºC per degree of latitude, $p<0.001$; Figure 14D).

Analysing the model for the optimal temperature of pollen performance (*Topt*), we see that the difference between *Topt* values in plants with different cultivation status and growth form was smaller as compared to their tolerance to low temperatures (Table 5; Figure 15). *Topt* in woody plants was slightly significantly higher as compared to the herbs (27.2 ± 0.6 ºC vs. 23.7 ± 0.6 ºC, respectively: Figure 15B), whereas this value was slightly but significantly lower in cultivated as in wild species (23.7 ± 0.6 ºC vs. 24.3 ± 0.6 ºC, respectively: Figure 15A). Latitude had a comparable significant, positive effect on *Topt* values in cultivated, wild and herbaceous species (increase towards the pole at the rate 0.02, 0.02 and 0.03 ºC per degree of latitude, $p<0.001$; Figures 15A and B, respectively).

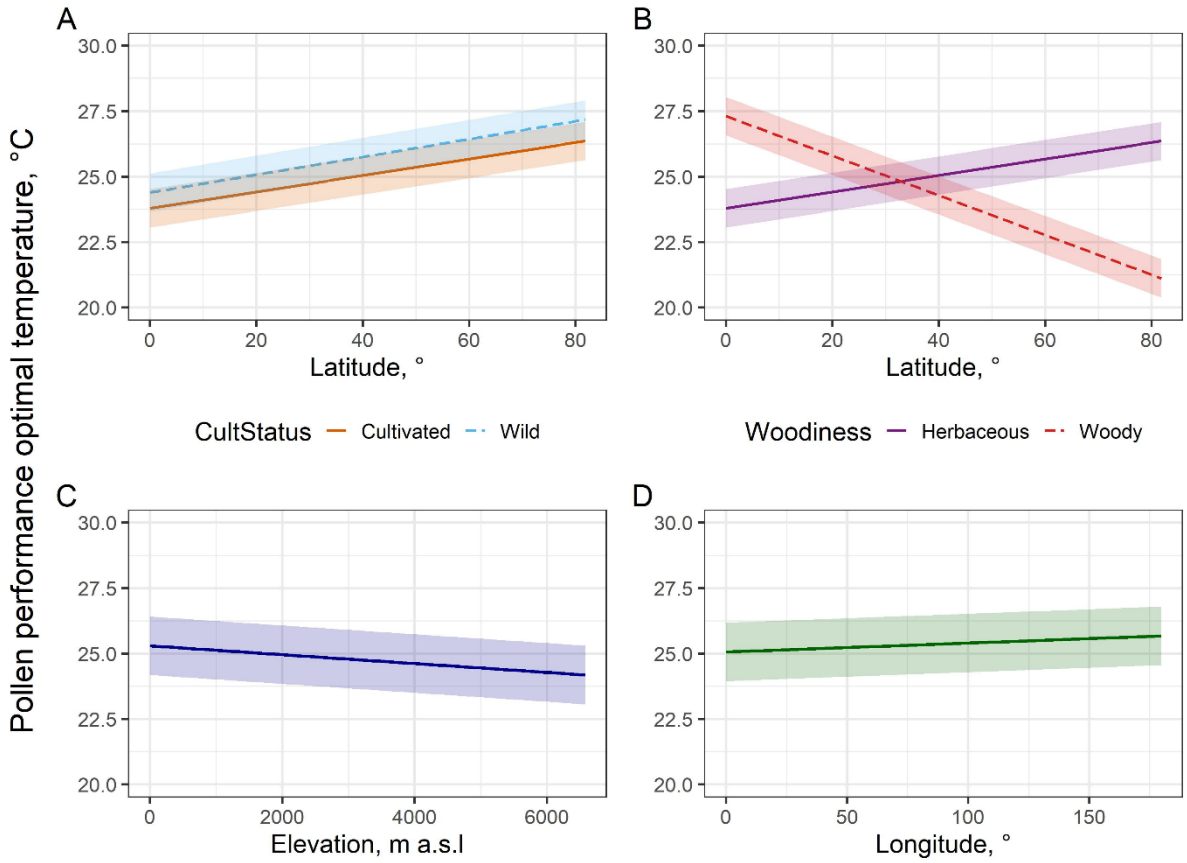


Figure 15: Variation of pollen optimal performance temperature (*Topt*) along gradients of latitude (A, B) longitude (D) and elevation (C). The panels A and B show variation of *Topt* values along latitudinal gradient among wild and cultivated plants (A) representing herbaceous and woody growth forms (B). Shaded areas denote 95% confidence interval.

Woody species displayed an opposite pattern with shrubs and trees occurring at high latitudes having considerably smaller *Topt* values (decrease in 0.07 ºC per degree of latitude, $p<0.001$; Figure 15B). Similarly, to the *Tmin*, we detected a significant negative elevational (decrease of 0.02 ºC per 100 m of elevation, $p<0.001$; Figure 15C) and positive longitudinal (0.003 ºC per degree of latitude, $p<0.001$; Figure 15D) trends in the optimal temperature of pollen performance.

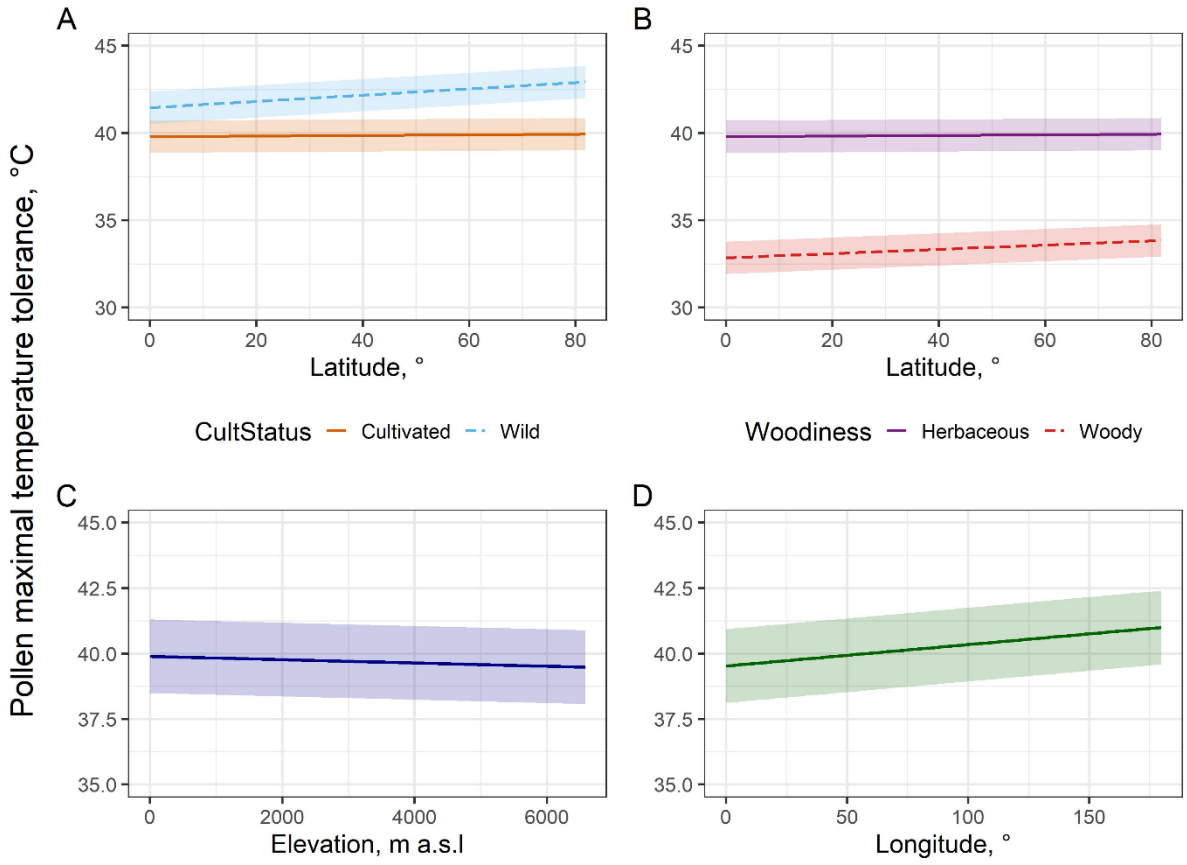


Figure 16: Variation of pollen heat tolerance (*Tmax*) along gradients of latitude (A, B) longitude (D) and elevation (C). The panels A and B show variation of *Tmax* values along latitudinal gradient among wild and cultivated plants (A) representing herbaceous and woody growth forms (B). Shaded areas denote 95% confidence interval.

On average, cultivated species had significantly lower pollen maximal temperature tolerance (*Tmax*) as compared to their wild counterparts (39.9 ± 0.1 vs. 42.3 ± 0.1 ºC; Figure 16B); the difference in the *Tmax* values was even larger in herbs and woody species (39.9 ± 0.1 vs. 33.4 ± 0.1 ºC, respectively; Figure 16B).

Species of all four groups showed a significant positive relationship with the latitudinal gradient (Figure 16A and B), however they differed in their slopes. *Tmax* in wild and woody species

increased in 0.02 ºC per degree of latitude, whereas the slope in cultivated and herbaceous species was 0.01 ºC per degree of latitude. We also detected a weak, yet significant negative elevational trend in *Tmax* (decrease of 0.01 ºC per 100 m of elevation; Figure 16C), as well as positive *Tmax*-longitude relationship (0.01 ºC per degree of latitude, $p < 0.001$; Figure 16D).

Table 5: Effects of latitude, longitude, elevation, and plant cultivation status and growth form on pollen thermal limits as determined from generalized linear mixed-effect models Bold values indicate significant treatment effects ($p<0.05$). SE – standard error. Marginal $R^2$ – the variance explained by the model fixed factors only (listed in the first column); conditional $R^2$ – the variances explained by the full model (i.e., the listed fixed factors plus phylogeny (family).

| | **Pollen cold tolerance (*Tmin*)** | | | **Optimal temperature of pollen performance (*Topt*)** | | | **Pollen heat tolerance (*Tmax*)** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Predictors** | Estimates | CI | p | Estimates | CI | p | Estimates | CI | p |
| Intercept | 9.58 | 8.32 – 10.83 | **<0.001** | 25.20 | 24.09 – 26.32 | **<0.001** | 39.88 | 38.47 – 41.28 | **<0.001** |
| Latitude | -0.25 | -0.27 – -0.22 | **<0.001** | 0.45 | 0.42 – 0.47 | **<0.001** | 0.03 | 0.00 – 0.05 | **0.029** |
| Cultivation status | -3.91 | -3.95 – -3.87 | **<0.001** | 0.72 | 0.68 – 0.76 | **<0.001** | 2.40 | 2.36 – 2.44 | **<0.001** |
| Growth form | -2.48 | -2.53 – -2.42 | **<0.001** | -1.33 | -1.40 – -1.27 | **<0.001** | -6.46 | -6.52 – -6.41 | **<0.001** |
| Elevation | -0.22 | -0.23 – -0.21 | **<0.001** | -0.12 | -0.13 – -0.11 | **<0.001** | -0.04 | -0.05 – -0.03 | **<0.001** |
| Longitude | 0.20 | 0.19 – 0.21 | **<0.001** | 0.15 | 0.14 – 0.16 | **<0.001** | 0.36 | 0.35 – 0.37 | **<0.001** |
| Latitude*Cultivation status | 0.37 | 0.34 – 0.39 | **<0.001** | 0.04 | 0.01 – 0.07 | **0.01** | 0.23 | 0.21 – 0.26 | **<0.001** |
| Latitude*Growth form | -0.33 | -0.35 – -0.30 | **<0.001** | -1.52 | -1.55 – -1.49 | **<0.001** | 0.15 | 0.12 – 0.18 | **<0.001** |
| Marginal $R^2$ | 0.14 | | | 0.03 | | | 0.21 | | |
| Conditional $R^2$ | 0.76 | | | 0.59 | | | 0.80 | | |

## Discussion

Traditionally, research on temperature limits of plant performance has been mainly focused more on vegetative and/or experimentally accessible organs, such as leaves (Geange et al., 2021), stems (Sakai and Larcher, 2012) or seeds (Sentinella et al., 2020) rather than on gametophytes (Rosbakh et al., 2018). While there is a substantial body of experimental work addressing pollen response to temperature stress (e.g., Raja et al., 2019; Chaturvedi et al., 2021; Tushabe et al., 2023; Zebro et al., 2023), these findings have seldom been consolidated and systematically analyzed across various scales. Our study bridges this gap by presenting the first comprehensive assessment of thermal limits of pollen performance and their drivers across multiple populations and species of wild and cultivated plants occurring worldwide.

*Temperature limits of pollen performance*

Our analysis reveals that, generally, pollen performance is limited by the temperatures ranging from 6.1 ºC (*Tmin*) to 39.5 ºC (*Tmax*), with 24.8 ºC being the optimal temperature of pollen germination and pollen tube growth (*Topt*). Also, several plant species in our data set have seemingly evolved pollen that can tolerate relatively low (the lowest *Tmin* value of -5 ºC) and high (the highest *Tmax* value of 50 ºC) temperatures. Although we lacked comprehensive data to test it formally, these findings suggest narrower pollen thermal tolerance breadth as compared to vegetative tissues. In general, plants can maintain their growth and development over a wide range of temperatures, approximately between -10 and +45 °C (Larcher, 2003; Luo, 2011; Nievola et al., 2017; Lancaster and Humphreys, 2020), while tissues of species growing in the most extreme biomes can survival temperatures between -60 °C and + 60 °C for short durations (Larcher, 2003; Nievola et al., 2017; Geange et al., 2021). We attribute the narrower pollen temperature tolerance to the lack of protective tissue in pollen grains, their comparatively small size and short lifespan, haploid set of chromosomes, and general sensitivity of cells to high temperature stress (Bedinger, 1992; Dafni and

Firmage, 2000; Araújo et al., 2013; Lohani et al., 2020). Consequently, these features explain why most of the *Tmin* values are above 0 ºC (germinated pollen grain and growing pollen tubes have limited opportunities to repair frost damage; (Wagner et al., 2016) and below 40 ºC (adaptive changes in lipid composition of membranes and production of heat shock proteins are impaired at temperatures around and above 45 ºC (Araújo et al., 2013). Thus, to circumvent the detrimental impacts of cold and heat stress on pollen germination and growth, plants coordinate the timing of anthesis with temperature conditions, both seasonal and diurnal, which are ideal/optimal for pollen performance. In essence, pollen adaptation to extreme temperatures is unnecessary since anthesis does not occur during freezing winters or scorching summers.

*Pollen and leaves share similar tolerance to low and high temperatures.*

Analysing the potential correlation between temperature tolerance in vegetative tissues and pollen in the subset of species (n = 13 for *Tmin* and n = 17 for *Tmax*; Figure 11), we see that plants with leaves adapted to extreme low and high temperatures, respectively, also tend to produce pollen grains with comparable temperature tolerance. Despite their distinct roles in the life cycle of plants, leaves and pollen share several characteristics at the molecular level that could contribute to their respective adaptive responses to environmental stresses. Such key molecular similarities reflect their common evolutionary heritage and shared biological processes that include antioxidant defence systems to regulate the levels of reactive oxygen species (Gill and Tuteja, 2010; Das and Roychoudhury, 2014), synthesis of protective proteins (e.g., heat shock and antifreeze proteins, Lee and Lee, 2003; Waters, 2013) and adjustments in cell membrane lipid composition (Narayanan et al., 2018). On the other hand, the relatively weak relationship between the leaf and pollen heat tolerance suggests that these two tissues might employ different mechanisms reducing the negative effects of high temperature exposure. Particularly, vegetative organs as complex, multicellular, and interconnected formations have many more opportunities to respond to the heat stress modifying their anatomy (e.g., presence

of trichomes reducing heat absorption), morphology (e.g., reduced leaf size and altered leaf orientation) and physiology (e.g., stomatal regulation and more efficient transport of water and metabolites among the organs) as compared to simply organized single-celled pollen grains. Thus, it remains unclear whether plant vegetative heat-tolerance can be used for reliable predictions of pollen thermotolerance, a key trait in, for example, in biogeography (Rosbakh and Poschlod, 2016; Rosbakh et al., 2018) or plant breeding (Kakani et al., 2002; Kakani et al., 2005).

*Intraspecific variability in pollen temperature tolerance*

The assessment of intraspecific variability in pollen cardinal temperatures within the subset of eleven cultivated species tolerance revealed a notable degree of plasticity in pollen temperature tolerance. Although we were not able to ascertain the specific climate conditions under which these cultivars were grown, we assumed that this variability primarily stems from adaptations to local growing conditions, facilitated by active breeding process towards better tolerance to temperature extremes (e.g., Kakani et al., 2002; Kakani et al., 2005). It is plausible that cultivars developed for and cultivated in colder and warmer regions are more likely to display enhanced tolerance to low and high temperatures, respectively (Gajanayake et al., 2011; Morrison et al., 2016; Ranasinghe et al., 2018).

The question of whether the observed intraspecific variability in pollen temperature tolerance is primarily driven by genetic differences, phenotypic plasticity, or a combination of both, remains unanswered due to the lack of corresponding research. Future investigations, such as plant cultivation in controlled environments or common gardens, combined with population genetic studies, are needed to shed light on this complex issue.

One of the most interesting findings of the study was the significantly larger variation in pollen cold tolerances across different species populations, while *Topt* and *Tmax* values were more stable. This observation aligns with previous research on intraspecific pollen performance of individual species

(Kakani et al., 2005; Salem et al., 2007; Singh et al., 2008) and broader studies on whole-plant temperature tolerance spanning multiple species (Araújo et al., 2013; Lancaster and Humphreys, 2020; Bennett et al., 2021; Geange et al., 2021). Specifically, the consistency of *Tmax* values across the study populations supports the theory that plant heat tolerance is constrained by destabilizing impact of temperatures surpassing 45 ºC on cell membranes and proteins, whereas plants demonstrate greater flexibility in their adaptive response to low temperatures (Araújo et al., 2013; Bennett et al., 2021). Additionally, the asymmetry in pollen tolerance to low and high temperatures may be augmented by different selective pressure on thermal limits; maximum habitat temperatures tend to exhibit less variation across contemporary biomes compared to minimum temperatures (Bennett et al., 2021). Finally, the spread of agriculture from warmer to colder climates has exerted additional selection pressure on pollen cold tolerance. The increased variability of pollen cold tolerance might reflect the crop breeding efforts at high latitudes/elevations aimed at adapting the studied species to novel, colder climates (Kakani et al., 2002; Kakani et al., 2005). Irrespective the underlying cause, the relatively lower plasticity of pollen heat tolerance raises concerns about the limited adaptative potential of plant sexual regeneration, particularly the male gametophyte performance, to the increasing high-temperature stress results from the recent climate warming. In long term, the impaired fruit and seed production could have adverse effects on plant population dynamics (Turnbull et al., 2000), plant-granivore interactions (Lewis et al., 2014; Bogdziewicz et al., 2016) and, ultimately, the food security for human population (Seppelt et al., 2022).

*Phylogenetic patterns in pollen thermal limits*

Gaining insights whether and to what extent pollen thermal limits are conserved across broad taxonomic groups can enhance our ability to predict how plant sexual regeneration might be impacted by current and future climates. Often, species preserve the ecological traits and environmental distributions inherited from their ancestors, with the tendency for closely related

species to share similar values for a given trait than distantly related species (Crisp et al., 2009; Burns and Strauss, 2011; Liu et al., 2015). Analysing the distribution of pollen cardinal temperatures across the phylogenetic tree, we revealed that pollen cold tolerance was to some extent phylogenetically conserved at the family level. This trend supports the 'deep-time climate legacies' hypothesis, suggesting that species whose ancestors originated in relatively colder paleoclimates tend to exhibit a better tolerance to colder temperatures compared to species with warm thermal ancestry (Bennett et al., 2021). Alternatively, closely related lineages often occur in spatially proximate regions, potentially leading them to inhabit more similar environments by chance, compared to more distantly related species (Lancaster and Humphreys, 2020). On contrary, pollen heat tolerance appears to be independent of climate ancestry, as evidenced by the lack of significant phylogenetic signal in the *Topt* and *Tmax* values. This finding corroborates another tenet of the 'deep-time climate legacies' hypothesis, suggesting that physiological constraints (e.g., cell membrane protein functioning at the temperatures above 45 ºC; see above) restrict the evolution of heat tolerance in living organisms (Hamilton, 1973). Yet, it is important to treat these findings with caution, as our data set includes a relatively small number of species (<200 species out of 300K of total angiosperm diversity) and strongly biased towards a few plant families, primarily representing species from temperate climates (e.g., Caryophyllaceae, Rosaceae). Thus, more research on pollen germination and tube growth is needed to assess pollen performance under different temperatures globally, particularly in wild plants occurring at lower latitudes (Rosbakh et al., 2018). This expanded research effort will shed more light on evolution of pollen temperature tolerance.

*Drivers of interspecific pollen thermal limits*

The analysis of species-specific variation in pollen cardinal temperatures revealed the significant influence of cultivation status, growth form, and local climate adaptations on pollen thermal limits. Notably, cultivated species exhibited a significantly reduced pollen thermal range compared to their

wild counterparts, as evidenced by *Tmin* - *Tmax* values of 10.3 – 39.9 ºC for cultivated species and 5.2 – 42.3 ºC for wild species, respectively. This difference likely mirrors the historical and present distribution of agroecological zones, which have always been confined to the climates most favourable for plants growth and reproduction (Fischer et al., 2002; Wezel et al., 2020). Also, the limited pollen temperature tolerance in cultivated species may be attributed to the historical oversight of this trait in breeding programmes, in contrast to wild plants that have evolved withstanding frequent temperature extremes. Thus, the narrower pollen temperature tolerances in the focal cultivated species (e.g., soybean; Djanaguiraman et al., 2019, groundnut; Kakani et al., 2002, and sorghum; Singh et al., 2016), coupled with their seemingly low intraspecific variability in the heat tolerance (as discussed above), renders this plant group particularly vulnerable to global warming. This vulnerability is reinforced by abundant experimental evidence highlighting the adverse effects of heat stress on crop yields (e.g., Prasad and Djanaguiraman, 2014; Djanaguiraman et al., 2018).

