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Temperature dependence of pollen germination and tube growth in conifers relates to their distribution along an elevational gradient in Washington State, USA

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RUNNING TITLE:

Temperature dependence of conifer pollen germination and tube growth

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ABSTRACT

- *Background and Aims*: Pollen germination and tube growth are essential processes for successful fertilization. They are among the most temperature-vulnerable stages and subsequently affect seed production and determine population persistence and species distribution under climate change. Our study aims to investigate intra- and inter-specific variations in the temperature dependence of pollen germination and tube length growth and to explore how these variations differ for pollen from elevational gradients.
- *Methods*: We focused on three conifer species, *Pinus contorta*, *Picea engelmannii*, and *Pinus ponderosa*, with pollen collected from 350 to 2200m elevation in Washington State, USA. We conducted pollen viability tests at temperatures from 5 to 40°C in 5°C intervals. After testing for four days, we took images of these samples under a microscope to monitor pollen germination percentage (GP) and tube length (TL). We applied the Gamma function to describe the temperature dependence of GP and TL and estimated key parameters, including the optimal temperature for GP (T_{opt_GP}) and TL (T_{opt_TL}).
- *Key Results*: Results showed that pollen from three species and different elevations within a species have different GP, TL, $T_{opt\_GP,}$ and T_{opt_TL}. The population with a higher T_{opt_GP} would also have a higher T_{opt_TL}, while T_{opt_TL} was generally higher than T_{opt_GP}, i.e., a positive but not one-to-one relationship. However, only *Pinus contorta* showed that populations from higher elevations have lower T_{opt_GP} and T_{opt_TL} and vice versa. The variability in GP increased at extreme temperatures, whereas the variability in TL was greatest near T_{opt_TL}.
- *Conclusions*: Our study demonstrates the temperature dependences of three conifers across a wide range of temperatures. Pollen germination and tube growth are highly sensitive to temperature conditions and vary among species and elevations, affecting their reproduction

success during warming. Our findings can provide valuable insights to advance our understanding of how conifer pollen responds to rising temperatures.

KEYWORDS: Conifer, *Pinus contorta*, *Picea engelmannii*, *Pinus ponderosa*, Pollen germination, Pollen tube length, Temperature dependence, Intra- and inter-specific variation, Elevational gradient, Climate change

1. INTRODUCTION

Temperature is a major climatic factor limiting plant species' geographical distributions (Rosbakh & Poschlod, 2016) and is expected to increase globally from 1.4°C (low GHG emission scenario) to 4.4°C (very high GHG emission scenario) with frequent temperature extremes by the end of this century (Calvin et al., 2023). Reproductive phases, e.g., pollen germination, pollen tube growth, and fruit set, are among the most temperature-sensitive and vulnerable stages (Bykova et al., 2012; Hedhly et al., 2009; Kakani et al., 2005; Zinn et al., 2010). The impact of climate change on reproduction could be a major limitation on tree distributions (Morin et al., 2007). However, species distribution models rarely consider temperature-induced reproductive failures (Bykova et al., 2012). When high-temperature stress is applied separately on male and female gametes before pollination, it is frequently observed that pollen is often the most vulnerable link in the reproductive cycle (Zinn et al., 2010). The ability and speed of pollen germination and the rate of pollen tube growth under different temperatures are traits that can shape the genetic structure and adaptation of the next generations (Pasonen et al., 1999, 2000, 2001, 2002; Skogsmyr & Lankinen, 2002). Therefore, determining the temperature dependence of conifer pollen germination and pollen

tube growth from different conifer species and populations is essential to evaluate species' response to temperature for predicting and mitigating the impacts of climate change on plant populations and ecosystems. Some studies have shown that pollination is one of the ecological factors that can contribute to range limits (Dawson-Glass & Hargreaves, 2022), and pollen germination test in vitro is a good predictor of total seed and percent filled seed in loblolly pine (Moody & Jett, 1990) and Douglas-fir (Webber & Bonnet-Masimbert, 1993). Therefore, pollen viability represents an effective way to study temperature-induced reproductive failure and its potential effects on population dynamics and species distribution (Rosbakh et al., 2018; Rosbakh & Poschlod, 2016).