We found significant differences between growth forms in the pollen temperature tolerance. Pollen of woody species displayed a slightly better tolerance to low temperatures, with average *Tmin* values of 8.9 ºC compared to 10.3 ºC for herbs. Conversely, the pollen of herbaceous plants demonstrated enhanced performance at significantly higher temperatures, with *Tmax* values of 39.9 ºC, as opposed to 33.4 ºC in woody species. This difference in *Tmin* and *Tmax* between the two growth forms was even more pronounced in regions with thermally variable climates of higher latitudes (as detailed below). We attribute this pattern to the distinct thermal conditions experienced by woody and herbaceous flowers during anthesis, owing to their contrasting position on the stem at varying heights above the ground. The flowers, and consequently pollen, of taller trees and shrubs are directly exposed to ambient air, subjecting them to greater variability in daily air temperatures (and potentially seasonal fluctuations, given the longer lifespan of pollen; Dafni and Firmage, 2000). In contrast, flowers of herbaceous benefit from buffering against these fluctuations by snow cover, soil,

and surrounding vegetation (Raunkiaer, 1934). In this context, the improved cold tolerance of tree pollen at higher latitudes might reflect overall plant adaptation to daily and seasonal temperature drops in early spring when many temperate and boreal, wind-pollinated trees and shrubs (e.g., *Alnus*, *Betula*, *Fagus* and *Pinus*) flower. Additionally, it could be an adaptation to occasional frost events, such as radiation frosts during still nights with a clear sky (Sakai and Larcher, 2012). Respectively, the relatively slow air movement close to the ground couple with efficient heat accumulation capacity of vegetation (Geiger et al., 2009; Körner, 2022), often results in situations where pollen during presentation, dispersal and germination, experience temperatures considerably higher than ambient conditions (Dietrich and Körner, 2014). This environmental selection pressure likely contributes to the observed superior heat tolerance in pollen of herbaceous plants.

Our results further confirmed our hypothesis that climate is one of the main drivers of pollen thermal limits. In general, pollen of species growing at high elevations and latitudes tended to have better cold and heat tolerance compared to their counterparts inhabiting lower elevations and latitudes. This geographic trend likely stems from the gradual transition towards climates with higher temperature fluctuations. Specifically, there is a substantial increase in daily thermal amplitudes towards higher latitudes and elevations, with flowering plants of these habitats at anthesis experiencing occasional nocturnal freezing conditions and periods of daily temperature spikes exceeding 30-40 ºC (Geiger et al., 2009; Körner, 2022). Consequently, the pollen of such species must evolve a comparatively broad thermal tolerance to cope with the large range of temperatures pollen can experience at anthesis. In contrast, pollen originating from stable thermal habitats can function effectively with narrower temperature tolerance (‘climate variability hypothesis’; Stevens, 1989; Dafni and Firmage, 2000; Cuesta et al., 2020; Lancaster and Humphreys, 2020). Notably, the considerably different slopes of the regression lines (Figures 14 and 16) indicate that the broader pollen thermal niche in climates characterised by fluctuating temperatures is primarily achieved through the greater tolerance

to cold temperatures, with a lesser contribution from pollen heat tolerance, potentially due to the physiological constraints discussed earlier.

**Conclusions and perspectives**

In conclusion, our study of pollen temperature tolerance has revealed several crucial insights into the thermal limitations of pollen performance. First, we revealed that pollen performance is predominantly constrained by temperatures ranging from 6.2 °C to 39.5 °C, with an optimal temperature for germination and pollen tube growth at 24.8 °C, with a few examples that can tolerate temperatures as low as -5 °C and as high as 50 °C. The correlation between temperature tolerance in leaves and pollen suggests shared molecular adaptations, but the weaker relationship for heat tolerance implies different mechanisms may be at play in these two tissues.

Second, the analysis of pollen thermal limits in eleven cultivated species revealed notable plasticity in intraspecific pollen temperature tolerance (particularly at the 'cold' end), most likely influenced by local growing conditions and/or breeding process. The smaller variation in pollen heat tolerance across the study cultivars (populations) therefore raises serious concerns about the adaptive potential of plant sexual regeneration in the face of increasing high-temperature stress from climate warming.

Third, climate emerged as a significant driver of pollen thermal limits, with pollen of species in higher elevations and latitudes displaying better cold and heat tolerance. Pollen of cultivated species exhibited narrower temperature tolerances compared to their wild counterparts, highlighting potential vulnerabilities of crops to global warming. Further, we revealed considerable differences in temperature tolerance between woody and herbaceous plants that we attribute to unique thermal conditions during anthesis pollen of the corresponding plant experiences. Finally, phylogenetic analysis indicates conservation in pollen cold tolerance at the family level, while heat tolerance appears independent of evolutionary history.

These findings have two profound ecological implications. First, as pollen represents the most temperature-sensitive stage in the plant reproductive cycle, regeneration by seed in species at high elevations and latitudes appears to be more resilient to the temperature extremes associated with their habitats, a crucial adaptation in the face of global climate change. However, the relatively low tolerance to heat, particularly in cultivated species at lower elevations and latitudes, highlight the potential challenge for modern agriculture posed by anthropogenic warming influences on the local climates. This reenforces the argument that screening for pollen heat tolerance should be integral to plant breeding programs, aiming to develop new genotypes that can withstand high temperature conditions (Kakani et al., 2002; Kakani et al., 2005).

Second, the close link between the pollen thermal limits and climate suggests that pollen temperature tolerance could serve as an alternative mechanism explaining species distribution patterns (Rosbakh and Poschlod, 2016; Rosbakh et al., 2018). Specifically, the restriction of pollen germination and tube growth by temperature exceeding its physiological limits should ultimately result in poor seed production. Consequently, the reduction in reproductive output will in turn affect a species distribution, limiting its capacity to expand its geographical range or to maintain existing populations (Grubb, 1977; Pigott and Huntley, 1981; Turnbull et al., 2000). Therefore, integrating knowledge on pollen thermal limits into future studies, a key precursor to seed production, will advance our understanding of species distribution along climatic gradients and enhance our ability to predict the effects of anthropogenic climate change on plant geographic ranges.

While our study sheds new light on critical aspects of pollen temperature tolerance, it is imperative to acknowledge its limited scope. The most surprising finding of the study is that, despite its critical importance for plant science, the data on pollen thermal limits were available for only ca. 200 species only (i.e., 0.06% of the global vascular plant diversity). Hence, more work is essential to measure pollen thermal limits across the world's flora, especially in the non-temperate regions. Additionally, future research should consider the precise temperature conditions pollen experiences during

anthesis. The latitude, longitude and elevation used in this study as proxies for local temperature conditions are coarse and mask local-scale variation in temperatues. Finally, other potential drivers of pollen thermal limits, such as flower morphology, flowering phenology and pollination type should be considered in subsequent studies.

# Chapter 4: Negative effects of high-temperature stress on gametophyte performance and their consequences for seed reproduction in wild plants


## Abstract

Plant regeneration by seeds is highly sensitive to temperature stress, particularly in the gametophyte stage. However, most of the existing research has focused on one single stage of gametophyte development and/or conducted using cultivated or a very few model species. Thus, it is unclear whether the results of such studies can be applied to natural populations.

To fill this gap, we investigated a) the effects of chronic heat stress (CHS; 17 days) at 35/30 °C (moderate stress) and 40/35 °C (severe stress) on gametophyte performance, and b) how these effects translated into seed quantity and quality. We measured six traits related to male (anther length, pollen production and size) and female (ovary length, ovule production, and size) gametophyte performance and leaf chlorophyll fluorescence (Fv/Fm) in four wild *Silene* species. The ripe seeds of the treated plants were used to measure seed mass and seed production; the seed germination was characterized in terms of germination percentage, speed, and synchrony.

Fv/Fm decreased significantly in both heat treatments, confirming a negative effect of CHS on overall plant performance. All male gametophyte traits decreased significantly in both CHS treatments compared to the control. The length and size of the ovary were significantly smaller in the 40/35 °C treatment than in the 35/30 °C treatment and the control, while ovule production decreased significantly in both CHS treatments compared to the control. The negative effects on gametophyte performance translated into significantly fewer seeds in the 35/30 °C and 40/35 °C treatments compared to the control. CHS treatments did not affect the seed mass. The final germination percentage differed weakly significantly between the severe treatment and the control but did not

show any negative impacts by heat stress, whereas seed germination was significantly faster in the treated plants, both moderate and severe. Germination synchrony was not affected by heat treatments.

The high sensitivity of gametophytes in vascular plants to high-temperature stress implies that climate change-associated heat waves can significantly impact seed reproduction in wild plants. The altered seed quantity could have potential consequences for the long-term survival of the wild plant populations and the performance of the granivores.

Keywords: climate change; gametophyte; seed; temperature stress; wild plant

## Introduction

The close relationship between the environment and the success of plant reproduction has long been known. Numerous studies have shown that exposure of plants at various plant reproductive phases to (a)biotic stress almost always results in altered seed quantity and quality (Wahid et al., 2007; Hedhly, 2011; Fahad et al., 2017). In the last two decades, interest in the possible effects of high temperatures on plant performance has increased considerably, due to recent climate change (Gitay et al., 2002; Solomon et al., 2007; Hedhly et al., 2009). To begin with, some published evidence has indicated that global warming can have positive effects on sexual reproduction in certain plants (Hedhly et al., 2009; Raza et al., 2019). These effects include longer and warmer flowering periods (e.g., 0.6-day longer flowering per 1 °C of warming, Miller-Rushing et al., 2007; Zhou et al., 2022), along with elevated carbon dioxide levels (e.g., a 2.3-day boost in spring wheat growth at 550 ppm, Streck, 2005). These changes are expected to enhance photosynthesis, potentially increasing plant growth and seed production (Reyes-Fox et al., 2014; Dusenge et al., 2019; Bhargava and Mitra, 2021). However, any potential benefits may be offset by other negative impacts of climate change (Orsenigo et al., 2014; Hatfield and Prueger, 2015). For example, the accelerated rise of air temperatures has also resulted in the increased severity, frequency, and duration of extreme temperature fluctuations (e.g., cold snaps and heat waves, Orth et al., 2016; Ummenhofer and Meehl, 2017; Cardell et al., 2020). These extreme weather events are known to have negative effects on flower bud formation, flowering, fruit ripening, and seed germination, resulting in lower seed yields and seed quality (Hatfield and Prueger, 2015; Raza et al., 2019; Yadav et al., 2022). In turn, altered rates of viable seed production could have profound ecological consequences. For instance, low seed input can lead to a decline in the abundance of seed plant populations and ultimately to the extinction of the species (Turnbull et al., 2000; Willis et al., 2008). Furthermore, in natural plant populations, fluctuations in fruit and seed production can affect other trophic levels, given the complex

interactions between plant reproduction and the diets of many animal species (Lewis et al., 2014; Bogdziewicz et al., 2016). Finally, a low seed yield in crop production could have negative consequences for food security (for example, estimated crop yields are expected to decline 30% by 2050; Bapna et al., 2019).

In general, plants can maintain their vegetative growth and development over a relatively wide range of temperatures, approximately between −10 and +60 °C (Luo, 2011; Nievola et al., 2017). However, a remarkable temperature sensitivity has been attributed to the gametophytic phase (that is, the stages from gametophyte formation to fertilization) that has a much narrower range of optimal temperatures for functioning between 10 and 30 °C (Luo, 2011; Źróbek-Sokolnik, 2012; Nievola et al., 2017). Experimental and observational studies have shown that even slight increases in temperature above the optimum during the gametophytic phase can alter plant reproductive ability by negatively affecting micro and macrosporogenesis, reducing the number and viability of pollen grains deposited on the stigma, subsequent fertilization of the ovules, and increasing rates of embryo abortion (Hedhly et al., 2009; Zinn et al., 2010; Hedhly, 2011; Arshad et al., 2017). Importantly, among gametophytic organs and tissues of angiosperms, pistil-protected ovules are more tolerant to abiotic stress than pollen grains and anthers (Zinn et al., 2010; Hedhly, 2011; Raja et al., 2019). The increased sensitivity of pollen compared to ovules is due to its comparatively small size, haploid set of chromosomes, lack of protective tissue, and direct exposure to the environment at anthesis (Bedinger, 1992; Pacini and Dolferus, 2016; Lohani et al., 2020).

Despite the large body of research on the effects of high-temperature stress on angiosperm gametophyte performance, there remain several gaps and biases. To begin with, the emphasis is often on the effects of individual gametophyte stages, either on microgametogenesis (see, e.g., Elsahookie et al., 2021), microsporogenesis (see, e.g., Porch and Jahn, 2001; Iovane and Aronne, 2022), megasporogenesis (see, e.g., Shi et al., 2022); or gamete fusion (see, e.g., Dupuis and Dumas, 1990;

Snider and Oosterhuis, 2012). Although convenient in terms of labor and time costs, such studies often do not provide a comprehensive picture of high-temperature effects on overall regeneration by seed. First, different stages of gametophytes differ in their thermotolerance (Hedhly, 2011; Raja et al., 2019); therefore, the negative effects of temperature on one stage may be amplified (Wang et al., 2017) or overcompensated (Raja et al., 2019) on the others. Second, not every study has considered the downstream consequences of temperature stress-altered performance of gametophytes on seed quality and quantity (e.g., Morrison and Stewart, 2002; Prasad and Djanaguiraman, 2014; Ye et al., 2015; Mácová et al., 2022). Due to several compensatory mechanisms (e.g., increased production of heat shock proteins, increased antioxidant capacity, changes in gene expression; Hasanuzzaman et al., 2013; Chaturvedi et al., 2021), pollen and ovule performance altered by heat stress are not necessarily translated into lower seed quality and quantity (Cross et al., 2003; Jiang et al., 2020; Choudhary et al., 2022).

Finally, the commonly accepted high sensitivity of gametophytes to temperature stress is based mainly on the experimental work carried out with cultivated species (e.g., rice, cotton, tomato; Lohani et al., 2020) or a few model species cultivated indoors (e.g., *Arabidopsis thaliana*; Bac-Molenaar et al., 2015). The experimental populations used in such studies might be either preselected to specific temperature conditions (i.e., the cultivars of cultivated species; Xu et al., 2021; Alsamir et al., 2021) or lack specific adaptations to the environmental conditions of the growing sites/chambers (e.g., mutants in model species; Stephenson and Bertin, 1983; Lyndon, 1992). On the contrary, studies on gametophyte performance under temperature stress in wild species are extremely scarce and are limited to plants grown under conditions with unclear levels of stress exposure to extreme temperatures (e.g., Steinacher and Wagner, 2012; Wagner et al., 2016; Rosbakh and Poschlod, 2016). Thus, it is unclear whether the results of such studies can be applied to natural populations.

Here, we address these knowledge gaps by assessing how exposure of wild herbaceous plants in the reproductive stage to heat stress affects their gametophyte performance at various stages and how these effects are translated into seed quantity and quality. Specifically, in a fully controlled experiment, we exposed individuals of four wild *Silene* species (*S. coeli-rosa*, *S. gallica*, *S. laeta*, and *S. noctiflora*) to chronic heat stress (CHS) treatments at 35/30 °C (moderate CHS) and 40/35 °C (severe CHS) for 17 days. To estimate the effects of CHS on *Silene* reproduction by seed, we measured six plant traits related to the performance of male gametophytes (anther length, pollen production, and size) and female gametophytes (ovary length, ovule production, and size). We also measured leaf chlorophyll fluorescence and flowering phenology to assess the effects of treatments on overall plant performance. The ripe seeds of the treated plants were used to measure seed mass and seed production, and to study their germination performance. The experimental data set was analyzed using linear mixed-effect models, to answer the following questions:

1) Are both the male and female gametophytes in wild plants sensitive to high-temperature stress and, if so, to what extent? Similar to previous research on cultivated species (see, e.g., Lohani et al., 2020), we anticipated that high temperature stress in wild plants during the reproductive phase will negatively affect both male and female gametophyte performance. Existing studies have indicated that heat stress can cause abnormalities in pollen/ovule development, resulting in decreased fertilization success. However, natural plant populations might show differences in response to heat stress because they react differently to environmental stressors due to natural selection pressure than cultivated species that undergo selective breeding (Lippmann et al., 2019). Furthermore, we expected that the negative effects of heat stress would be stronger in the traits of male gametophytes than their female counterparts, due to the lack of protective tissue and direct exposure to the environment at anthesis in pollen (Bedinger, 1992; Pacini and Dolferus, 2016).

2) Are the negative effects on gametophyte performance translated into lower seed quality and quantity? Since heat stress negatively affects plant growth and development, with particularly strong effects on sporogenesis (Djanaguiraman et al., 2018), we expected stressed plants to produce fewer ovules and pollen of lower quality, which would ultimately lead to reduced seed mass and production and altered germination patterns.

## Material and methods

*Study species and experimental setup*

We selected four herbaceous species of the genus *Silene* (campion or catchfly, Caryophyllaceae) that commonly occur in a range of different climates and non-forest habitats in Europe (Table 6). The genus *Silene* has been traditionally used in ecological studies (Alatalo and Totland, 1997; Ferrarini et al., 2019; Kahl et al., 2019), because campions are easy to breed and have short life cycles, making experimental studies convenient (Bernasconi et al., 2009). The selected *Silene* species all have hermaphroditic flowers (i.e., both male and female reproductive organs in the same flower) with a short period to reach sexual maturity from seed of about seven to nine weeks. Only *Silene laeta* is a short-lived perennial, while the other *Silene* species (*S. coeli-rosa*, *S. gallica*, and *S. noctiflora*) are annual.

Table 6: The characteristics of the study species based on (Brickell, 1996; Phillips and Rix, 2002; Pilkington, 2007; Farris et al., 2013).

| Species | Distribution | Ecology | Origin of the seed material |
|---|---|---|---|
| ***Silene coeli-rosa* (L.) Godr.** | Southwestern Europe, North Africa, and the Canary Islands | Occurs in wastelands, railway yards and in damp, grassy places such as riverbeds | Saint Florent, Corsica, France |
| ***Silene gallica* L.** | Native to Europe, North Africa, and Western Asia | Occurs in dry, open habitats, often along roadsides and wastelands | Rocher de Roquebrune, France |
| ***Silene laeta* (Aiton) Godr.** | Native to North Africa and Southwestern Europe | On wet soils, sometimes in stagnant water | Compomoro Corsica, France |
| ***Silene noctiflora* L.** | Native to Eurasia | Arable land on dry, sandy, and calcareous soils | Blaufelden, Germany |

The study was carried out in the greenhouse of the University of Regensburg, Germany, from November 2021 to July 2022, under fully controlled conditions. The experimental plants were propagated from seeds collected in the natural habitats of the species (*Silene coeli-rosa*, *Silene gallica*, and *Silene laeta*) or cultivation conditions similar to those of the natural (*Silene noctiflora*). Several hundreds of seeds of each *Silene* species were initially seeded in trays (60 cm × 40 cm) filled with substrate (three parts of low-nutrient planting soil [CL Pikier- Einheitserde], three parts of coarse sand [Geser GmbH, Germany], and one part of dry compost soil [CL Topf Einheitserde]). The juvenile plants (approximately two weeks after germination) were transplanted into 9 cm pots, three

plants per pot, filled with the same soil substrate. After a growing period of about one month, the plants were again separated and repotted into individual 9 cm pots (i.e., one plant per pot) (see Appendix 2, Figure A1). At each repotting step, we selected only healthy plants and those in a similar phenological state, to ensure similar treatment effects on all individuals tested (see below). The pots were randomly rearranged and regularly watered within the greenhouse during germination and cultivation. All plants were grown in similar greenhouse conditions with day/night temperatures of 20/15 °C and natural illumination supported with additional lighting (Osram Plantastar 400 W, Osram, China) to achieve a photoperiod of 12 h in winter months (November – April). To promote blooming in *Silene laeta* we applied growth and flowering fertilizer ('Wuxal Super', Wuxal, Germany).

The experimental setup consisted of six similar grow chambers (Homebox Vista Medium, HOMEbox, Germany; Appendix 2, Figure A2): two chambers with day/night temperatures of 35/30 °C (moderate chronic heat stress [CHS]), two chambers with 40/35 °C (severe CHS) and two used as a control (30/25 °C) (Appendix 2, Figure A3). The chambers were equipped with two heating mats ('Fyto heat Deluxe', Schilling Phytotechnik GmbH, Italy, and 'Heating mat aluminium', Bio Green, Germany), to heat the chambers to test temperatures, and three photosynthesis-powered LED lamps ('Sanlight Flex 20 W′, SANlight, Austria) for illumination. Thermostats ('Thermo2′, Bio Green, Germany) in each box and a control panel ('dnt RoomLogg Pro', dnt, China) were used to measure and maintain the temperature at a constant level.

For heat treatments, we selected a total of 36 plants per species in a similar phenological stage (first visible flower bud). The plants were randomly divided into three groups of 12 individuals and grown under the three temperature conditions in a random block design for 17 days. The duration of treatment was selected to ensure that pollen and flowers of different stages of development received stress treatment (Mesihovic et al., 2016). Further, it corresponds with the observed and predicted heat

waves that European plants experience or will experience during the sexual reproduction process (Lin et al., 2022; Lhotka and Kyselý, 2022).

To avoid the drought stress associated with high temperatures, a steady state of soil humidity was maintained during application of heat stress by regular watering. Subsequently, all plants were returned to the greenhouse at constant temperatures of 20/15 °C, and natural illumination was supported by additional lights (Osram Plantastar 400 W, Osram, China) to allow seed ripening.

*Plant trait measurements*

To evaluate the effects of treatments on overall plant performance, we measured leaf chlorophyll fluorescence ('Pocket PEA', Hansatech, Germany), on the last day (17) of treatments. Leaf clips were applied to ten mature leaves of ten different individuals per heat treatment and species to allow dark adaptation. After an adaptation period of about 20 min (Maxwell and Johnson, 2000), the photosynthetic rate was measured and the maximum quantum yield (efficiency) of PS II photochemistry (Fv/Fm) was calculated by the tool.

*Flowering phenology*

To estimate the effects of heat treatment on flower development during the experiment, we also made regular (every three or four days, i.e., a total of six observation days) phenological observations of all treated plants. On each observational day, we recorded the number of individuals at the peak of flowering (i.e., 50% of all flowers on an individual plant are open).

*Gametophyte traits*

To estimate the effects of heat treatment on sexual reproduction, we measured six traits related to male (anther length, pollen production, and size) and female (ovary length, ovule production, and size) gametophyte performance. For measurements, we selected from ten to twelve open flowers in full anthesis per species per treatment and control on day 17 of the heat treatment and preserved

them in 70% ethanol for further analysis. In the laboratory, the flowers were dissected under a stereomicroscope to randomly select eight ovaries and eight anthers to be photographed for measurements. Scaled images of ovaries and anthers were used to measure their length using ImageJ software (Schneider et al., 2012). Anther length is defined as the average of the lengths of both thecae (Vries, 1974) while the ovary length is defined as the sum of proximal length, placenta length and distal length (Damodharan et al., 2016).

The ovaries were further dissected under the stereomicroscope, ovule images were taken, and the number of ovules was counted using ImageJ software (Schneider et al., 2012). The same images were used to measure the size of the ovules with the help of ImageJ software. To determine the size and number of pollen, a whole anther per replicate was placed in 100 µl of deionized water in an Eppendorf tube. The anther was then crushed and vortexed to achieve uniform pollen dispersion, and 5 µl of the solution was pipetted onto a microscope slide. Scaled images of the solution were taken under a microscope (Nikon Eclipse TS100); the images were analyzed for pollen size and number in ImageJ software. Pollen count in 5 µl was used to estimate the number of pollen grains released per anther of stock solution (100 µl).

*Seed mass and production*

To estimate the downstream consequences of the effects of heat stress on gametophyte performance, we also measured seed mass and seed production (number of seeds produced per individual plant) in treated plants. To achieve this, the treated plants were left in the greenhouse for about three months until full seed maturation; the plants were bagged with organza bags to prevent seed loss. For the measurements, we collected all seeds from ten plants per species and treatment, which were consequently counted and weighed. The average weight of an individual seed was calculated as the total weight of seeds divided by the number of seeds per individual. After the measurements, the seeds were stored at 4 °C prior to the seed germination experiments to preserve their viability.

*Seed germination traits*

*Silene* seeds germinated in climate chambers (Panasonic, MIR-254) at 22/14 ºC (photoperiod 14/10 h of light/dark regime), in five replicates with 20 seeds each. Germination was scored every other day for 15 days (most seeds germinated during the first week of the trial). The viability of the non-germinated seeds was tested using the 'cut' test: seeds with a white, firm embryo and endosperm were considered viable (Ooi et al., 2004). Non-viable seeds were excluded from the analysis.

Seed germination was characterized by (1) final germination percentage (FGP; seed ability to complete the germination process), (2) mean germination time (MGT; a proxy for germination speed with lower values indicating faster germination), and (3) germination synchrony. Germination synchrony was estimated by calculating the Z synchronization index (Lozano-Isla et al., 2019), which varies from 0 (events of seed germination were evenly spread throughout the entire incubation period) to 1 (all seeds germinated at the same time). The three traits were calculated for each replicate (Petri dish) in each treatment.

*Statistical analysis*

All data analyzes were performed with R software version 4.2.0 (R Core Team, 2023).

The effects of heat treatments on plant performance traits were analyzed using generalized linear mixed effect models conducted with the help of the *lme4* and *lmertest* packages in R (Bates et al., 2015; Kuznetsova et al., 2017). In each model, the trait of interest (Fv/Fm, gametophyte, or seed trait) was included as a response variable, heat as fixed effects, and species identity and experimental block (tray) as random factors. Differences among treatment effects were estimated with the help of the post hoc Tukey test ($p<0.05$), implemented in the packages *emmeans* and *multcomp* (Hothorn et al., 2008; Lenth, 2023). GLM for FGP data included family 'binomial' (logistic regression), while the remaining traits were analyzed using family 'Gaussian' ('simple' linear mixed effect models).

Trait data of anther length, pollen number, pollen size, and seed mass were first log-transformed as log (x + 1) to improve the normality of residuals. All the model assumptions were met in all the cases.

**Results**

*Leaf chlorophyll fluorescence*

CHS treatments had a significant negative effect on overall plant performance (measured as photosynthesis capacity via leaf chlorophyll fluorescence) in experimental plants, with the strongest effects in severe CHS (mean Fv/Fm values: control = 0.81, 35/30 ºC = 0.74, 40/35 ºC = 0.67; Figure 17, Table 7).

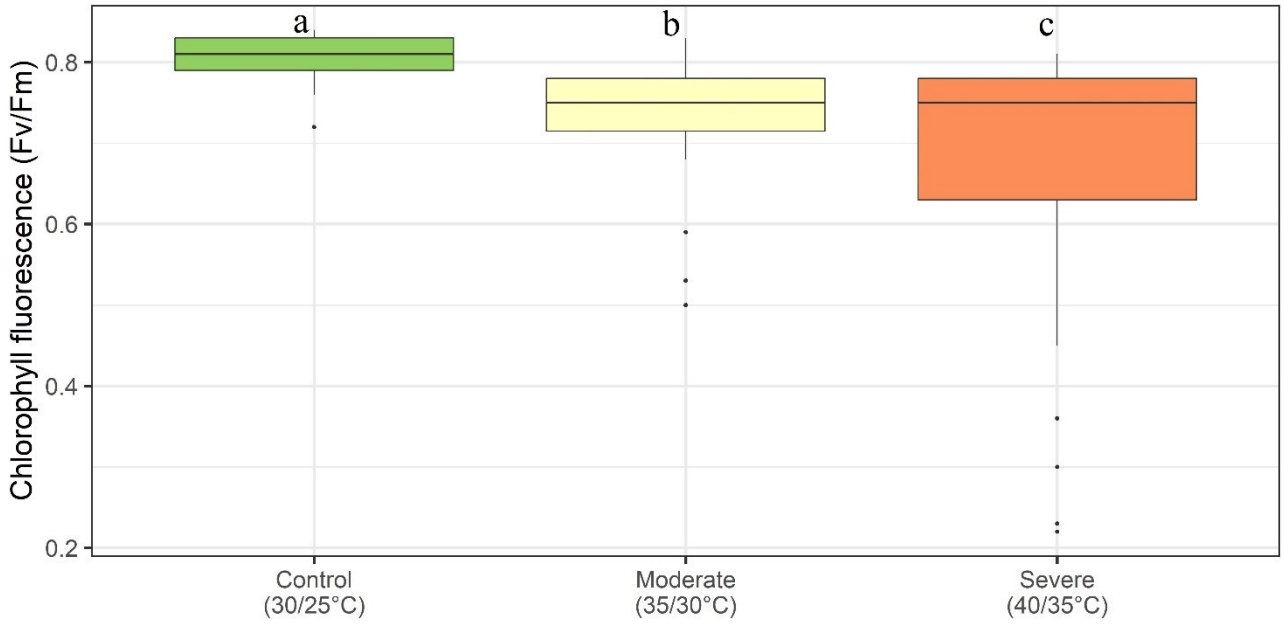


Figure 17: Effects of chronic heat stress treatments on leaf chlorophyll fluorescence (Fv/Fm) in the four *Silene* species. Letters indicate statistical differences between the control and two chronic heat stress treatments (moderate – 35/30ºC and severe – 40/35ºC) as deduced from generalized linear mixed effect models and post-hoc Tukey test ($p < 0.05$).

Table 7: Results of the linear mixed effects models and post-hoc Tukey tests for the chronic heat stress effects on leaf chlorophyll fluorescence (Fv/Fm), female and male gametophyte, and seed traits. Bold values indicate significant treatment effects ($p<0.05$). Different letters indicate significant differences between the control and two treatments as induced by the Tukey Post-hoc test ($p<0.05$). SE – standard error.

| | **Fv/Fm** | | **Ovary length (mm)** | | **Ovule count** | | **Ovule size (µm)** | | **Anther length (mm)** | | **Pollen count** | | **Pollen size (µm)** | | **Seed count** | | **Seed weight (mg)** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | Mean | SE± | Mean | SE± | Mean | SE± | Mean | SE± | Mean | SE± | Mean | SE± | Mean | SE± | Mean | SE± | Mean | SE± |
| 30/25°C (control) | 0.81 a | 0.02 | 1.38 a | 0.33 | 111 a | 22.1 | 93.86 a | 10.5 | 0.66 a | 0.09 | 1782 a | 788 | 44.69 a | 2.85 | 1368 a | 348 | 0.44 a | 0.22 |
| 35/30°C (moderate heat stress) | **0.74** b | 0.02 | 1.22 a | 0.33 | **81** b | 22.1 | 94.69 a | 10.7 | **0.39** b | 0.09 | **1003** b | 788 | **37.68** b | 2.91 | **796** b | 348 | 0.43 a | 0.22 |
| 40/35°C (severe heat stress) | **0.67** c | 0.02 | **0.87** b | 0.33 | **50** c | 22.1 | **84.45** b | 10.8 | **0.14** c | 0.09 | **50** c | 788 | **23.58** c | 3.01 | **744** b | 349 | 0.41 a | 0.22 |

*Flowering phenology*

In general, most of the individuals flowered during the 17-day heat treatments, except for a few species x treatment combinations where flowering was delayed. Specifically, *S. gallica* and *S. laeta* plants did not show differences in flowering phenology in all three treatments (Figure 18). In both species, flowering began immediately, increasing exponentially in *S. laeta* and gradually in *S. gallica* throughout the observation period. In *Silene coeli-rosa*, the individuals in all the treatments started to flower approximately on the sixth day. Subsequently, flowering gradually increased in the 30/25 °C and 35/30 °C treatments, while it was generally low in the 40/35 °C treatment. In *Silene noctiflora*, flowering increased exponentially in the control (30/25 °C), gradually increased at 35/30 °C, while flowering generally ceased in the treatment at 40/35 °C (Figure 18).

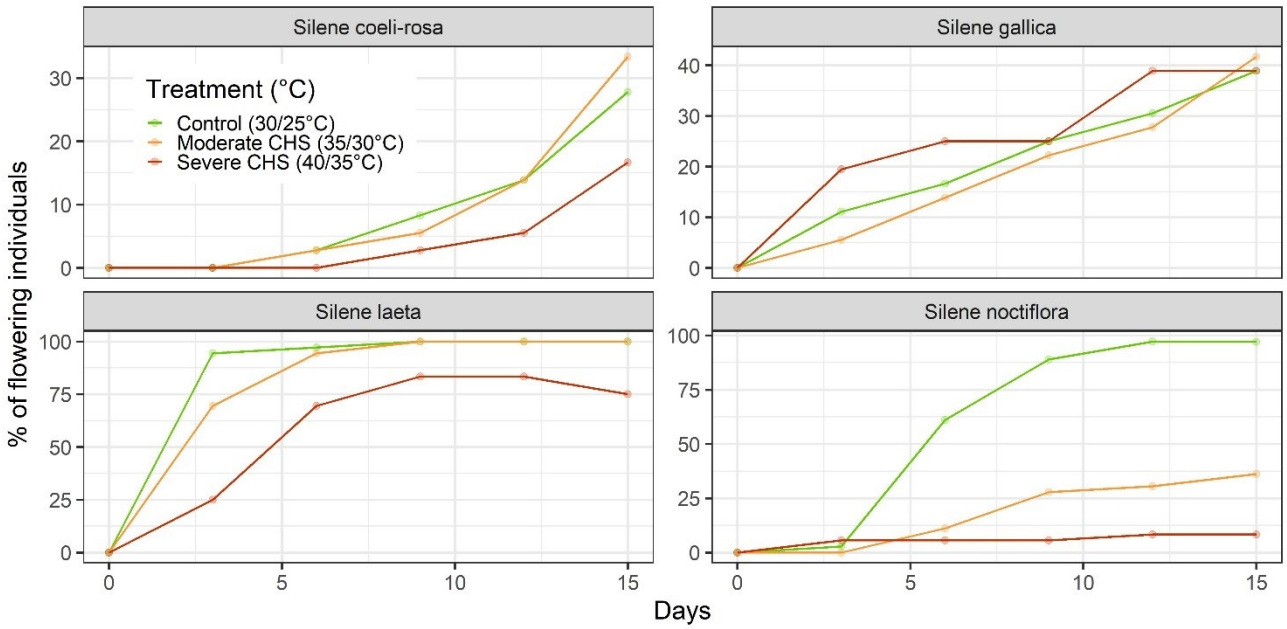


Figure 18: Percentage of flowering individuals observed during chronic heat stress treatments in the four *Silene* species.

*Gametophyte traits*

*Female gametophyte traits*

The effects of CHS treatments on ovary length exhibited a significant decrease in the 40/35 °C treatment (mean ovary length = 0.87 mm) compared to the 35/30 °C treatment (1.22 mm) and the control (1.38 mm) (Figure 19A, Table 7). Similar effects were also detected for the mean ovule size, with significantly smaller ovules produced in the 40/35 °C treatment (control: 94 µm, 35/30 °C: 95 µm, 40/35 °C: 84 µm; Figure 19C, Table 7). Similarly, ovule production was strongly negatively affected by CHS treatments, being the lowest in the 40/35 °C treatment (control: 111 ovules, 35/30 °C: 81, and 40/35 °C: 50; Figure 19B, Table 7).

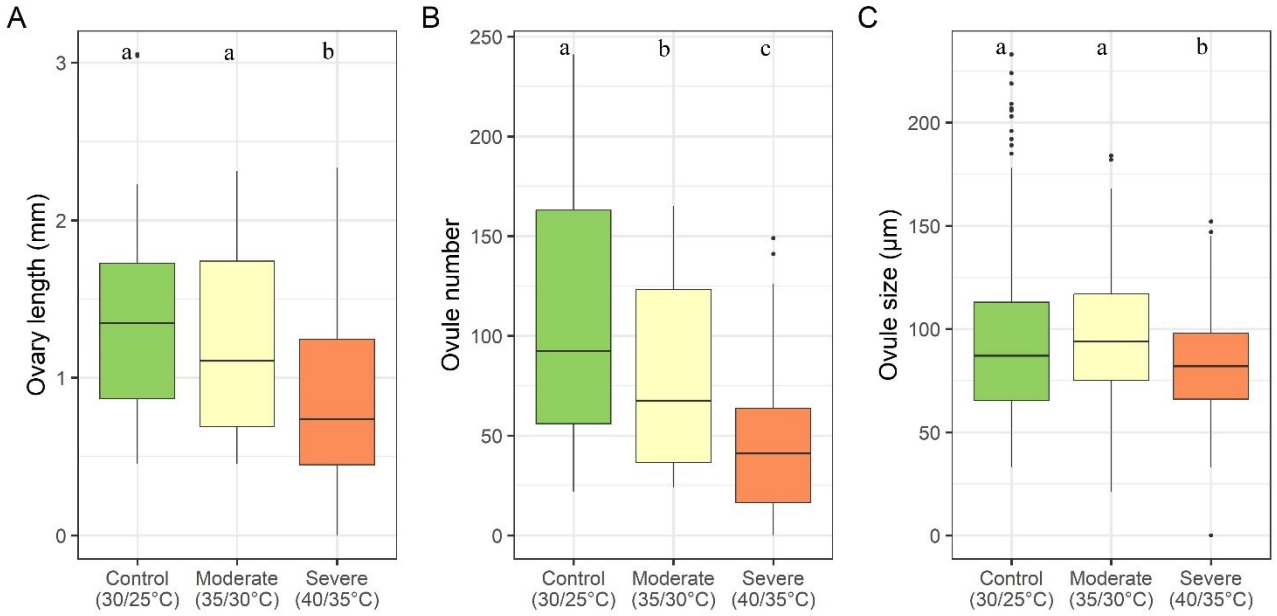


Figure 19: Effects of chronic heat stress treatments on mean ovary length (A), ovule number (B), and ovule size (C) in the four *Silene* species. Letters indicate statistical differences between the control and two chronic heat stress treatments (moderate – 35/30ºC and severe – 40/35ºC) as deduced from generalized linear mixed effect models and post-hoc Tukey test ($p < 0.05$).

*Male gametophyte traits*

The three male gametophyte traits showed a significant negative response to heat stress treatments, with significantly larger effect sizes in the 40/35 °C treatment compared to the control and the 35/30 °C treatment (Figure 20, Table 7). Specifically, mean anther length (0.14 mm), pollen number (50) and pollen size (23.58 µm) were significantly lower in the 40/35 °C treatment than in the 35/30 °C treatment (0.39 mm, 1003, and 37.68 µm), and significantly lower in the 35/30 °C treatment than in the control (0.66 mm, 1782 and 44.69 µm, respectively).

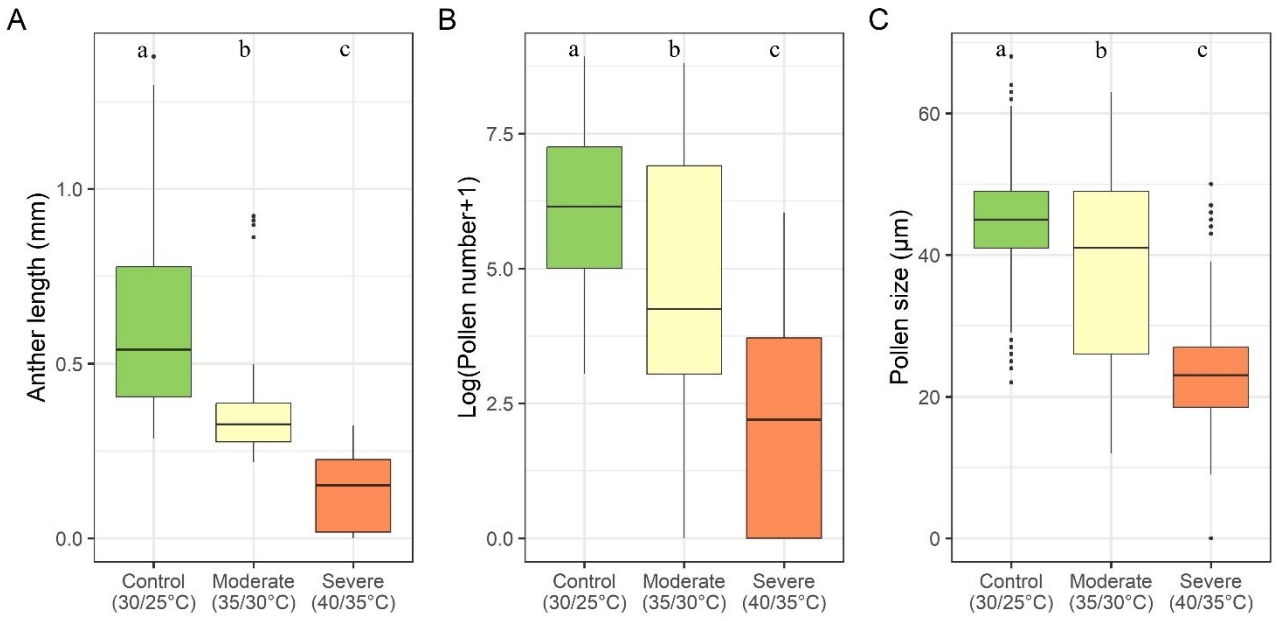


Figure 20: Effects of chronic heat stress on mean anther length (A), pollen number (B), and pollen size (C) in the four *Silene* species. Letters indicate statistical differences between the control and two chronic heat stress treatments (moderate – 35/30ºC and severe – 40/35ºC) as deduced from generalized linear mixed effect models and post-hoc Tukey test ($p < 0.05$).

*Seed traits*

*Seed mass and production*

Both heat treatments resulted in a significantly lower number of seeds produced (35/30 °C treatment, 796 seeds; 40/35 °C, 744) than in the control (1368) (Figure 21A, Table 7). There were no significant differences in the mean seed mass across all species exposed to the different CHS treatments (Figure 21B, Table 7).

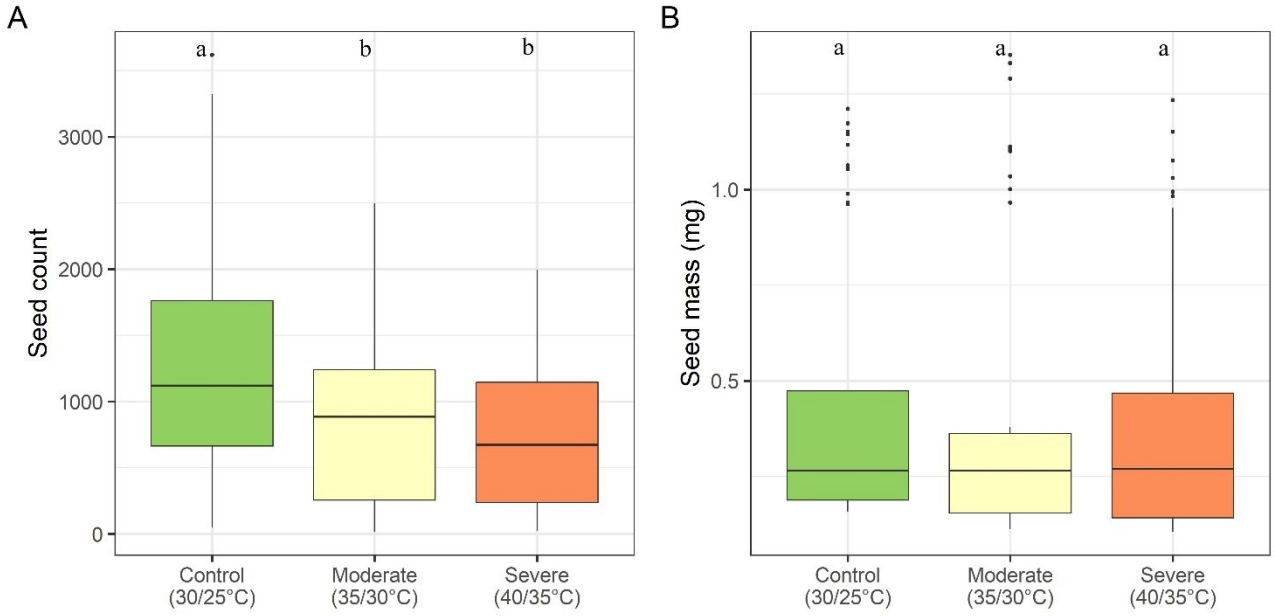


Figure 21: Effects of chronic heat stress treatments on mean seed count (A) and seed mass (B) in the four *Silene* species. Letters indicate statistical differences between the control and two chronic heat stress treatments (moderate – 35/30ºC and severe – 40/35ºC) as deduced from generalized linear mixed effect models and post-hoc Tukey test ($p < 0.05$).

*Seed germination traits*

In general, most of the *Silene* seeds, regardless of species or treatment, germinated quickly and with high final percentages, as high germination was already achieved on the fifth day of incubation

(Figure 22A). The GLM revealed only a small, yet significant difference between the final germination percentage control (91%) and the severe heat treatment (96%). No clear treatment effects were detected in this trait. The seed germination speed (Figure 22C) was positively affected by both treatments (mean germination time for control 5.3 days, moderate stress – 5.0 days, and severe stress – 4.9 days), although the effects sizes were relatively small. We did not detect significant differences in seed germination synchrony.

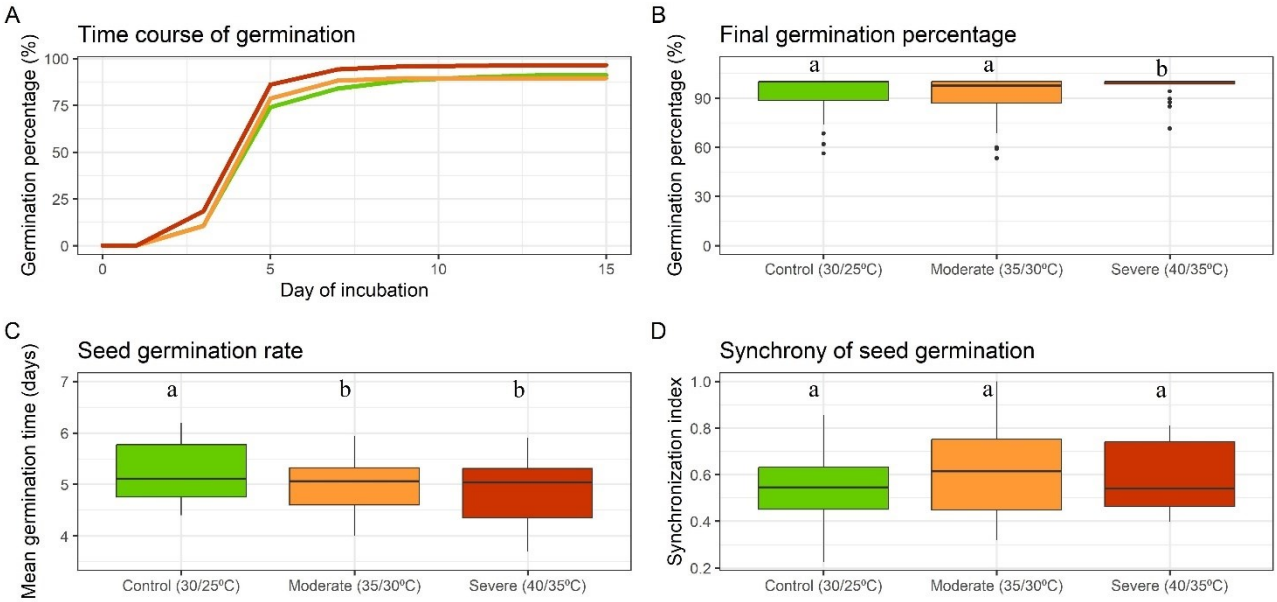


Figure 22: Effects of chronic heat stress treatments on time course of germination (A), final germination percentage (B), seed germination speed (C), and germination synchrony (D). Letters indicate statistical differences between the control and two chronic heat stress treatments (moderate – 35/30ºC and severe – 40/35ºC) as deduced from generalized linear mixed effect models and post-hoc Tukey test ($p < 0.05$).

## Discussion

*Heat stress negatively affects gametophyte performance in wild plants*

Confirming our expectation, chronic heat stress (CHS) treatments had significant negative effects on the traits of female and male gametophytes in the four wild *Silene* species. Except for ovary length and ovule size in the moderate CHS (35/30 °C), the treated plants had smaller anthers and ovaries that contained smaller numbers of pollen grains and ovules, respectively, of smaller sizes. These negative effects were more pronounced under severe CHS (40/35 °C). Therefore, our study confirms previous findings for cultivated and model species that heat stress is an essential factor that negatively affects the gametophytic performance of vascular plants (Prasad et al., 2008; Raja et al., 2019; Hedhly et al., 2020). These findings further suggest that exposure of plants occurring in natural populations to heat stress (for example, during climate change-associated heat waves) at the flowering stage can considerably affect reproductive success (Zinn et al., 2010; Mesihovic et al., 2016; Hedhly et al., 2020).

Chlorophyll fluorescence measurements revealed that photosynthetically active vegetative tissues were also negatively affected by heat stress treatments. This reduced the overall performance of the treated *Silene* plants and could also have indirect negative effects on the anther/pollen and ovary/ovule traits. Although not measured in this study, the low Fv/Fm values measured could indicate that the photosynthetic apparatus and the PSII complex were damaged by heat stress and did not function at their maximum capacity (Sharma et al., 2015; Shanker et al., 2022). Reduced photosynthetic rates could limit the production, transport, and allocation of photosynthetic assimilates from leaves to gametophytic tissues and organs (Sharma et al., 2012; Poudyal et al., 2018). In turn, reduced carbohydrate supply to pollen grains and pistils results in low energy production (Adenosine triphosphate (ATP)) and resources required for normal growth, development, and fertilization of female and male gametophytes (Snider et al., 2009). Additionally, the heat stress

could significantly increase dark respiration (Timlin et al., 2006) and photorespiration (Jiao and Grodzinski, 1996), which further depletes the carbohydrate supply for developing gametophytes.

The poor performance of the female and male gametophytes under heat stress could be due to direct and indirect high-temperature effects on plant sexual reproduction. As for the former, heat stress can directly affect enzymatic activity (e.g., reduced starch and/or sugar biosynthesis), and metabolic processes (e.g., glycolysis, a main metabolic pathway primarily responsible for capturing energy in the form of ATP) required for optimal gametophyte development (Hasanuzzaman et al., 2013; Kumar et al., 2022). Disruption of these processes affects the normal production and accumulation of key components, such as carbohydrates, proteins, and lipids, required for cell growth and function (Wahid et al., 2007; Sehgal et al., 2018). Furthermore, in the female gametophyte, heat stress can cause a decline in auxin levels, a hormone that promotes cell growth and division in the ovary (Ruan et al., 2012; Wang et al., 2021; Kaur et al., 2021). This can lead to a significant decrease in ovary length (see, e.g., Wang et al., 2021), as also observed in our study with severe CHS treatment (Figure 19A). The heat stress-induced disruption of normal ovary development appears to transfer the negative effects to the ovules, resulting in their reduced production and size (Figure 19B and C, Table 7). Moreover, the meiotic phase during ovule production is highly sensitive to heat stress (e.g., Prasad and Djanaguiraman, 2014; Shi et al., 2022; Choudhary et al., 2022) which potentially contributes to the significant reduction in ovule number at both moderate and severe CHS treatments (Figure 19B).

Similarly, in the performance of male gametophytes, heat stress can affect the resources required during the critical stages of anther development and differentiation (Chaturvedi et al., 2021). Disrupting normal processes of anther formation can lead to premature anther maturation and dehiscence before they reach their full size (length; Snider and Oosterhuis, 2012; Raja et al., 2019). Anther length is a critical determinant in pollen production, since longer anthers typically produce

more pollen grains (Harder and Barrett, 1993). The shorter anthers that produced fewer and smaller pollen grains than in the control (Figure 20), as observed in the heat stressed *Silene* plants, can confirm these observations made in cultivated species. Finally, at particularly high temperatures, heat stress can lead to rapid damage and death of gametophyte cells, especially during sporogenesis, resulting in a decrease in the overall number and size of pollen and ovules (Ahuja et al., 2010; Li et al., 2018; Iovane and Aronne, 2022), a possible explanation for the stronger negative effects of severe CHS (40/35 °C) applied to the test *Silene* species.

*The male gametophyte is more sensitive to heat stress compared to its female counterpart*

When comparing the effects of heat stress on the characteristics of female and male gametophytes, we revealed that anthers and pollen grains in *Silene* species were more sensitive to CHS than ovaries and ovules in the same flowers. According to our expectations, all male generative traits, anther length, pollen production, and size, were strongly negatively affected by moderate (35/30 °C) and severe (40/35 °C) CHS treatments, while female gametophyte traits, ovary length, and ovule size were significantly affected only by severe CHS treatment (40/35 °C). These findings are in line with previous studies showing that the male gametophyte is more sensitive to heat stress than their female counterparts (Zinn et al., 2010; Hedhly, 2011; Jagadish, 2020). The relatively thicker ovary tissues of the female gametophyte provide a protective environment that protects it from moderate abiotic stresses (Zinn et al., 2010; Hedhly, 2011). However, the lack of protective tissue in pollen makes them more sensitive to even mild abiotic stress (Bedinger, 1992; Pacini and Dolferus, 2016; Lohani et al., 2020).

*Heat stress effects on gametophyte performance are translated into lower seed quantity but not quality*

The negative downstream consequences of gametophyte temperature stress-altered performance on seed quality and quantity have long been suggested but have been tested in a limited number of

experimental studies, almost exclusively on crops or model species (e.g., *Arabidopsis thaliana*, Huang et al., 2014; wheat, Prasad and Djanaguiraman, 2014; rice, Ye et al., 2015; sorghum, Djanaguiraman et al., 2018). One of the most interesting results of our study is the significant reduction in seed production in wild *Silene* species (Figure 21A) exposed to CHS. We attribute these results mainly to the fact that stressed plants produced less pollen and ovules in the 35/30 °C and 40/35 °C CHS treatments (Figures 19B and 20B), which reduced the chances of successful pollination and fertilization. Furthermore, heat stress could impair pollen germination and tube growth (Walters and Isaacs, 2023), reduce pollen or ovule viability and/or stigma receptivity, leading to floret infertility (Nguyen et al., 2013; Prasad and Djanaguiraman, 2014). Alternatively, the lowered overall plant performance (that is, the lower photosynthetic rates in both heat treatments; Figure 17) could have also contributed to the lower number of seeds, since the plant's ability to produce and store the necessary resources needed for seed production was reduced (see, e.g., Poudyal et al., 2018; Sommer et al., 2023).

In contrast to seed production, the average seed mass was not affected by the different CHS treatments (Figure 21B). Several studies have shown that the environmentally induced reduction in seed production may be compensated by the increase in seed mass, due to the seed number-weight trade-off (by e.g., increasing availability of more assimilates to developing seeds; Huang et al., 2017). However, the negative effects of CHS on ovules (low number and size; Figure 19) probably imposed a limit to the trade-off between seed mass and number (see, e.g., Lázaro and Larrinaga, 2018) resulting in the unaffected seed mass in four focal *Silene* species. Although seed numbers are generally more directly affected by reproductive processes and seed set, seed mass is a product of seed-filling rate and duration (Prasad et al., 2008). In our study, seed filling and maturation of treated *Silene* plants occurred after the CHS treatment ended leaving these states unaffected by heat stress. In addition, seed mass is largely dependent on the availability of photosynthetic reserves (e.g., water

and nutrients) during the seed-filling stage (Sehgal et al., 2018). Since water and nutrients were not limiting factors throughout our study, the lower number of seeds probably did not affect the source-sink balance of resources and thus did not have a major impact on seed mass (see, e.g., Wardlaw et al., 1980). Moreover, as an evolutionary adaptation, seed mass is generally more conservative than seed number for most species (Sadras, 2007).

Analyzing the seed germination process of the treated plants, we found no significant effects of heat treatment on the establishment of *Silene* seedlings. Although we detected significant differences in the final germination percentage (+5% Figure 22B) and the germination speed (full germination 0.3–0.4 days earlier in both heat treatments than in the control, Figure 22C), we assume that the detected deviations, most likely, would not have any impact on plant establishment in wild populations. We attribute the almost unchanged seed germination process in the experimental plants, similarly to the unaltered seed mass patterns, to the fact that seed filling and maturation were not affected by the heat treatments.

**Conclusions**

In conclusion, our study adds a new important piece of evidence that heat waves associated with climate change can have significant and complex impacts on the reproductive process in wild plants. Specifically, we demonstrated that heat stress during gametogenesis can have negative cascading effects on seed quantity. These findings further imply that producing fewer seeds in a warming climate would inevitably result in a lower number of individuals that can; disperse to colonize new areas (Soons and Heil, 2002), successfully germinate and recruit in time and space (Jakobsson and Eriksson, 2000), and persist in changing environmental conditions (Long et al., 2015). This can decrease the species' ability to persist and expand its range, resulting in a more concentrated population with smaller population size and reduced genetic diversity over time (Long et al., 2015; Schierenbeck, 2017). Limited dispersal and persistent abilities alone or in combination with low

genetic variation can limit the ability of a population to adapt to changing environmental conditions or to resist diseases and pests, making them more vulnerable to extinction (Aitken et al., 2008; Schierenbeck, 2017).

Finally, we admit that our findings cannot be extended to all wild plant species due to the study's limited scope (we only examined four herbaceous species within one genus, which inhabit diverse climates and non-forest habitats in Europe). Thus, caution should be exercised in applying these results to wild plants in general. Consequently, additional research should be conducted to establish a more comprehensive understanding of the impacts of heat waves on wild plants, encompassing a broader array of genotypes and/or species.

# Chapter 5: Adaptation and acclimation of gametophytic traits to heat stress in a widely distributed wild plant along a steep climatic gradient


## Abstract

Climate change-induced heat waves often result in reduced seed yields and quality via high-temperature effects in the gametophytic phase. Surprisingly, the ability of pollen and ovules, particularly among wild plant populations, to adapt or acclimate to heat stress remains poorly understood.

To address this gap, we examined the adaptive and acclimation potential of six gametophytic traits in eleven distinct populations of wild *Silene vulgaris* across a temperature gradient in Europe.

First, we cultivated plants in a common garden to reveal how their gametophytic traits adapt to local conditions. Next, we assessed the acclimation potential of these traits to heat stress by subjecting flowering plants to two chronic heat stress (CHS) treatments: moderate (35/30 °C) and severe (40/35 °C), for 18 days.

Findings from the common garden experiment indicated no intraspecific variation in gametophytic traits across the temperature gradient, suggesting that these traits may not influence the plant's sexual adaptation to its local habitat. Plants originating from colder climates exhibited larger seed production and mass than those from warmer climates.

During CHS treatments, the female gametophyte demonstrated a greater ability to acclimate compared to the male gametophyte. Moderate CHS led to larger ovaries with more, large-sized ovules, while severe CHS reduced ovule numbers but increased their size. In contrast, both CHS treatments decreased pollen grain numbers, size, and anther length, with severe CHS causing more significant reductions. These reductions in gametophytic traits ultimately translated to lower seed quantities and quality which may threaten the long-term survival of wild plant populations.

Under both CHS treatments, the acclimation potential did not vary among plant populations along the temperature gradient, except for pollen size under severe CHS, with larger pollen size in warmer climates than in colder regions.

Our findings suggest that the lack of adaptation and acclimation mechanisms in the gametophytic traits of wild *Silene vulgaris* populations indicates that the plants may rely on alternative strategies, such as shifts in flowering time and phenotypic plasticity, to cope with climate change-induced heat waves.

Keywords: *Silene vulgaris*, climate change, local adaptation, acclimation, gametophytes, seed, heat waves, climatic gradient

## Introduction

In the face of rising global temperatures, heat waves have become increasingly severe and frequent, impacting the growth and development of plants. The sensitivity of crucial stages in plant development, such as the gametophytic phase, to heat stress can negatively affect reproductive processes (Hedhly, 2011; Sinha et al., 2021; Yadav et al., 2022). Particularly, the heat-wave effects on pistils, anthers and, therefore, ovules and pollen grains, often leads to diminished seed yields and compromised quality (Hatfield and Prueger, 2015; Raza et al., 2019; Tushabe et al., 2023). Consequently, the altered reproductive performance not only jeopardizes food security by causing significant reductions in crop yields (Kumar, 2016; Seppelt et al., 2022) but also carries ecological consequences. As for the former, estimated crop yields are expected to decline 30% by 2050 (Bapna et al., 2019). As for the latter, altered seed production might cause declines in plant population sizes and disrupt plant-animal interaction across various trophic levels associated with natural populations (Willis et al., 2008; Bogdziewicz et al., 2016).

The ability of species to endure consequences of heat waves mainly relies on either migration to suitable habitats or local adaptation (Pecl et al., 2017; Åkesson et al., 2021). In most cases, migration is not a viable option, due to extensive seed dispersal distances (Ellis, 2015) or lack of suitable new habitats (Rumpf et al., 2018). In this regard, local adaptation and acclimation emerge as crucial survival strategies (Kleine et al., 2021; Wadgymar et al., 2022). While local adaptation involves genetic changes over generations to better suit specific environments (Rehfeldt et al., 2002; Savolainen et al., 2007), acclimation entails short-term physiological adjustments to cope with immediate environmental changes within a plant's lifetime (Kleine et al., 2021). Both local adaptation and acclimation can enhance heat tolerance through various plant traits. These include maintaining essential physiological processes (e.g., efficient water use or improved photosynthetic mechanisms (Vincent et al., 2020)), phenotypic traits (e.g., early, or delayed flowering and or

changes in leaf and root morphology (Cook et al., 2012)), and genetic adaptations (e.g., increased production of heat shock proteins (Hasanuzzaman et al., 2013)). As for plant sexual reproduction, previous research has shown that species and populations from climates experiencing frequent and/or severe heat waves tend to have higher heat tolerance of their gametophytic traits (Lancaster and Humphreys, 2020; McDonald et al., 2023). Notably, these studies suggest varying tolerance levels, with species from colder regions showing greater cold tolerance, and those from warmer areas displaying higher thermal tolerance (Rosbakh et al., 2016; Lancaster and Humphreys, 2020; Sentinella et al., 2020). Yet, the contribution and relative importance of adaptive changes and acclimation in plant gametophytic responses to heat stress still remains poorly understood. To begin with, existing studies have mainly focused on estimating intra- and interspecific variation of gametophytic traits related to heat tolerance across complex ecological gradients (Di Biase et al., 2021; Kang et al., 2022; Amimi et al., 2023). Although such studies inform us about potential range of plant gametophytic heat tolerance, there is often a challenge in distinguishing the relative contribution of local adaptation and acclimation, because plants usually grow under field conditions that are difficult to control. This limitation can be lifted by cultivating plants representing different populations and species under controlled conditions (i.e., common garden experiment; Kahl et al., 2019; Tushabe et al., 2023), yet such experimental research has been limited either to cultivated species (see e.g., Hedhly, 2011) or indoor-grown model species (e.g., *Arabidopsis thaliana*; Scheepens et al., 2018). Importantly, just a few of them have considered ovule and pollen traits (e.g., Flores-Rentería et al., 2018), with most of the studies focusing on flowering phenology (e.g., Scheepens and Stöcklin, 2013; Arnold et al., 2022) and/or seed traits (e.g., Zhou et al., 2021; Amimi et al., 2023). As a result, our understanding of adaptability and acclimation of gametophytic traits, which are the most sensitive to temperature stress (Hedhly, 2011), in wild plant populations is still lacking.

Here, we close these gaps by testing the adaptive and acclimation potential of six gametophytic traits measured to heat stress in eleven populations of wild *Silene vulgaris* (bladder campion), occurring along a steep climatic gradient of temperature in Europe. In the first part of our experimental study, we cultivated plants in a common garden, to reveal potential long-term adaptations in the gametophytic traits to the local growing conditions. We hypothesized that plants originating from colder climates tend to produce smaller anthers and ovaries with fewer and smaller-sized pollen and ovules, respectively ($H_1$). The relatively smaller investment into gametophytic tissues of cold-adapted plants should be therefore reflected in lower seed production, both in terms of size and number, compared to their counterparts from warmer climates. This hypothesis is based on the assumption that plant regeneration is constrained in cold environments due to the resource-intensive nature of flowers, gametophytes, and seeds, acting as resource sinks (e.g., Obeso, 2002).

Next, we investigated the acclimation potential of the same gametophytic traits across the study populations by exposing the flowering plants to experimental chronic heat stress (CHS) treatments (35/30 °C [moderate] and 40/35 °C [severe], both lasting 18 days). The moderate CHS treatment represents typical heat waves that plants, especially those in the southern regions, may have already encountered (Table 1; Lhotka and Kyselý, 2022). In contrast, the severe stress represents potential future heat waves that the plants may not have acclimated or adapted to yet (Lin et al., 2022). In response to moderate CHS treatment (35/30 °C), we expected that gametophytic traits in plant populations from warmer climates, experiencing more frequent heat waves, would exhibit a better ability to acclimate ($H_2$). This is attributed to the presence of acclimation mechanisms developed in response to previous encounters with similar temperatures (e.g., McDonald et al., 2023), enabling these plants to sustain the production of normal-sized anthers, ovaries, pollen, and ovules, resulting in unaltered seed production. Conversely, in plants from colder climates where heat waves are less common, we expected a reduced capacity for acclimation in gametophytic traits due to the lack of

previous exposure to such temperatures and the consequent lack of pre-acclimation mechanisms (Nievola et al., 2017). As a result, these plants might produce smaller anthers and ovaries containing fewer and smaller-sized pollen and ovules respectively, potentially resulting in altered seed production and size.

Under severe CHS treatment (40/35 °C), we hypothesized that all populations would show reduced acclimation potential ($H_3$). The rarity of exposure to such extreme stress conditions in these plants hinders the development of acclimation mechanisms. Moreover, the physiological constraints imposed by temperatures at 40 °C including the disruption of cellular membranes, protein denaturation, oxidative stress, and impaired gene regulation further contribute to the inability of plants to acclimate to such extreme temperatures (e.g., Araújo et al., 2013; Hasanuzzaman et al., 2013). This is expected to have negative impacts on gametophytic traits, such as reduced pollen and ovule production, ultimately resulting in reduced seed quantity and quality.

## Methods and materials

### *Study species and experimental setup*

*Silene vulgaris* (Moench) Garcke (Caryophyllaceae), commonly known as bladder campion, is a short-lived herbaceous perennial plant widely distributed across diverse geographical regions, ranging from subarctic to temperate zones (Rabinowitz et al., 1986). It is indigenous to Europe, Asia, and North Africa, with a global presence due to introductions in different regions (USDA NRCS, 2023). Its primary habitats include open grasslands, meadows, woodland edges, as well as disturbed areas such as roadsides, waste places, and metalliferous soils (Marsden-Jones and Turrill, 1957; Friedrich, 1979). The plant exhibits a clump-forming growth habit, with erect or sprawling stems that can reach heights of up to sixty centimeters.

The genus *Silene* has been widely utilized in ecological research due to its favorable attributes, including ease of cultivation and short life cycles, facilitating experimental investigations (Alatalo and Totland, 1997; Bernasconi et al., 2009; Ferrarini et al., 2019; Kahl et al., 2019). *Silene vulgaris* particularly provides a unique opportunity to explore how wild plants locally adapt and vary along thermal gradients (e.g., Kahl et al., 2019).

The seeds were collected from eleven populations of *Silene vulgaris* across locations in Austria, Czech Republic, Germany, France, Spain, and Sweden (see Table 8, Figure 23). These populations represent a climatic gradient of *S. vulgaris* in Europe, exhibiting plant sexual reproduction adaptations to varying temperature conditions. All selected populations have hermaphroditic flowers (i.e., both male and female reproductive organs within a single flower) and a short seed-to-maturity period of about seven weeks.

Table 8: Origins of the eleven European *Silene vulgaris* populations, including the mean annual temperatures in their respective locations, the mean number of days with temperatures at or exceeding 35 °C and 40 °C, the mean temperature during the flowering period, and the months of flowering period. The climatic data are means from 2000 to 2020, derived from the European climatic database. The flowering period data are sourced from GBIF occurrence records.

| Population | Country | Site | Latitude | Longitude | Mean annual temperature (°C) | Mean days ≥35 (°C) | Mean days ≥40 (°C) | Mean temperature of flowering period (°C) | Flowering period (months) |
|---|---|---|---|---|---|---|---|---|---|
| SE98 | Austria | Innsbruck | 47°16'14.6"N | 11°24'39.7"E | 5.7 | 3 | 0 | 26 | June-August |
| SE84 | Sweden | Vickleby | 56°34′37.1″N | 16°27′39.5″E | 7.6 | 0 | 0 | 20.5 | June-August |
| SE31 | Czech | Brno | 49°11'45.4"N | 16°36'31.8"E | 8.4 | 4 | 0 | 24 .7 | May-July |
| SE91 | Germany | Konstanz | 47°40'42.5"N | 9°10'30.0"E | 9.2 | 4 | 0 | 24 | May-July |
| SE86 | France | Normandie | 49°16′06.0″N | 01°37′39.0″E | 10.3 | 3 | 1 | 22.2 | May-July |
| SE49 | Spain | La Gueria Carrocera | 43°19'02.0"N | 5°39'45.6"W | 11.4 | 0 | 0 | 21.3 | May-July |
| SE83 | France | La Noue du Bourg | 46°39′28.0″N | 01°23′13.6″W | 12 | 2 | 0 | 20 | April-June |
| SE35 | Spain | Madrid | 40°24'55.0"N | 3°42'45.2"W | 13.8 | 28 | 2 | 24.5 | April-June |
| SE72 | Spain | La Sentiu de Sió | 41°48'18.7"N | 0°52'43.6"E | 14.4 | 22 | 3 | 25 | April-June |
| SE80 | Spain | La Palma | 28°46′58.0″N | 17°56′22.0″W | 14.5 | 0 | 0 | 18 | April-June |
| SE82 | Spain | Alcanó | 41°29′29.4″N | 00°26′27.3″E | 15 | 25 | 2 | 26 | April-June |

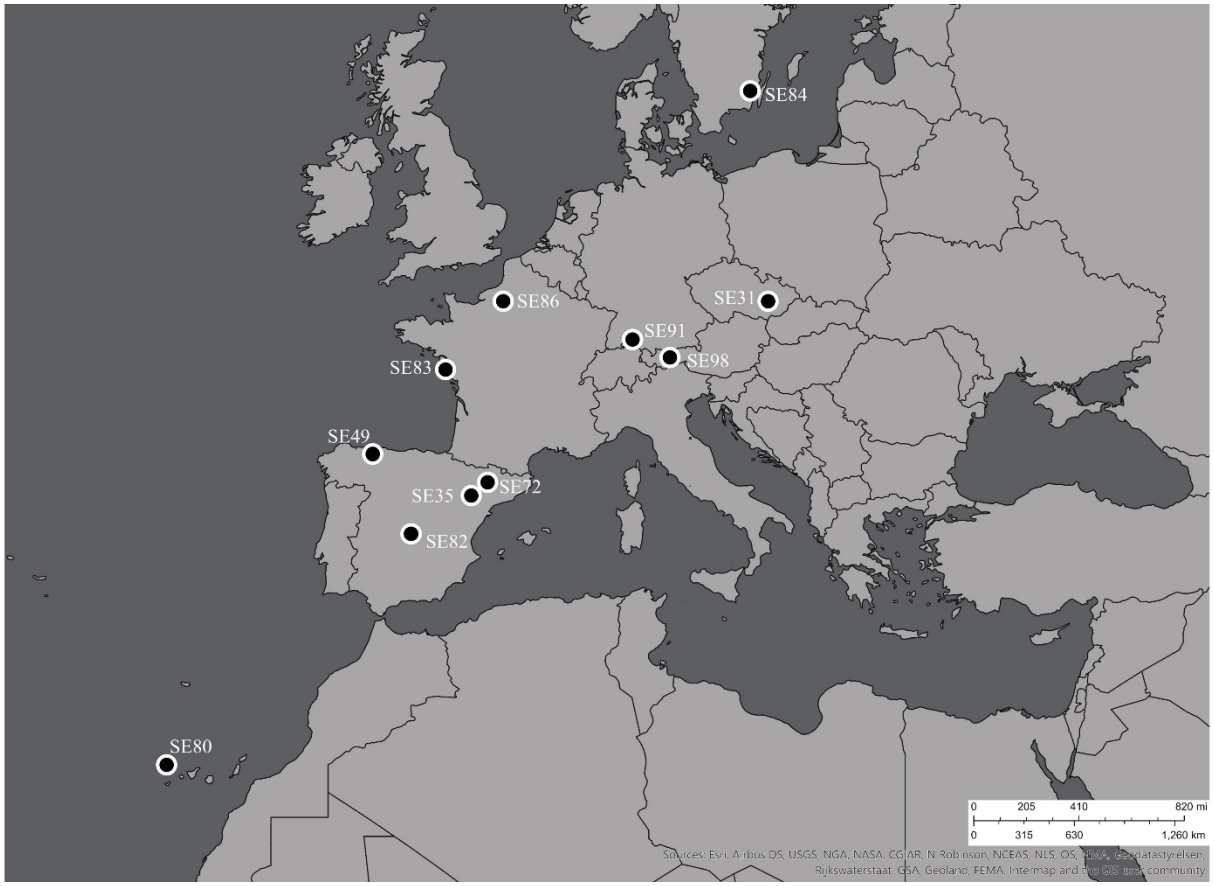


Figure 23: Sampling sites of the eleven *Silene vulgaris* populations across six different European countries. Map created with Esri ArcGIS Online.

The experiment was conducted in a greenhouse from November 2022 to July 2023, under fully controlled conditions. To eliminate potential maternal effects, we cultivated F1 plants from the seeds collected in the field from each *S. vulgaris* population. The plants were cultivated in trays (60 cm × 40 cm) containing a substrate comprised of three parts low-nutrient planting soil (CL Pikier-Einheitserde), three parts coarse sand (Geser GmbH, Germany), and one-part dry compost soil (CL Topf Einheitserde). Plants from distinct populations were bagged before the onset of flowering to prevent cross-pollination.

Thereafter, the F1 seeds were planted in a similar soil substrate as described above. Approximately three weeks after germination, the young plants were individually transplanted into 9 cm pots. Only healthy plants in comparable phenological states were chosen during the repotting process to ensure consistent treatment effects across all tested individuals.

Within the greenhouse, the pots were randomly rearranged and consistently watered during germination and cultivation. All plants experienced similar greenhouse conditions, with day/night temperatures of 20/15 °C and natural illumination supplemented by additional lighting (Osram Plantastar 400 W, Osram, China) to achieve a 12-hour photoperiod in winter months (November to April). This setup served as the common garden, to investigate the adaptation of gametophytic traits to local growing conditions.

After the emergence of the initial flower bud, plants at similar phenological stage were randomly selected for the CHS experiment, following a random-block design.

The setup for the experiment consisted of six identical grow chambers (Homebox Vista Medium, HOMEbox, Germany). Each chamber was equipped with two heating mats ('Fyto heat Deluxe', Schilling Phytotechnik GmbH, Italy, and 'Heating mat aluminium', Bio Green, Germany) to raise the temperature to specified levels. In addition, three photosynthesis-powered LED lamps ('Sanlight Flex 20W', SANlight, Austria) were used for illumination. Thermostats ('Thermo2', Bio Green, Germany) in each chamber and a control panel ('dnt RoomLogg Pro', dnt, China) were used for temperature measurement and regulation, ensuring a consistent environment.

Two chambers were maintained at day/night temperatures of 35/30 °C, representing moderate chronic heat stress (CHS), while another two chambers were set at 40/35 °C, indicating severe CHS. Two chambers at 20/15 °C served as the control group. The moderate and severe CHS chambers were used to investigate the acclimation potential of the gametophytic traits, aligning with observed

and projected heat waves that European plants are currently experiencing or are expected to encounter during the process of sexual reproduction (Lin et al., 2022; Lhotka and Kyselý, 2022). In each chamber, 10 to 24 plants per population were cultivated for 18 days, ensuring stress treatment at the different developmental stages of pollen and flowers (Mesihovic et al. 2016).

To prevent drought stress linked to elevated temperatures, we maintained a consistent soil moisture level by regular watering during the application of heat stress. After the heat treatments, the treated plants were brought back to the greenhouse under constant temperatures of 20/15 °C, and natural light was supplemented with additional lights (Osram Plantastar 400W, Osram, China) to facilitate seed maturation.

During cultivation, the plants in the control group had an aphid infestation and therefore received treatment with Karate zeon (Karate Zeon, Syngenta, Switzerland).

*Plant trait measurements*

To assess the impact of the treatments on the overall plant performance, we measured leaf chlorophyll fluorescence (expressed as the maximum quantum yield (efficiency) of PS II photochemistry (Fv/Fm)) using Pocket PEA tool (Hansatech, Germany) on the final day (day 18) of the treatment period.

*Gametophytic traits*

To assess the impact of heat treatment on sexual reproduction, we measured six traits related to both male (anther length, pollen production, and size) and female (ovary length, ovule production, and size) gametophytic performance. On day 18 of the heat treatments, we selected open flowers at full anthesis per accession per treatment and control, preserving them in 70% ethanol for further analysis. In the laboratory, ovaries and anthers were randomly selected from the dissected flowers and

photographed for measurements under a stereomicroscope. ImageJ software (Schneider et al., 2012) was then used to measure anther length (average of both thecae lengths (Vries, 1974)) and ovary length (sum of proximal length, placenta length, and distal length (Damodharan et al., 2016)).

The ovaries were further dissected under the stereomicroscope, and ovule images were captured and counted using ImageJ. Ovule size was also determined with the same software. For pollen size and number, one entire anther per replicate was crushed in 100 µl of deionized water, and 5 µl of the solution was pipetted onto a microscope slide. Scaled images were taken and analysed under a Nikon Eclipse TS100 microscope, and ImageJ was used to determine pollen size and number. The pollen count in the 5 µl solution was then utilized to estimate the overall number of pollen grains released per anther in the stock solution (100 µl).

*Seed traits*

To evaluate the subsequent impacts of heat stress on gametophytic performance, we measured seed mass and seed production in the treated plants. To accomplish this, the treated plants were kept in the greenhouse for approximately three months until complete seed maturation. During this period, the plants were self-hand-pollinated to ensure the reproductive success of plants. Additionally, the plants were covered with organza bags to prevent any loss of seeds. Due to low seed production, it was not feasible to collect seeds from individual plants. Instead, all seeds from each accession were combined, and the corresponding seed mass and number were determined.

*Data analysis*

Data analyses were conducted using R software version 4.3.0 (R Core Team, 2023).

*Temperature conditions in seed collection sites*

To characterize the temperature conditions that each study population experiences during fertilization across the sampled gradient, we calculated the average temperature of the flowering period and identified occurrences of heat waves. To begin with, we extracted species occurrence data per population from the Global Biodiversity Information Facility (GBIF). Our initial assumption was that the GBIF occurrence data were derived from images and herbarium specimens of flowering individuals. Visual inspection of randomly selected images and digitalized herbarium specimens confirmed that assumption at the subsequent stage. Only human observations and preserved specimens with georeferenced locations from the years 2000 to 2020 were considered. This time frame was chosen due to the relatively short lifespan of *Silene vulgaris*, making it more relevant to gather information during the 21$^{st}$ century when significant global climate changes have occurred. The data included details such as the year, month, and days of observation. The flowering period was determined as the month with the highest number of observations, along with the month prior and after it (a total of three months).

Based on the flowering phenology data, we obtained the maximum temperature values for the respective locations within the period from 2000 to 2020. This extraction was performed using the R package *easy climate* (Cruz-Alonso et al., 2023), which relies on high-resolution (1 km) daily climate data sourced from the European climatic database. The mean temperature of the flowering period (MTFP) was computed based on temperatures recorded in the month with the highest number of observations, along with the month prior and after it.

To determine the occurrence of heat waves, we calculated the mean number of days with temperatures at or exceeding 35 °C and 40 °C per accession at the corresponding locations over the period from 2000 to 2020.

*Statistical analysis*

To estimate the variability of local adaptation and acclimation potential of the gametophytic traits within study populations across the climatic gradient, we fitted a linear mixed-effects model using the package *lme4* (Bates et al., 2015). The model used one of the measured traits (e.g., chlorophyll fluorescence, gametophyte, or seed trait) as the response variable. The fixed effects included the mean temperature of the flowering period (MTFP), moderate and severe CHS treatments, the control temperature, and their interaction (MTFP*Treatments). To account for individual variability in the response of gametophytic traits, the origin of every plant population accession was included in all models as a random intercept. We visualized how the relationship between mean temperature of the flowering period and gametophytic traits varies across the different heat stress treatments and the control with an interaction plot created using the *interactions* package in R (Long, 2019). Local adaptation was assessed by examining the variability in the control regression slope across the temperature gradient (MTFP). Acclimation was determined by considering both the overall treatment effects and the differences in regression lines between the control and CHS treatments.

Differences in local adaptation and acclimation of gametophytic traits among plant groups from different thermal environments were estimated with the help of the post hoc Tukey test ($p<0.05$), implemented in the packages *emmeans* and *multcomp* (Hothorn et al., 2008; Lenth, 2023).

**Results**

*Leaf chlorophyll fluorescence*

The application of both CHS treatments had a significant negative impact on the overall vegetative plant performance regardless plant origin, with the strongest effects in severe CHS treatment (mean Fv/Fm values: control = 0.82, moderate CHS = 0.795, and severe CHS = 0.774; Figure 24, Table 9).

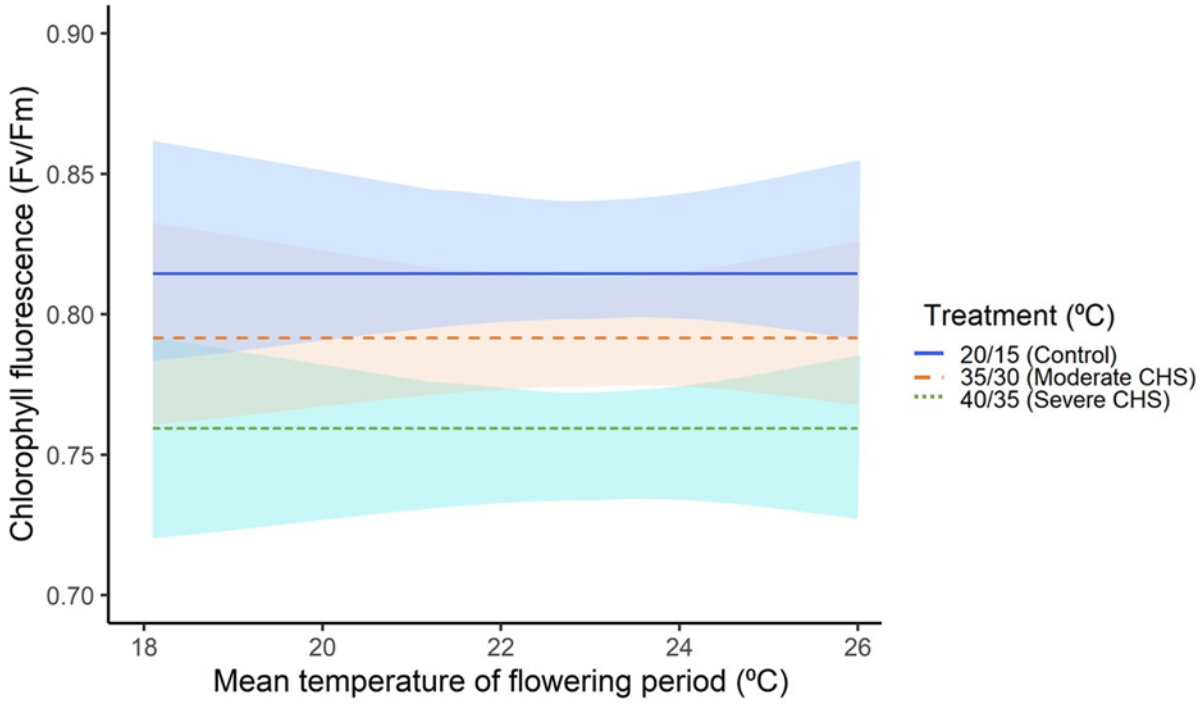


Figure 24: Variation in chlorophyll fluorescence (Fv/Fm) values in eleven *Silene vulgaris* populations under two chronic heat stress treatments (moderate and severe), along with a control group, across mean temperature of flowering period. Shaded areas indicate a 95% confidence interval.

*Female gametophytic traits*

In the common garden experiment ($H_1$), there were no differences in female gametophytic traits (ovary length, ovule production, and ovule size) in plants from different climates across the temperature gradient, as the slopes of the corresponding regression lines were not significantly different from zero (Figure 25, Table 9).

When exposed to moderate CHS treatments ($H_2$), plants showed a higher ability to acclimate by producing significantly increased ovary length (3.91 mm) and larger-sized ovules (0.31 mm) compared to the control (3.64 mm and 0.27 mm, respectively; Figure 25A and C; Table 9).

In response to severe CHS treatment ($H_3$), plants exhibited a significant decrease in ovule production (66 ovules), suggesting a lack of acclimation, in contrast to both moderate CHS treatment (94) and the control (88). Contrary, ovule size in the plants showed a significantly higher ability to acclimate to severe CHS treatments, as shown by their average ovule size of 0.29 mm compared to the control (0.27 mm; Figure 25B and C; Table 9).

In both moderate and severe CHS treatments, the acclimation potential of female gametophytic traits in plants from different climates showed no significant differences along the temperature gradient, as the slopes of the corresponding regression lines were not significantly different from zero (Figure 25, Table 9).

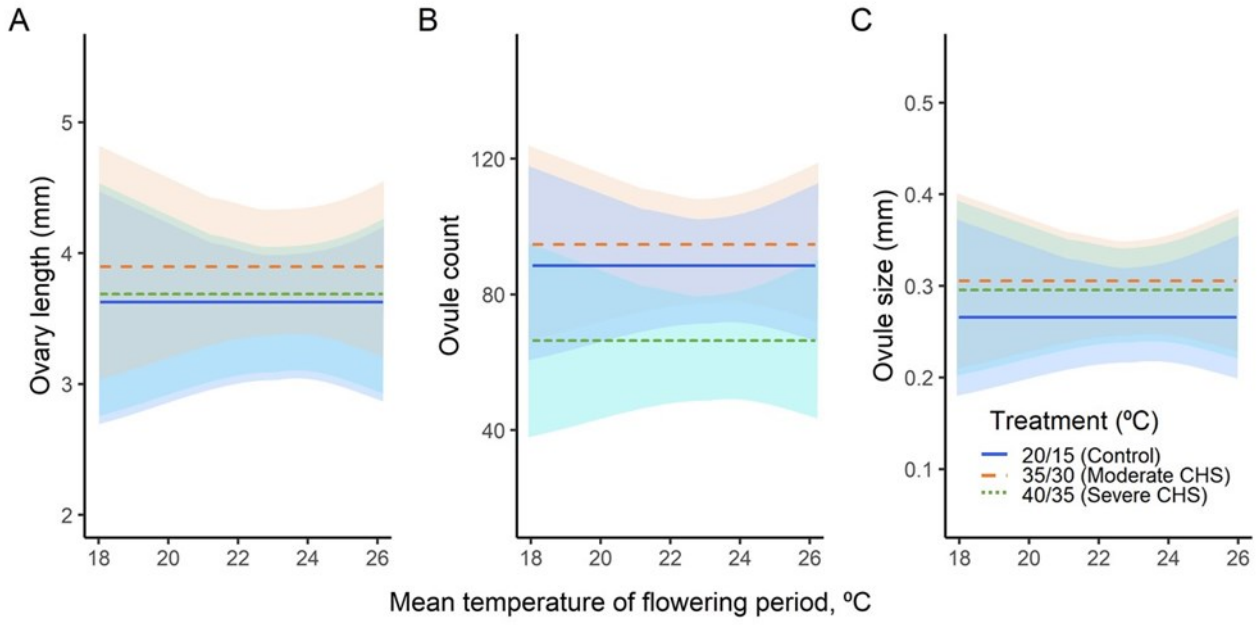


Figure 25: Variations of ovary length (A), ovule production (B), and ovule size (C) in eleven *Silene vulgaris* populations under two chronic heat stress treatments (moderate and severe), along with a control group, across mean temperature of flowering period. Shaded areas indicate a 95% confidence interval. Regression lines parallel to the x-axis indicate lack of statistical differences among the study populations.

*Male gametophytic traits*

In the common garden ($H_1$), plants originating from both warm and cold climates showed no differences in male gametophytic traits along the temperature gradient, as the slopes of the corresponding regression lines were not significantly different from zero (Figure 26, Table 9).

The acclimation potential of all three male gametophytic traits measured showed a significant negative response to both heat stress treatments, with significantly larger effect sizes in the severe CHS treatment ($H_3$) compared to the moderate CHS ($H_2$) and the control (Figure 26, Table 9). Specifically, in moderate CHS, plants showed significantly decreased ability to acclimate (i.e., anther length (2.2 mm), pollen production (1315), and pollen size (0.036 mm) compared to the control (2.5 mm, 2522 pollen, and 0.043 mm respectively).

Under severe stress conditions ($H_3$), the acclimation potential of male traits was further significantly lowered, with the mean anther length decreasing to 2.0 mm, pollen production reducing to 649, and pollen size decreasing to 0.033 mm, in contrast to the control values of 2.5 mm, 2522 pollen, and 0.043, respectively.

Plants originating from different climates did not show any significant differences in their acclimation potential in both CHS treatments along the temperature gradient, except for pollen size, which exhibited a significant positive correlation with the temperature gradient under severe CHS treatment ($p<0.05$; Figure 26C, Table 9). Cold-adapted plants showed the strongest negative effects. Plants from warmer climates demonstrated greater acclimation ability, with a 0.0015 mm increase in pollen size per degree of temperature rise.

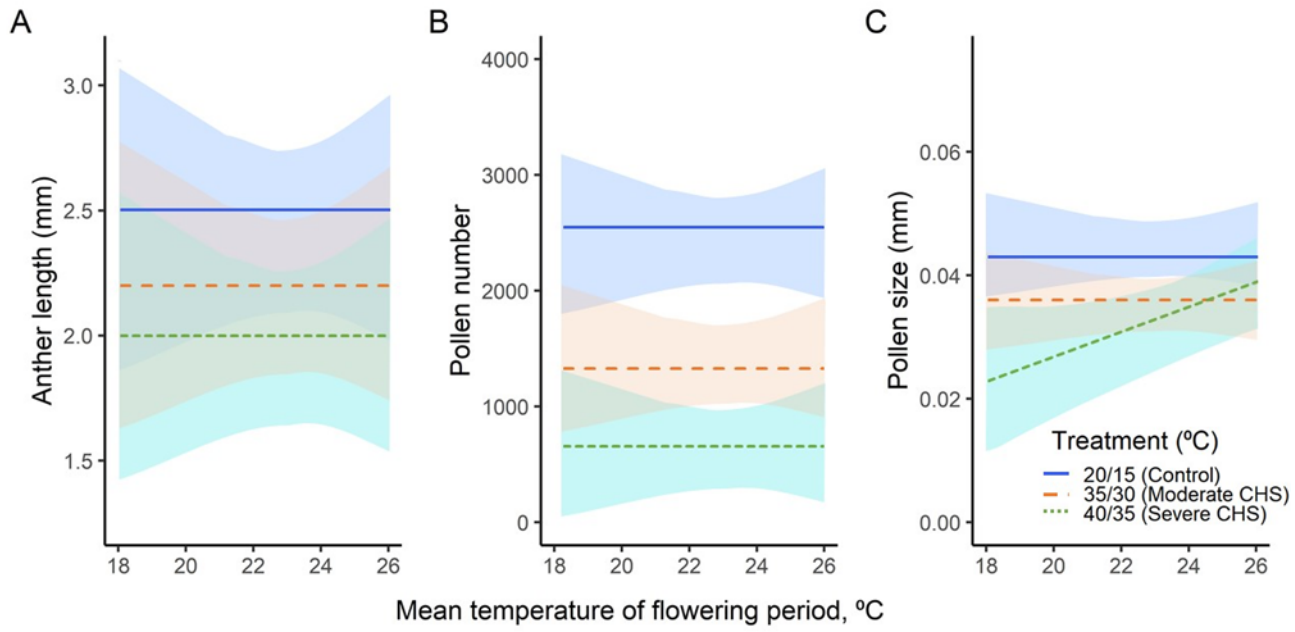


Figure 26: Variations of anther length (A), pollen production (B), and pollen size (C) in eleven *Silene vulgaris* populations under two chronic heat stress treatments (moderate and severe), along with a control group, across mean temperature of flowering period. Shaded areas indicate a 95% confidence interval. Regression lines parallel to the x-axis indicate lack of statistical differences among the study populations.

*Seed traits*

In the common garden, plants from warmer climates exhibited a significant negative decline in seed production and mass, with a reduction of 74 seeds and 0.1 g (seed mass) per degree of increasing temperature. Conversely, plants originating from colder climates tend to yield more of larger seeds ($p<0.05$; Figure 27, Table 9).

Both CHS treatments applied to the plants resulted in a significant decrease in both mean seed production and seed mass compared to the control group. Specifically, plants subjected to moderate CHS treatment yielded 13 seeds with a seed mass of 0.02 g, whereas those exposed to severe CHS

treatment produced 56 seeds with a seed mass of 0.07 g, in contrast to the control which produced 221 seeds with a seed mass of 0.3 g (Figure 27, Table 9).

Importantly, the resulting seed production and mass from plants across different populations exposed to both CHS treatments showed no differences across the temperature gradient, as the slopes of the corresponding regression lines were not significantly different from zero (Figure 27, Table 9).

Notably, warm-climate populations, characterized by their tendency to yield small seeds in smaller quantities, experienced less pronounced negative impacts on seed production and mass under high temperatures compared to plants in regions with lower temperatures.

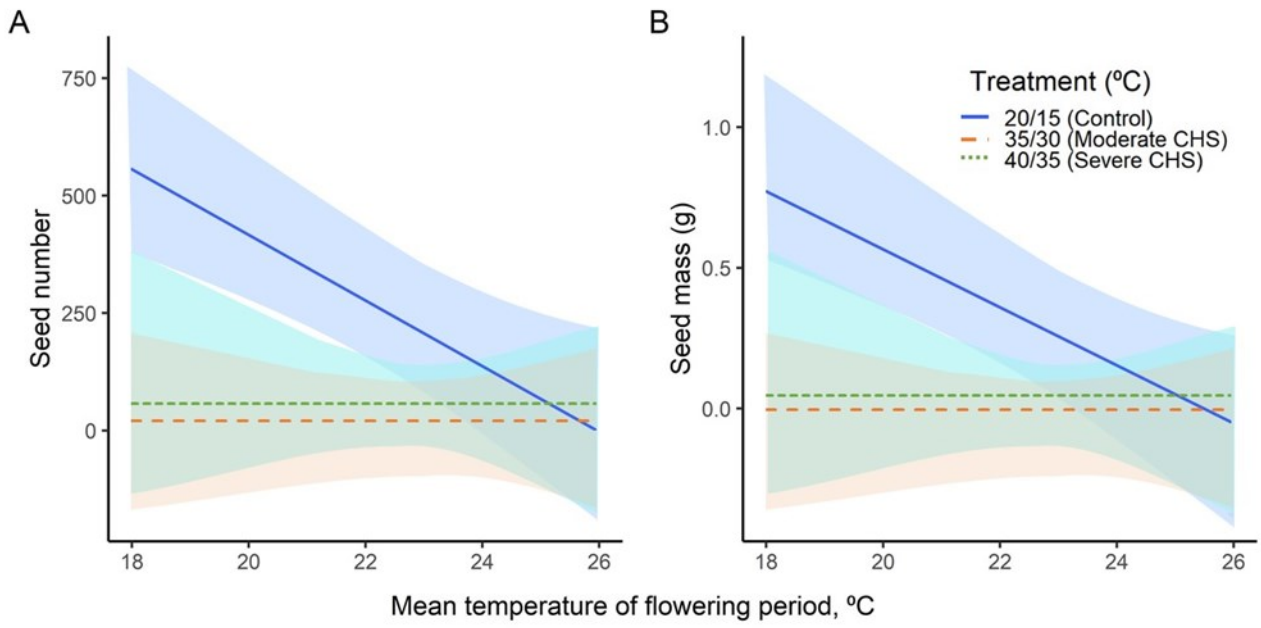


Figure 27: Variations in seed production (A) and seed mass (B) in eleven *Silene vulgaris* populations under two chronic heat stress treatments (moderate and severe), along with a control group, across mean temperature of flowering period. Shaded areas indicate a 95% confidence interval. Regression lines parallel to the x-axis indicate lack of statistical differences among the study populations.

Table 9: The adaptive (slope of the control regression) and acclimation potential (overall treatment effects and regression slopes) of traits measured (leaf chlorophyll fluorescence [Fv/Fm], female and male gametophyte, and seed traits) in the eleven wild *Silene vulgaris* populations. Results determined from generalized linear mixed-effect models and post-hoc Tukey tests. Bold values indicate significant treatment/slope effects ($p<0.05$). Different letters indicate significant differences between the control and two treatments as induced by the Tukey Post-hoc test ($p<0.05$). SE – standard error.

| Measured traits | Estimates | Control | 35/30°C | 40/35°C | Intercept |
|---|---|---|---|---|---|
| Fv/Fm | Mean | 0.82 a | **0.795** b | **0.774** c | |
| | SE± | 0.03 | 0.04 | 0.05 | **0.86** |
| | Slope | -0.002 | 0.0025 | 0.0026 | |
| Ovary length (mm) | Mean | 3.64 a | **3.91**b | 3.70 ab | |
| | SE± | 0.89 | 0.87 | 1.23 | **4.05** |
| | Slope | -0.023 | -0.037 | -0.061 | |
| Ovule count | Mean | 88 a | 94 a | **66** b | |
| | SE± | 33.09 | 32.40 | 38.95 | 57 |
| | Slope | 1.32 | -0.87 | -2.49 | |
| Ovule size (mm) | Mean | 0.27 a | **0.31** b | **0.29** bc | |
| | SE± | 0.08 | 0.09 | 0.12 | **0.36** |
| | Slope | -0.004 | -0.0065 | -0.0029 | |
| Anther length (mm) | Mean | 2.5 a | **2.2** b | **2.0** c | |
| | SE± | 0.66 | 0.56 | 0.87 | 2.15 |
| | Slope | 0.0157 | 0.0433 | -0.0613 | |
| Pollen count | Mean | 2522 a | **1315** b | **649** c | |
| | SE± | 878.34 | 832.31 | 583.58 | 2252 |
| | Slope | 9.35 | -5.62 | -20.13 | |
| Pollen size (mm) | Mean | 0.043 a | **0.036** b | **0.033** c | |
| | SE± | 0.01 | 0.01 | 0.01 | **0.04** |
| | Slope | 4.01E-05 | -3.17E-04 | **0.0015** | |
| Seed count | Mean | 221 a | **13** b | **56** b | |
| | SE± | 329.54 | 17.12 | 67.63 | **1894** |
| | Slope | **-73.55** | -4.88 | -17.51 | |
| Seed weight (g) | Mean | 0.3 a | **0.02** b | **0.07** b | |
| | SE± | 0.55 | 0.03 | 0.10 | **3.08** |
| | Slope | **-0.123** | -0.0067 | -0.026 | |

## Discussion

*Common garden experiment reveals no intraspecific variation in gametophytic traits ($H_1$)*

Contrary to our expectations, we found no significant differences in all male and female gametophytic traits measured across the temperature gradient. This finding suggests that these traits in *Silene vulgaris* do not play any role in the plant sexual adaptation to the specific conditions of their local origins. This contradicts the commonly observed variation in other traits such as vegetative traits like canopy height (Jónsdóttir et al., 2023) and specific leaf area (Rosbakh et al., 2015). While research indicates that numerous plant species exhibit local adaptation, some argue that local adaptation might be less prevalent than commonly thought (e.g., Leimu and Fischer, 2008; Hereford, 2009). For instance, a study by Ebeling et al. (2011) on *Buddleja davidii*, an ornamental shrub, found no evidence of clinal variation in growth and reproductive traits among the different populations.

The lack of clinal variation observed in the gametophytic traits assessed in our study could be attributed to several factors. Firstly, rather than altering the size and number of anthers, ovaries, pollen grains, and ovules, plants may adapt the physiology and biochemistry of these organs. For example, previous studies have shown that warm-adapted pollen tends to have higher concentrations of sugars such as glucose and fructose, more unsaturated fatty acids in lipids, and increased expression of heat-shock proteins and antioxidants to counteract heat stress (Nievola, et al., 2017; Rieu, et al., 2017). On the other hand, cold-adapted pollen tends to have lower sugar levels, increased proportions of saturated fatty acids, and synthesizes cold shock proteins or antifreeze proteins to cope with low temperatures (Satyakam et al., 2022; Jahed et al., 2023). Consequently, these adjustments rarely influence the morphological characteristics of both male and female gametophytes, as could be the case in our study. Secondly, plants possess the ability to adjust their phenological timing to optimize resource utilization under local temperature conditions, thereby

minimizing variations in gametophytic traits. For instance, a study on *Arabidopsis lyrata* revealed that southern populations flowered earlier than their northern counterparts (Riihimäki and Savolainen, 2004). Similarly, in our study, populations from southern Europe (e.g., Spain) exhibited early flowering, whereas northern populations (e.g., Sweden) flowered later (see Table 8). This difference could be a strategy for southern populations to evade heat stress associated with high temperatures in later months, while the northern populations may benefit from warmer periods (e.g., Rauschkolb et al., 2023). If plants from diverse locations have adapted to distinct phenological timings in their native environments, it suggests that their reproductive processes are already adjusted to their local conditions.

Lastly, the gametophytic traits might be phylogenetically constrained, i.e., evolutionarily conserved across related species, and thus are not subject to intraspecific variation (e.g., Emilio et al., 2021). Therefore, the lack of clinal variation in our study could be attributed to a combination of physiological/biochemical adaptation, phenological adjustments, and phylogenetic constraints.

Although gametophytic traits did not exhibit clinal variation, significant differences were observed in seed production and mass along the temperature gradient (Figure 27; Table 9). Plants originating from colder climates produced more seeds of greater mass compared to those from warmer climates. This could be attributed to the influence of priority sinks on plant growth patterns, with seeds having the highest sink strength (Wardlaw, 1990; Obeso, 2002). In colder climates, where growth and survival conditions are less favorable, plants tend to allocate more resources towards seed production as an adaptation strategy. This results in the production of larger seeds for better establishment in the unfavorable climatic conditions (Moles and Westoby, 2006; Zhou et al., 2021; Celebias and Bogdziewicz, 2023). On the contrary, within warmer southern populations, plants tend to produce smaller seeds as an adaptation to cope with the stress of higher temperatures (e.g., Zhou et al., 2021). This occurs especially as they approach the edge of their distribution range, where plants are near

their tolerance limits. Consequently, these plants allocate fewer resources to seed production, prioritizing other survival mechanisms like efficient water use and growth (e.g., Huot et al., 2014; Lauder et al., 2019; Zhou et al., 2021).

*The acclimation potential of gametophytic traits*

*Overall treatment effects*

Acclimation plays a pivotal role in enabling plants to enhance their tolerance to environmental extremes by directly modifying their physiology or morphology (Sumner et al., 2022). The gametophytes of *Silene vulgaris* in our study demonstrated the capacity for acclimation. The results revealed a higher ability to acclimate in female gametophytic traits under both moderate and severe CHS treatments with the male gametophyte exhibiting a reduced capacity to acclimate across both stress treatments.

Female gametophytes exposed to moderate heat stress ($H_2$), showed larger ovaries, and produced a larger number of larger ovules, whereas male gametophytes produced fewer and smaller pollen grains, with shorter anthers (Figures 25 and 26). The longer ovaries likely provide more space for ovules to develop, and larger ovules may contain more resources, potentially enhancing support for embryo development and increasing the likelihood of viable seed production (e.g., Strelin and Aizen, 2018; Wilkinson et al., 2019). The observed higher ability of female gametophytic traits to acclimate suggests potential trade-offs between female and male traits, where more resources are allocated to female reproductive structures to mitigate the adverse effects of the heat stress on male gametophytes. A study by Gillet and Gregorius (2020) shows that maximizing fertilization success requires more investment in ovule production than in pollen. Moreover, heat stress appeared to directly hinder physiological processes critical for optimal male gametophyte development, leading shorter anthers that produced fewer and smaller pollen grains compared to the control group (Figure 26; Hasanuzzaman et al., 2013; Kumar et al., 2022). Consequently, the reduced number and size of

pollen grains decreased the likelihood of successful pollination and fertilization, ultimately resulting in lower seed quantity and quality (Figure 27; Huang et al., 2014; Tushabe et al., 2023).

Averaged over all populations, under severe stress conditions ($H_3$), fewer but large-sized ovules, less pollen of smaller sizes, with shorter anthers were produced. These alterations in gametophyte morphology likely stem from adjustments in resource allocation to withstand the stress (Ruan et al., 2013; Brock et al., 2017). The fewer yet larger ovules, indicate a shift in resources allocation towards producing fewer but potentially more resilient or viable ovules. This reallocation suggests that larger ovules may have a higher chance of survival or successful fertilization in stressful environments (Gillet and Gregorius, 2020). Conversely, the decrease in pollen production, smaller pollen size, and shorter anther length indicates a reduced investment in male gametophytic structures, likely due to resource scarcity and physiological constraints imposed by severe stress, hindering pollen formation and viability (Müller and Rieu, 2016; Gillet and Gregorius, 2020; Chaturvedi et al., 2021). A similar study showed that male reproduction of *Pinus edulis*, was negatively affected by high temperatures at 40 $^{o}$C, with stronger effects during pollen germination (Flores-Rentería et al., 2018).

Under both CHS treatments, female gametophytic traits showed greater resilience to short-term heat stress compared to their male counterparts. These finding further confirm the observation that male traits are more susceptible to stress (e.g., Zinn et al., 2010; Chaturvedi et al., 2021; Tushabe et al., 2023). Female gametophytes with thicker ovary tissues are better protected from abiotic stresses (Zinn et al., 2010; Hedhly, 2011), whereas pollen lacks this protection, making it more sensitive to even mild stressors (Bedinger, 1992; Pacini and Dolferus, 2016; Lohani et al., 2020). Furthermore, pollen and tapetum cells show a high demand for energy, indicated by the numerous mitochondria in their cells. Consequently, depletion in energy reserves such as starch and sugar accumulation typically observed under stress conditions, may affect pollen more than other cells (Müller and Rieu, 2016). Additionally, it is proposed that prioritizing the development of the female gametophyte under stressful conditions rather than male gametes could be advantageous (Müller and Rieu, 2016).

This strategy could promote outcrossing, leading to greater genetic diversity among progeny and enhancing the chances of genetic adaptation to unfavourable environments (Boyko et al., 2010; Beaudry et al., 2020).

Our study further found that the gametophytic acclimation responses were not effective in coping with both CHS treatments, resulting in a significant decrease in both seed size and number. It is also possible that the heat treatments were too intense for these adaptive mechanisms to counteract the stress. This reduction in ovule and pollen production, resulted in fewer and lower-quality seeds, also reported in previous studies (e.g., Huang et al., 2014; Prasad and Djanaguiraman, 2014; Djanaguiraman et al., 2018). Moreover, the lowered overall performance of the plants, as indicated by lower photosynthetic rates in both heat treatments (Figure 24), likely contributed to the reduced seed production, as the plants' capacity to generate and store the essential resources required for seed production was reduced (see, e.g., Poudyal et al., 2018; Sommer et al., 2023). Under high temperature stress, plants may allocate more resources towards growth or survival traits rather than reproduction, potentially enhancing plant fitness (Huot et al., 2014). Supporting evidence for this is found in studies showing a decrease in auxin levels in gametophytes during heat stress, while auxin levels increase in vegetative tissues (Sakata et al., 2010; Sharma et al., 2018).

*Acclimation potential of gametophytic traits along the temperature gradient*

All plants from different climates did not show any differences in their acclimation potential to both CHS treatments along the temperature gradient, except for pollen size under severe CHS treatment (Figure 26C). Specifically, plants from warmer climates showed a greater acclimation ability, with larger pollen size in higher temperatures compared to those from colder areas. High temperatures lead to increased evapotranspiration rates, which can rapidly desiccate pollen grains, reducing their viability and thus affecting successful pollination. Therefore, the optimal strategy for plants flowering under high temperatures is to produce fewer but larger pollen grains that are more resilient

to volume changes, as observed in our study and supported by Ejsmond et al. (2011). Their study on eight Rosaceae species found that the plants flowering in high temperatures and high evapotranspiration conditions produced significantly larger pollen grains compared to those in lower temperature and lower evapotranspiration conditions (Ejsmond et al., 2011). Moreover, producing larger pollen could also be a pre-adaptative strategy as larger pollen grains may have increased competitive advantages when interacting with the stigma (Ejsmond et al., 2015). Therefore, warm-adapted plants might prioritize resource allocation towards pollen size rather than pollen number.

The lack of differences in acclimation potential for the other traits across the temperature gradient may be due to similar factors explained in the common garden experiment (see above). These factors include plants prioritizing adaptations in gametophyte physiology and biochemistry over morphological changes in size and quantity (e.g., Nievola, et al., 2017; Rieu, et al., 2017), or adjusting their phenology timings to optimize resource utilization and enhance survival in diverse environments (e.g., Cook et al., 2012; Gugger et al., 2015). Additionally, gametophytic traits might be phylogenetically constrained, thus limiting variations across climatic gradients (e.g., Emilio et al., 2021).

**Conclusion**

The evaluation of the male (anther length, pollen production, and size) and female (ovary length, ovule production, and size) gametophytic traits of wild *Silene vulgaris* populations in response to heat stress revealed a lack of adaptation and/or acclimation mechanisms. This prompts the question of how gametophytes in natural plant populations, especially in southern regions, cope with heat stress. One plausible explanation is the presence of alternative mechanisms or adaptive strategies, such as alterations in flowering phenology, enabling survival without specific gametophytic adaptations/variations. However, these strategies have limitations due to physiological and, possibly, genetic constraints. For instance, inconsistencies in environmental cues for flowering or sudden

environmental changes can disrupt flowering timing, leading to mismatches with optimal gametophytic performance affecting reproductive success (e.g., Fu et al., 2022; Elmendorf and Hollister, 2023). The negative effects on the gametophyte performance can then be translated to reduced seed numbers and quantity, also observed in this study. Producing fewer seeds can lead to reduced dispersal, germination success, and adaptation abilities for plants (Jakobsson and Eriksson, 2000; Soons and Heil, 2002; Long et al., 2015). This could result in smaller, less diverse populations, making species more vulnerable to extinction due to limited abilities to adapt to changing conditions (Long et al., 2015; Schierenbeck, 2017). Consequently, further research into alternative adaptive strategies and mechanisms, including phenotypic plasticity, which was not accounted for in this study, is crucial for gaining insights into the resilience of natural plant populations in the face of ongoing climate change.

# Chapter 6: Conclusions and perspectives

The impacts of climate change are expected to adversely affect plant sexual reproduction, with broader implications for plant biology, agriculture, and conservation efforts. Temperature alterations are among the most crucial factors influencing plant reproduction, given their direct impact on physiological processes such as flowering timing, pollen development, and seed maturation (Hatfield and Prueger, 2015; Resentini et al., 2023). Yet, the understanding of how sudden temperature fluctuations induced by climate change affect the sexual reproductive processes of plants, both within and between species, and the mechanisms they employ to adapt to these changes, remains limited, particularly among wild plants (e.g., Kahl et al., 2019; Tushabe et al., 2023). Furthermore, the lack of integrating a wide range of methodologies, including field studies, experimental manipulations, molecular techniques, and meta-analyses, within a single study poses challenges for comprehensive exploration. Field studies, for instance, enable observations in natural settings where complex interactions of abiotic and biotic factors occur but pose difficulty in controlling multiple variables. Experiments under controlled laboratory and or green house conditions create ideal settings for studying the precise effects of temperature on specific traits but lack ecological realism to easily extrapolate results in natural settings. Molecular techniques are used to understand genetic or molecular pathways or mechanisms behind specific responses to temperature but overlook other ecological interactions. Meta-analyses that synthesize data from multiple studies help to combine results and identify patterns but largely depend on the quality of the studies and availability of data. Therefore, each method has its own advantages and limitations, requiring an integrative approach to achieve a more comprehensive understanding of how temperature impacts plant reproduction.

This thesis employed both experimental and meta-analytical approaches to address some of the gaps in gametophytic research and examine plant responses to high temperature stress, focusing on wild plant populations. Given the Ph.D. timeline and resource constraints largely caused by the COVID-

19 pandemic, it was not feasible to address, for example, molecular-scale aspects, which could have enhanced the results. Integration of these aspects are necessary in future studies.

The thesis begins by addressing a methodological gap, particularly concerning gametophytic research in wild plants, where the lack of *in vitro* pollen germination media (PGM) for most species and the scattered literature of available PGM protocols makes experimental work challenging (Chapter 2). Pollen germination media plays a vital role not only in examining plant gametophytic performance but also in understanding their responses to various abiotic factors such as temperature stress (e.g., Kakani et al., 2005). By compiling a 'cookbook' of optimized PGM recipes sourced from various studies representing diverse families, genera, and species, and establishing general guidelines for developing PGM tailored to specific species or taxonomic groups, this study provides a framework for future experimentation, particularly for species lacking such protocols. The dataset, already published, is accessible to the broader research community for further exploration.

Additionally, the physiological responses of pollen to temperature extremes and the underlying factors influencing its temperature limits, which remain poorly understood, were examined in Chapter 3. Analyzing data from 98 studies on temperature tolerance ranges for pollen germination and tube growth provided insights into the plasticity of pollen temperature tolerance at the intraspecific level. Furthermore, it highlighted how local growing conditions impact heat tolerance and the correlation between pollen thermal limits and species distribution patterns. These findings emphasized the vulnerability of pollen to temperature extremes, with significant implications for agriculture, species distribution dynamics, and conservation efforts.

Moving on to the wider ecological implications of temperature changes on the reproductive processes of plants, chapter 4 examined how heat stress affects both male and female gametophytes using seeds collected from species' natural habitats and cultivated in controlled greenhouse experiments. The results revealed a correlation between the physiological responses of the

gametophytes to heat stress and their ecological outcomes, such as effects on seed production and quality. While the primary focus was on *Silene* herbaceous species, the study emphasized the broader susceptibility of wild plant populations to heat waves induced by climate change, which can adversely affect population dynamics and genetic diversity.

Finally, the study explored the strategies employed by plants to cope with or respond to temperature fluctuations, particularly focusing on how gametophytic traits adapt and acclimate to heat stress across a temperature gradient. Through exposing eleven wild populations of *Silene vulgaris* to heat stress conditions, the study showed a lack of pre-local adaptation to heat stress, with limited acclimation responses in gametophytic traits (Chapter 5). The findings raise concerns regarding the capacity of plant populations to adjust to the ongoing challenges posed by climate change. It highlights the complex interaction between genetic, physiological, and environmental factors, emphasizing the need to further study plant resilience mechanisms and the alternative strategies they employ to cope with heat stress.

Generally, the thesis contributes to existing research and lays a basis for future investigations into how wild plant species respond to environmental stress, particularly high temperatures. However, the study's limitations regarding species coverage emphasize the need for further research to include a wider range of natural plant populations and species across diverse genotypes, habitats, and climate zones. It also highlights the need for future studies on species distribution dynamics to incorporate research on pollen temperature tolerance, given its significant role in plant species' reproductive success and overall fitness. Moreover, more studies on the ecological consequences of climate change should encompass other abiotic stressors such as precipitation, $CO_2$ levels, drought, and their interactions. This broader approach enables a more holistic understanding of adaptive responses in plant sexual reproduction processes.

The translation of research findings into practical applications, including informing breeding programs, agriculture, and conservation strategies, remains crucial for mitigating the impacts of climate change and supporting ongoing and future research endeavors.

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# Appendix

Chapter 3

Appendix 1, Table 1: Pollen thermal limits for 198 species in the dataset. *Tmin*, *Topt* and *Tmax* are minimal, optimal and maximal temperatures of pollen performance.

| Species | Family | Coordinates | CultStatus | Woodiness | *Tmin* | *Topt* | *Tmax* |
|---|---|---|---|---|---|---|---|
| Aconitum napellus L. | Ranunculaceae | 47.553399, 12.917786 | Wild | Herbaceous | 2.4 | 21.2 | 37.5 |
| Actinidia deliciosa (A.Chev.) C.F.Liang & A.R.Ferguson | Actinidiaceae | 35.380877, 139.469662 | Cultivated | Woody | 13.0 | 26.0 | - |
| Adonis ramosa Franch. | Ranunculaceae | 42.966667, 141.383333 | Wild | Herbaceous | - | 20.0 | - |
| Aesculus hippocastanum L. | Sapindaceae | 48.993013, 12.090839 | Wild | Woody | 0.0 | 17.1 | 37.5 |
| Agrostemma githago L. | Caryophyllaceae | 47.268167, 11.378364 | Wild | Herbaceous | 3.0 | - | - |
| Ajuga reptans L. | Lamiaceae | 49.161111, 11.960833 | Wild | Herbaceous | 1.0 | 19.9 | 40.0 |
| Allium ursinum L. | Amaryllidaceae | 47.268167, 11.378364 | Wild | Herbaceous | 0.0 | - | - |
| Allium victorialis ssp. platyphyllum Hultén | Amaryllidaceae | 43.070609, 141.340661 | Wild | Herbaceous | 0.0 | 21.0 | - |
| Allocasuarina verticillata (Lam.) L.A.S.Johnson | Casuarinaceae | -35.333333, 149.100000 | Wild | Woody | - | 12.5 | - |
| Alnus glutinosa (L.) Gaertn. | Betulaceae | 48.993013, 12.090839 | Wild | Woody | 0.0 | 31.0 | 35.0 |
| Amaryllis vittata L'Hér. | Amaryllidaceae | 30.901270, 75.807319 | Cultivated | Herbaceous | - | 28.0 | - |
| Anemone nemorosa L. | Ranunculaceae | 47.553399, 12.917786 | Wild | Herbaceous | 6.3 | 28.7 | 35.0 |
| Anemone pulsatilla L. | Ranunculaceae | 47.553399, 12.917786 | Wild | Herbaceous | 9.0 | 24.8 | 35.6 |
| Annona cherimola Mill. | Annonaceae | 36.756522, -4.043274 | Cultivated | Woody | - | 25.0 | - |
| Annona squamosa L. | Annonaceae | -15.830213, -43.268850 | Cultivated | Woody | 9.7 | 26.9 | 44.2 |
| Anthericum ramosum L. | Asparagaceae | 49.161111, 11.960833 | Wild | Herbaceous | 5.1 | 31.4 | 40.0 |
| Anthyllis vulneraria L. | Fabaceae | 49.161111, 11.960833 | Wild | Herbaceous | 0.0 | 26.4 | 40.0 |
| Arachis hypogaea L. | Fabaceae | 51.441541, -0.941859 | Cultivated | Herbaceous | 14.4 | 32.2 | 43.3 |
| Arceuthobium americanum Nutt. ex A.Gray | Viscaceae | 50.561020, -96.624182 | Wild | Herbaceous | - | 30.0 | - |

| **Species** | **Family** | **Coordinates** | **CultStatus** | **Woodiness** | ***Tmin*** | ***Topt*** | ***Tmax*** |
|---|---|---|---|---|---|---|---|
| Betula cordifolia Regel | Betulaceae | 45.978026, -66.588883 | Wild | Woody | 8.0 | 19.5 | - |
| Betula nana L. | Betulaceae | 48.993013, 12.090839 | Wild | Woody | 10.1 | 27.7 | 45.0 |
| Betula papyrifera Marshall | Betulaceae | 45.978026, -66.588883 | Wild | Woody | 5.0 | 17.0 | - |
| Betula pendula Roth | Betulaceae | 60.625930, 24.460296 | Wild | Woody | 5.0 | 26.8 | 30.0 |
| Brassica napus L. | Brassicaceae | 45.387526, -75.709143 | Cultivated | Herbaceous | 6.5 | 24.3 | 33.6 |
| Cajanus cajan (L.) Millsp. | Fabaceae | 29.151382, 75.706858 | Cultivated | Woody | 17.0 | 24.5 | - |
| Calendula arvensis M.Bieb. | Asteraceae | 40.537018, -3.682473 | Cultivated | Herbaceous | - | 32.5 | - |
| Caltha palustris L. | Ranunculaceae | 48.084976, 9.628400 | Wild | Herbaceous | 8.1 | 25.0 | 35.5 |
| Campanula alpina Jacq. | Campanulaceae | 47.553399, 12.917786 | Wild | Herbaceous | 0.0 | 17.1 | 34.0 |
| Campanula patula L. | Campanulaceae | 49.161111, 11.960833 | Wild | Herbaceous | -2.5 | 13.1 | 50.0 |
| Campanula rotundifolia L. | Campanulaceae | 49.161111, 11.960833 | Wild | Herbaceous | 4.2 | 21.9 | 39.9 |
| Campanula scheuchzeri Vill. | Campanulaceae | 47.553399, 12.917786 | Wild | Herbaceous | 0.0 | 17.1 | 37.8 |
| Campanula trachelium L. | Campanulaceae | 47.268167, 11.378364 | Wild | Herbaceous | 2.0 | - | - |
| Canarium schweinfurtii Engl. | Burseraceae | 3.857811, 11.500721 | Cultivated | Woody | - | 30.0 | - |
| Capsicum annuum L. | Solanaceae | 33.466667, -88.783333 | Cultivated | Herbaceous | 12.4 | 27.7 | 41.1 |
| Capsicum chacoense Hunz. | Solanaceae | 33.264276, -84.284063 | Cultivated | Herbaceous | 15.1 | 30.2 | 40.9 |
| Capsicum frutescens L. | Solanaceae | 33.264276, -84.284063 | Cultivated | Herbaceous | 15.1 | 32.5 | 40.0 |
| Capsicum pubescens Ruiz & Pav. | Solanaceae | 33.264276, -84.284063 | Cultivated | Herbaceous | 15.0 | 31.0 | 41.6 |
| Cardamine pratensis L. | Brassicaceae | 48.084976, 9.628400 | Wild | Herbaceous | 1.0 | 26.0 | 35.0 |
| Carex acuta L. | Cyperaceae | 48.084976, 9.628400 | Wild | Herbaceous | 7.5 | 30.1 | 39.9 |
| Carex brizoides L. | Cyperaceae | 48.084976, 9.628400 | Wild | Herbaceous | 15.0 | 30.3 | 40.0 |
| Carex caryophyllea Latourr. | Cyperaceae | 47.553399, 12.917786 | Wild | Herbaceous | 0.0 | 25.1 | 40.0 |

| Species | Family | Coordinates | CultStatus | Woodiness | *Tmin* | *Topt* | *Tmax* |
|---|---|---|---|---|---|---|---|
| Carex disticha Huds. | Cyperaceae | 48.084976, 9.628400 | Wild | Herbaceous | 4.6 | 27.8 | 40.0 |
| Carex firma Host | Cyperaceae | 47.553399, 12.917786 | Wild | Herbaceous | 0.1 | 19.9 | 35.4 |
| Carex flacca Schreb. | Cyperaceae | 47.553399, 12.917786 | Wild | Herbaceous | 4.3 | 27.7 | 39.0 |
| Carex humilis Leyss. | Cyperaceae | 49.161111, 11.960833 | Wild | Herbaceous | 2.5 | 31.0 | 40.0 |
| Carica papaya L. | Caricaceae | 31.992768, 34.817262 | Cultivated | Woody | 7.0 | 26.0 | - |
| Carpinus betulus L. | Betulaceae | 48.993013, 12.090839 | Wild | Woody | 0.0 | 27.0 | 35.0 |
| Carya illinoinensis (Wangenh.) K.Koch | Juglandaceae | 31.697433, -106.282503 | Cultivated | Woody | - | 28.5 | - |
| Cerastium arvense L. | Caryophyllaceae | 49.161111, 11.960833 | Wild | Herbaceous | 2.0 | 23.3 | 45.0 |
| Ceratonia siliqua L. | Fabaceae | 37.982494, 23.696112 | Cultivated | Woody | 10.0 | 25.0 | 40.0 |
| Chamaecytisus ratisbonensis (Schaeffer) Rothm. | Fabaceae | 49.161111, 11.960833 | Wild | Herbaceous | 6.0 | 24.3 | 40.0 |
| Chrysanthemum cinerariifolium (Trevir.) Vis. | Asteraceae | 51.984681, 5.665780 | Cultivated | Herbaceous | - | 28.5 | 43.0 |
| Cicer arietinum L. | Fabaceae | 29.166667, 75.766667 | Cultivated | Herbaceous | - | 20.0 | - |
| Citrus maxima (Burm.) Merr. | Rutaceae | 37.518413, 15.071618 | Cultivated | Herbaceous | - | 25.0 | - |
| Citrus medica L. | Rutaceae | 37.518413, 15.071618 | Cultivated | Woody | - | 25.0 | - |
| Citrus reticulata Blanco | Rutaceae | 37.518413, 15.071618 | Cultivated | Herbaceous | 15.0 | 25.0 | - |
| Citrus sinensis (L.) Osbeck | Rutaceae | -21.226930, -44.973701 | Cultivated | Woody | - | 25.0 | - |
| Cocos nucifera L. | Arecaceae | 7.566667, 80.383333 | Cultivated | Woody | 13.8 | 27.4 | 40.5 |
| Colchicum autumnale L. | Colchicaceae | 49.161111, 11.960833 | Wild | Herbaceous | 2.5 | 21.6 | 40.0 |
| Cornus alba L. | Cornaceae | 48.993013, 12.090839 | Wild | Woody | 14.6 | 24.3 | 38.5 |
| Corylus avellana L. | Betulaceae | 48.993013, 12.090839 | Wild | Woody | 0.0 | 19.8 | 34.0 |
| Crataegus laevigata (Poir.) DC. | Rosaceae | 48.993013, 12.090839 | Wild | Woody | 5.0 | 24.9 | 39.8 |
| Dianthus carthusianorum | Caryophyllaceae | 47.268167, 11.378364 | Wild | Herbaceous | 4.0 | - | - |

| Species | Family | Coordinates | CultStatus | Woodiness | *Tmin* | *Topt* | *Tmax* |
|---|---|---|---|---|---|---|---|
| Dianthus carthusianorum L. | Caryophyllaceae | 49.161111, 11.960833 | Wild | Herbaceous | 2.5 | 23.1 | 41.3 |
| Dianthus deltoides L. | Caryophyllaceae | 47.268167, 11.378364 | Wild | Herbaceous | 1.0 | 21.5 | 44.7 |
| Digitalis lutea L. | Plantaginaceae | | Wild | Herbaceous | 1.0 | - | - |
| Dimocarpus longan Lour. | Sapindaceae | | Cultivated | Woody | - | 31.3 | 35.0 |
| Echinopsis chamaecereus H.Friedrich & Glaetzle | Cactaceae | 40.985423, 29.052647 | Cultivated | Herbaceous | - | 30.0 | - |
| Epilobium parviflorum Schreb. | Onagraceae | 47.268167, 11.378364 | Wild | Herbaceous | 1.5 | - | - |
| Erica carnea L. | Ericaceae | 47.553399, 12.917786 | Wild | Herbaceous | 0.0 | 27.4 | 37.4 |
| Erysimum odoratum Baumg. | Brassicaceae | 49.161111, 11.960833 | Wild | Herbaceous | 5.0 | 24.8 | 40.0 |
| Fagus sylvatica L. | Fagaceae | 48.993013, 12.090839 | Wild | Woody | 4.3 | 27.6 | 35.0 |
| Festuca arundinacea Schreb. | Poaceae | 25.042539, 121.616135 | Cultivated | Herbaceous | - | 24.0 | - |
| Filipendula ulmaria (L.) Maxim. | Rosaceae | 48.084976, 9.628400 | Wild | Herbaceous | 17.5 | 22.8 | 40.0 |
| Fragaria × ananassa (Duchesne ex Weston) Duchesne ex Rozier | Rosaceae | 37.764722, 30.556667 | Cultivated | Herbaceous | - | 22.5 | - |
| Fragaria vesca L. | Rosaceae | 49.161111, 11.960833 | Wild | Herbaceous | 7.5 | 27.0 | 50.0 |
| Fraxinus excelsior L. | Oleaceae | 48.993013, 12.090839 | Wild | Woody | 15.0 | 24.6 | 35.0 |
| Galanthus nivalis L. | Amaryllidaceae | 47.268167, 11.378364 | Wild | Herbaceous | -4.0 | - | - |
| Galeopsis tetrahit L. | Lamiaceae | 49.161111, 11.960833 | Wild | Herbaceous | 0.0 | 19.9 | 39.3 |
| Galium album Mill. | Rubiaceae | 48.084976, 9.628400 | Wild | Herbaceous | 2.5 | 22.6 | 40.0 |
| Gentiana asclepiadea L. | Gentianaceae | 47.553399, 12.917786 | Wild | Herbaceous | 8.0 | 19.2 | 35.6 |
| Gentiana pannonica Scop. | Gentianaceae | 47.553399, 12.917786 | Wild | Herbaceous | 1.5 | 24.1 | 35.5 |
| Gentianella aspera (Hegetschw. & Heer) Dostál ex Skalický, Chrtek & Gill | Gentianaceae | 47.553399, 12.917786 | Wild | Herbaceous | 1.8 | 21.3 | 37.4 |
| Gentianella germanica (Willd.) | Gentianaceae | 47.200000, 11.450000 | Wild | Herbaceous | 3.0 | 27.6 | 42.4 |

| **Species** | **Family** | **Coordinates** | **CultStatus** | **Woodiness** | ***Tmin*** | ***Topt*** | ***Tmax*** |
|---|---|---|---|---|---|---|---|
| Geum rivale L. | Rosaceae | 48.084976, 9.628400 | Wild | Herbaceous | 2.1 | 21.9 | 39.9 |
| Globularia cordifolia L. | Plantaginaceae | 47.553399, 12.917786 | Wild | Herbaceous | 9.0 | 30.0 | 40.0 |
| Globularia elongata Hegetschw. | Plantaginaceae | 49.161111, 11.960833 | Wild | Herbaceous | 7.5 | 25.6 | 45.0 |
| Glycine max (L.) Merr. | Fabaceae | 33.466667, -88.783333 | Cultivated | Herbaceous | 11.6 | 32.6 | 47.1 |
| Gossypium hirsutum L. | Malvaceae | 33.466667, -88.783333 | Cultivated | Woody | 12.6 | 28.6 | 43.3 |
| Helleborus niger L. | Ranunculaceae | 47.553399, 12.917786 | Wild | Herbaceous | -0.3 | 19.0 | 33.9 |
| Hippocrepis comosa L. | Fabaceae | 49.161111, 11.960833 | Wild | Herbaceous | 0.0 | 23.5 | 39.8 |
| Hypericum maculatum Crantz | Hypericaceae | 48.084976, 9.628400 | Wild | Herbaceous | 6.0 | 27.4 | 44.5 |
| Hypericum perforatum L. | Hypericaceae | 49.161111, 11.960833 | Wild | Herbaceous | 7.5 | 26.7 | 40.0 |
| Impatiens sultanii Hook. f. | Balsaminaceae | 46.860221, -113.985208 | Cultivated | Herbaceous | - | 26.3 | - |
| Iris pseudacorus L. | Iridaceae | 48.084976, 9.628400 | Wild | Herbaceous | 8.0 | 31.3 | 40.0 |
| Juglans nigra L. | Juglandaceae | 38.538408, -121.761691 | Cultivated | Woody | 17.9 | 31.7 | 40.0 |
| Juglans regia L. | Juglandaceae | 38.538408, -121.761691 | Cultivated | Woody | 13.7 | 30.0 | 40.4 |
| Lathyrus pratensis L. | Fabaceae | 48.084976, 9.628400 | Wild | Herbaceous | 6.0 | 26.4 | 40.0 |
| Limonium perezii (Stapf) F.T. Hubb. | Plumbaginaceae | -40.385713, 175.613269 | Cultivated | Herbaceous | - | 27.5 | - |
| Linaria vulgaris Mill. | Plantaginaceae | 49.161111, 11.960833 | Wild | Herbaceous | 0.0 | 22.9 | 42.3 |
| Litchi chinensis Sonn. | Sapindaceae | 26.095690, 85.444124 | Cultivated | Woody | - | 27.2 | - |
| Lotus corniculatus L. | Fabaceae | 49.161111, 11.960833 | Wild | Herbaceous | 2.5 | 21.1 | 43.4 |
| Lychnis flos-cuculi L. | Caryophyllaceae | 48.084976, 9.628400 | Wild | Herbaceous | 3.0 | 25.0 | 40.0 |
| Lycopersicon esculentum Mill. | Solanaceae | 37.982837, 23.705388 | Cultivated | Herbaceous | - | 19.5 | - |
| Malus domestica Borkh. | Rosaceae | 36.274229, 128.452211 | Cultivated | Woody | - | 25.2 | 35.0 |
| Mangifera indica L. | Anacardiaceae | -26.642570, 152.939661 | Cultivated | Woody | - | 33.8 | - |
| Medicago sativa L. | Fabaceae | 49.161111, 11.960833 | Wild | Herbaceous | -2.5 | 21.9 | 45.0 |
| Naumburgia thyrsiflora (L.) Duby | Primulaceae | 48.084976, 9.628400 | Wild | Herbaceous | 8.0 | 25.2 | 38.6 |

| Species | Family | Coordinates | CultStatus | Woodiness | *Tmin* | *Topt* | *Tmax* |
|---|---|---|---|---|---|---|---|
| Oenothera biennis L. | Onagraceae | 47.268167, 11.378364 | Wild | Herbaceous | 5.0 | - | - |
| Olea europaea L. | Oleaceae | 35.496918, 23.998491 | Cultivated | Woody | - | 23.8 | - |
| Ononis repens L. | Fabaceae | 49.161111, 11.960833 | Wild | Herbaceous | 5.0 | 22.6 | 40.1 |
| Orobanche lutea Baumg. | Orobanchaceae | 49.161111, 11.960833 | Wild | Herbaceous | 7.5 | 27.5 | 39.6 |
| Oryza glaberrima Steud. | Poaceae | 14.168825, 121.255426 | Cultivated | Herbaceous | 8.0 | 28.6 | 42.2 |
| Oryza sativa L. | Poaceae | 14.168825, 121.255426 | Cultivated | Herbaceous | - | 19.0 | - |
| Parnassia palustris L. | Parnassiaceae | 47.553399, 12.917786 | Wild | Herbaceous | 3.3 | 31.4 | 34.0 |
| Pedicularis palustris L. | Orobanchaceae | 48.084976, 9.628400 | Wild | Herbaceous | 5.0 | 23.8 | 40.0 |
| Pennisetum glaucum (L.) R.Br. | Poaceae | 42.875877, -77.009449 | Cultivated | Herbaceous | 9.4 | 28.3 | 47.3 |
| Persea americana Mill. | Lauraceae | 35.494450, 24.048746 | Cultivated | Woody | 6.0 | 25.3 | - |
| Phaseolus vulgaris L. | Fabaceae | -27.473464, 153.024350 | Cultivated | Herbaceous | 4.5 | 12.0 | 38.3 |
| Phyteuma orbiculare L. | Campanulaceae | 47.553399, 12.917786 | Wild | Herbaceous | 0.0 | 19.0 | 32.2 |
| Pinus caribaea Morelet | Pinaceae | -29.618943, 30.397487 | Cultivated | Woody | - | - | 42.0 |
| Pinus elliottii Engelm. | Pinaceae | -29.618943, 30.397487 | Cultivated | Woody | - | 31.5 | 40.0 |
| Pinus greggii var. australis Donahue & Lopez Upton | Pinaceae | -25.596891, 30.287957 | Cultivated | Woody | - | 32.0 | 40.0 |
| Pinus nigra J.F.Arnold | Pinaceae | 55.680243, 12.572341 | Cultivated | Woody | - | 31.0 | - |
| Pinus patula Schiede ex Schltdl. & Cham. | Pinaceae | -29.476106, 30.179503 | Cultivated | Woody | - | 33.5 | 41.5 |
| Pinus tecunumanii F.Schwerdtf. ex Eguiluz & J.P.Perry | Pinaceae | -28.576657, 31.394541 | Cultivated | Woody | - | 32.0 | 42.0 |
| Pistacia atlantica Desf. | Anacardiaceae | 37.056249, 37.340029 | Cultivated | Woody | 7.0 | 23.3 | 40.7 |
| Pistacia khinjuk Stocks | Anacardiaceae | 37.056249, 37.340029 | Cultivated | Herbaceous | 7.0 | 24.3 | 40.1 |
| Pistacia palaestina Boiss. | Anacardiaceae | 37.056249, 37.340029 | Cultivated | Herbaceous | 7.7 | 24.0 | 39.0 |
| Pistacia terebinthus L. | Anacardiaceae | 37.056249, 37.340029 | Cultivated | Woody | 7.2 | 24.3 | 40.5 |
| Pistacia vera L. | Anacardiaceae | 37.056249, 37.340029 | Cultivated | Woody | 6.7 | 25.0 | 40.7 |

| Species | Family | Coordinates | CultStatus | Woodiness | Tmin | Topt | Tmax |
|---|---|---|---|---|---|---|---|
| Plantago lanceolata L. | Plantaginaceae | 49.161111, 11.960833 | Wild | Herbaceous | 5.0 | 22.4 | 38.0 |
| Plantago media L. | Plantaginaceae | 49.161111, 11.960833 | Wild | Herbaceous | 7.5 | 20.7 | 42.5 |
| Platanus × hispanica Münchh. | Platanaceae | 48.993013, 12.090839 | Wild | Herbaceous | 7.6 | 26.8 | 37.4 |
| Polygala chamaebuxus L. | Polygalaceae | 49.161111, 11.960833 | Wild | Woody | -5.0 | 21.2 | 40.0 |
| Primula minima L. | Primulaceae | 47.553399, 12.917786 | Wild | Herbaceous | 0.0 | 21.5 | 34.0 |
| Primula veris L. | Primulaceae | 49.161111, 11.960833 | Wild | Herbaceous | 7.2 | 22.7 | 34.4 |
| Prunus arabica (Olivier) Meikle | Rosaceae | 32.352828, 50.826086 | Cultivated | Herbaceous | 18.2 | 24.6 | 45.3 |
| Prunus armeniaca L. | Rosaceae | 39.899781, 41.244156 | Cultivated | Woody | - | 11.3 | - |
| Prunus avium (L.) L. | Rosaceae | 39.899781, 41.244156 | Wild | Woody | 1.3 | 17.3 | 31.4 |
| Prunus dulcis (Mill.) D.A.Webb | Rosaceae | 32.352828, 50.826086 | Cultivated | Woody | 12.7 | 21.8 | 41.8 |
| Prunus eleaegnifolia Mill. | Rosaceae | 32.352828, 50.826086 | Cultivated | Herbaceous | 11.6 | 24.4 | 42.4 |
| Prunus glauca (Browicz) A.E.Murray | Rosaceae | 32.352828, 50.826086 | Cultivated | Herbaceous | 15.6 | 22.5 | 46.0 |
| Prunus lycioides (Spach) C.K.Schneid. | Rosaceae | 32.352828, 50.826086 | Cultivated | Herbaceous | 15.0 | 26.7 | 43.7 |
| Prunus mume (Siebold) Siebold & Zucc. | Rosaceae | 34.672248, 135.732512 | Cultivated | Herbaceous | - | 23.5 | - |
| Prunus orientalis (Mill.) Koehne | Rosaceae | 32.352828, 50.826086 | Cultivated | Herbaceous | 13.3 | 22.7 | 42.6 |
| Prunus persica (L.) Batsch | Rosaceae | -28.950000, -51.616667 | Cultivated | Woody | 1.3 | 24.1 | - |
| Prunus scoparia (Spach) C.K.Schneid. | Rosaceae | 32.352828, 50.826086 | Cultivated | Herbaceous | 16.7 | 23.0 | 46.2 |
| Prunus spinosa L. | Rosaceae | 48.993013, 12.090839 | Wild | Woody | 7.9 | 26.9 | 32.5 |
| Pulsatilla vulgaris Mill. | Ranunculaceae | 49.161111, 11.960833 | Wild | Herbaceous | 8.0 | 24.3 | 39.1 |
| Pyrus calleryana Decne. | Rosaceae | 35.500000, 133.700000 | Wild | Woody | 10.0 | - | - |
| Pyrus communis L. | Rosaceae | 35.500000, 133.700000 | Wild | Woody | - | 17.5 | - |

| Species | Family | Coordinates | CultStatus | Woodiness | *Tmin* | *Topt* | *Tmax* |
|---|---|---|---|---|---|---|---|
| Pyrus pyrifolia (Burm.f.) Nakai | Rosaceae | 35.500000, 133.700000 | Cultivated | Woody | 7.9 | 17.6 | - |
| Quercus robur L. | Fagaceae | 48.993013, 12.090839 | Wild | Woody | 5.0 | 27.1 | 40.0 |
| Ranunculus acris L. | Ranunculaceae | 48.084976, 9.628400 | Wild | Herbaceous | 9.9 | 28.6 | 35.3 |
| Ranunculus bulbosus L. | Ranunculaceae | 49.161111, 11.960833 | Wild | Herbaceous | 5.5 | 21.3 | 35.3 |
| Ranunculus repens L. | Ranunculaceae | 48.084976, 9.628400 | Wild | Herbaceous | 12.0 | 26.2 | 39.0 |
| Ranunculus serpens ssp.polyanthemophyllus (W.Koch & H.E.Hess) Kerguélen | Ranunculaceae | 47.553399, 12.917786 | Wild | Herbaceous | 5.0 | 22.3 | 35.0 |
| Rumex acetosa L. | Polygonaceae | 48.084976, 9.628400 | Wild | Herbaceous | 5.0 | 18.7 | 40.0 |
| Saintpaulia ionantha H.Wendl. | Gesneriaceae | 22.663806, 120.608478 | Cultivated | Herbaceous | 4.6 | 25.4 | 46.5 |
| Salix caprea L. | Salicaceae | 48.993013, 12.090839 | Wild | Woody | 0.0 | 27.7 | 42.1 |
| Salix purpurea L. | Salicaceae | 48.993013, 12.090839 | Wild | Woody | 6.0 | 25.5 | 39.3 |
| Sambucus nigra L. | Adoxaceae | 48.993013, 12.090839 | Wild | Woody | 7.5 | 26.1 | 37.2 |
| Saxifraga bryoides L. | Saxifragaceae | 46.983333, 11.116667 | Wild | Herbaceous | - | - | 33.5 |
| Saxifraga caesia L. | Saxifragaceae | 47.700000, 11.383333 | Wild | Herbaceous | - | - | 35.0 |
| Securigera varia (L.) Lassen | Fabaceae | 49.161111, 11.960833 | Wild | Herbaceous | 3.2 | 19.9 | 42.4 |
| Sesleria albicans Kit. | Poaceae | 49.161111, 11.960833 | Wild | Herbaceous | -2.5 | 22.6 | 39.3 |
| Silene flos-cuculi (L.) Greuter & Burdet | Caryophyllaceae | 47.553399, 12.917786 | Wild | Herbaceous | 9.0 | 21.6 | 34.0 |
| Simmondsia chinensis (Link) C.K. Schneid. | Simmondsiaceae | 32.225870, -111.116872 | Cultivated | Woody | 5.0 | 27.5 | 40.0 |
| Soldanella alpina L. | Primulaceae | 47.553399, 12.917786 | Wild | Herbaceous | 2.3 | 21.0 | 33.8 |
| Sorbus aucuparia L. | Rosaceae | 48.993013, 12.090839 | Wild | Woody | 3.0 | 21.5 | 42.7 |
| Sorghum bicolor (L.) Moench | Poaceae | -27.383333, 153.100000 | Cultivated | Herbaceous | 11.4 | 32.3 | 40.5 |
| Stachys recta L. | Lamiaceae | 49.161111, 11.960833 | Wild | Herbaceous | 0.0 | 17.8 | 43.4 |
| Symplocarpus renifolius Schott ex Tzvelev | Araceae | 36.650000, 137.833333 | Cultivated | Herbaceous | - | 23.0 | - |
| Teucrium montanum L. | Lamiaceae | 49.161111, 11.960833 | Wild | Herbaceous | 12.5 | 30.4 | 45.0 |
| Theobroma cacao L. | Malvaceae | 12.528042, 74.968487 | Cultivated | Woody | - | 35.0 | - |

| **Species** | **Family** | **Coordinates** | **CultStatus** | **Woodiness** | ***Tmin*** | ***Topt*** | ***Tmax*** |
|---|---|---|---|---|---|---|---|
| Tilia cordata Mill. | Malvaceae | 48.993013, 12.090839 | Wild | Woody | 9.4 | 26.1 | 37.5 |
| Tilia platyphyllos Scop. | Malvaceae | 48.993013, 12.090839 | Wild | Woody | 8.4 | 23.7 | 32.5 |
| Trifolium pratense L. | Fabaceae | 38.087842, -84.491573 | Cultivated | Herbaceous | - | 30.0 | - |
| Triticum aestivum L. | Poaceae | 28.634335, 77.152373 | Cultivated | Herbaceous | - | - | - |
| Trollius europaeus L. | Ranunculaceae | 47.553399, 12.917786 | Wild | Herbaceous | 6.3 | 30.7 | 34.0 |
| Typha latifolia L. | Typhaceae | 48.084976, 9.628400 | Wild | Herbaceous | 7.0 | 30.7 | 42.5 |
| Vicia cracca L. | Fabaceae | 48.084976, 9.628400 | Wild | Herbaceous | 2.5 | 16.7 | 44.4 |
| Vicia faba L. | Fabaceae | 51.450000, -0.933333 | Cultivated | Herbaceous | - | 22.0 | 32.0 |
| Vigna radiata (L.) R.Wilczek | Fabaceae | 26.494173, 80.272028 | Cultivated | Herbaceous | - | - | 37.0 |
| Zea mays L. | Poaceae | 43.009730, -81.273734 | Cultivated | Herbaceous | 10.0 | 28.0 | 40.5 |
| Zingiber mioga (Thunb.) Roscoe | Zingiberaceae | 26.251283, 127.764878 | Cultivated | Herbaceous | - | 12.5 | - |
| Zingiber officinale Roscoe | Zingiberaceae | 26.251283, 127.764878 | Cultivated | Herbaceous | - | 18.5 | |

Chapter 4

Appendix 2

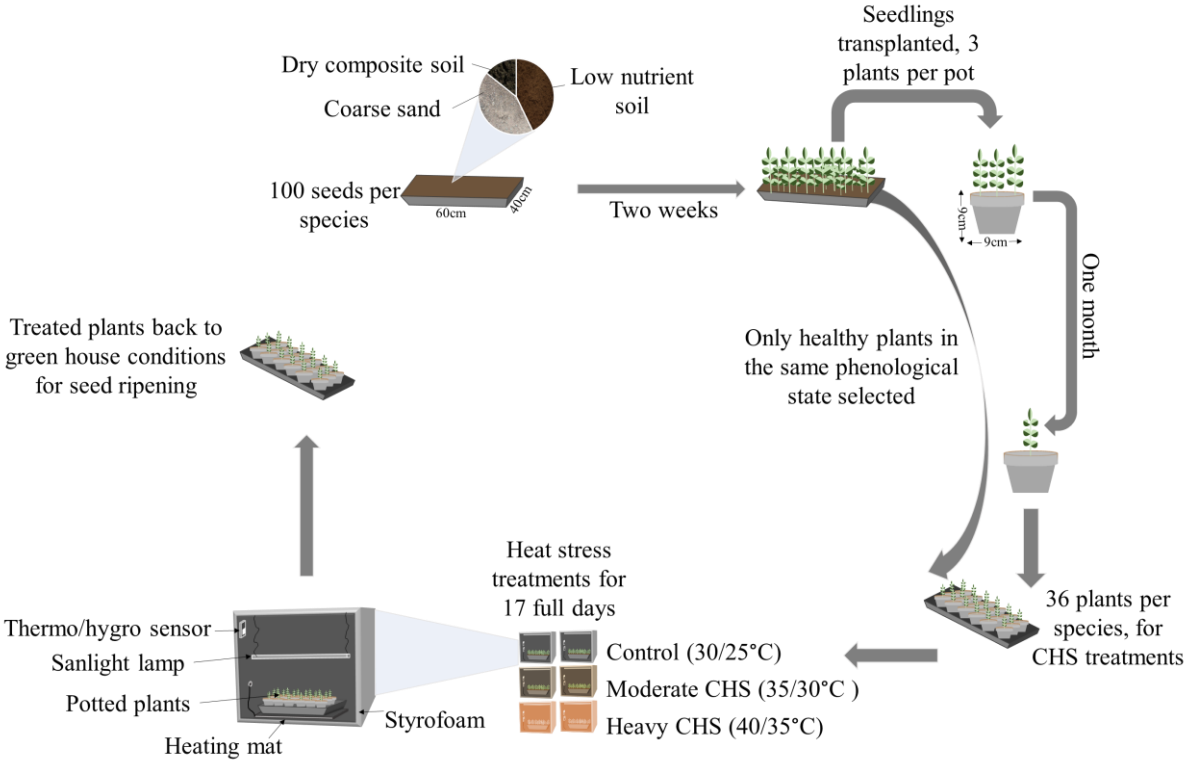


Figure A1: Schematic illustration of the experimental design of this study

Figure A2: Grow chambers used for the experiment in the greenhouse

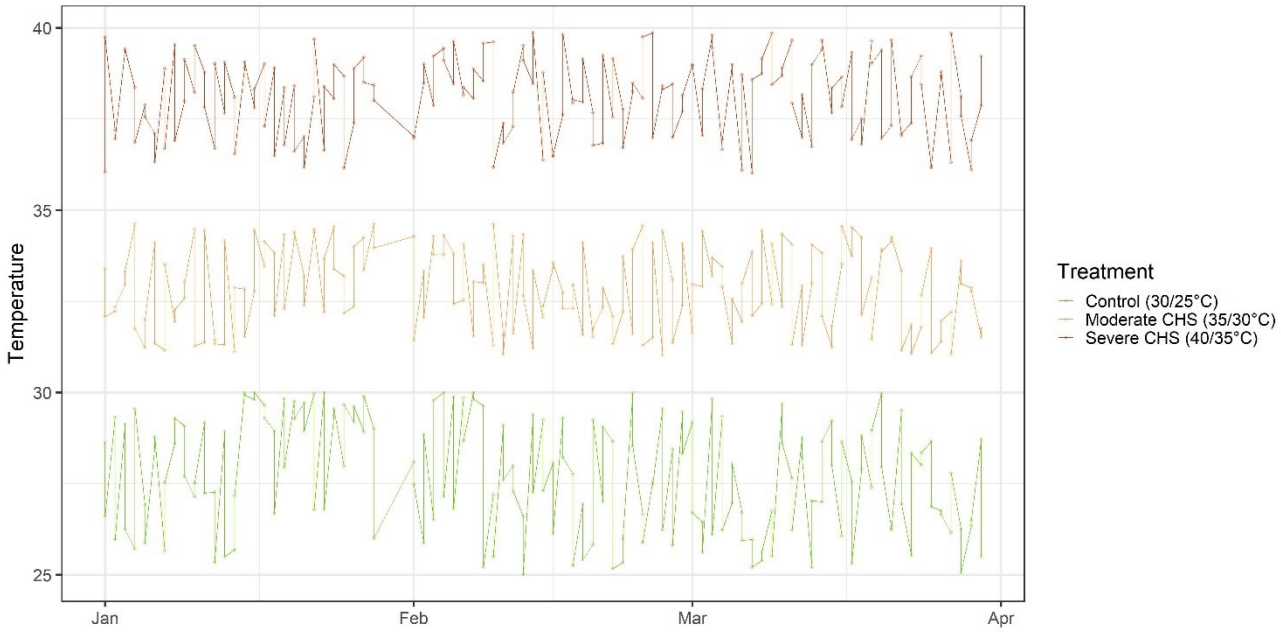


Figure A3: Control and chronic heat stress treatments in grow chambers during the experiment for the four species of *Silene*