Temperature stresses on the pollen in angiosperms focusing on crops and fruit trees have been studied frequently (e.g., Hedhly et al., 2004, 2005b, 2009; Kakani et al., 2002; Liu et al., 2023; Thakur et al., 2010; Zinn et al., 2010) due to the risk to food security. However, very little is known about the susceptibility of conifer pollen to warm temperatures and whether they can adapt or acclimate to heat stress within populations (Flores-Rentería et al., 2018). Most studies of conifer pollen (Owens et al., 1998; Owens & Simpson, 1986), including *Pinus spp.* (e.g., Moody & Jett, 1990; Parantainen & Pasonen, 2004; Parantainen & Pulkkinen, 2002; Siregar & Sweet, 2000), *Pseudotsuga menziesii* (Mirb.) Franco (e.g., Dumont-BéBoux & Von Aderkas, 1997; Owens et al., 1981; Webber, 1987; Webber & Bonnet-Masimbert, 1993), *Picea engelmannii* Parry ex Engelm. (Owens et al., 1987; Webber, 1995), and *Thuja plicata* Donn ex D. Don (Colangeli & Owens, 1990), were conducted decades ago for seed production purposes in seed orchards, tree improvement, and breeding programs (Owens et al., 1998). Few studies investigated how conifer pollen responds to warming or whether the warming would affect reproduction. The conclusions did

not provide sufficient information on the species' persistence in the future because these pollen were collected in the orchards, and the tests were done in vitro. Current understanding of pollination in conifers reflects the advances made in the past few decades (Breygina et al., 2021; Dumont-BéBoux & Von Aderkas, 1997; Fernando et al., 1997, 2005; Owens et al., 1981, 1987, 1998) but critical knowledge gaps remain to be filled including the temperature dependence of pollen germination and tube growth. Conifer pollen and pollen tubes exhibit numerous distinctive traits absent in flowering plants; examples include reduced rate and extended period of growth, extremely delayed sperm formation, no cytokinesis following sperm formation, a pollen tube wall made up primarily of cellulose, and distinct cytoskeletal control and organelle zonation (Fernando et al., 2005). Little is known about whether these trait differences will result in different temperature response rates or directions in conifers. Thus, the study on the temperature dependence of pollen germination and tube development in conifers provides valuable insights into a lesser-studied form of sexual reproduction (Fernando et al., 2005).

Temperature ranges and optima for reproduction are known to vary among species and cultivars and reflect the adaptation of species to average temperature during the flowering period (Hedhly et al., 2004, 2005a; Pham et al., 2015). It has been suggested that pollen of species from habitats with a higher mean annual temperature are adapted to germinate and grow under relatively high temperatures (Pasonen et al., 2000) and that pollen of crop species and cultivars flowering under relatively high temperatures germinates and grows tubes at relatively high temperatures (Kakani et al., 2005; Luza et al., 1987). All these results indicate a consistently strong correlation between habitat temperature and the temperature requirements of pollen germination and tube growth and are potentially important

contributors to the climatic restriction of plant species distributions. Some studies in high-mountain flowering plants (Rosbakh & Poschlod, 2016; Steinacher & Wagner, 2012) and temperate or tropical deciduous trees (Luza et al., 1987; Pasonen et al., 2000) have shown that pollen germination and tube growth has diverse optimal, cold- and heat-limited temperatures. Some conifer pollen studies have also shown that different species or the same species from different habitats have very different optimal germination temperatures; for example, seven *Pinus* species from South Africa have optimal germination at 32°C (Nel et al., 2005), while Scots pine from Finland has germination percentage from 62 to 92% at 20°C (Parantainen & Pulkkinen, 2002). In addition, the pollen germination and tube growth of Scots pine from northern populations is greater at higher temperatures, whereas pollen from southern populations is unaffected (Varis et al., 2011). These inter- and intra-specific variations may result in different responses in successful fertilization and consequently, seed production under climate change since pollen germination and tube growth are highly temperature-dependent. Improved knowledge of the thermal requirement in pollen germination, a precursor to seed production, could enhance our understanding of species distributions along climatic gradients and our ability to predict how climate change might affect plant community composition (Rosbakh & Poschlod, 2016).

Environmental conditions vary along latitudinal and elevational gradients. In general, temperature decreases from low to high elevation and latitude, allowing for the elevational and latitudinal gradients to be used as proxies for studying plant species' responses to temperature (Rosbakh & Poschlod, 2016; Wu et al., 2019). An overarching goal of this study was to determine the temperature dependence of pollen germination and tube growth in three conifers with high economic and ecological significance and unique niches vulnerable to

climate change due to various complex and interacting reasons; for example, increasing frequency, extent, and severity of disturbance caused by climate change and changing climate that is faster than trees can adapt or migrate. Our aim was to explore the following research questions intra- and inter-specifically: (1) how temperature dependence of pollen germination and tube growth varies among pollen collected from different elevations, (2) whether and how the optimal temperatures for pollen germination (T_{opt_GP}) and tube growth (T_{opt_TL}) are related to pollen collection elevations or to the temperatures when pollen sheds, and (3) if and how the T_{opt_GP} and T_{opt_TL} are related. The germination or tube growth responses to temperature may vary among populations across species' geographical distribution since they have acclimated or adapted to local climatic conditions or habitats (Chamorro et al., 2018). Therefore, our hypotheses for each research question are: (H_1) The pollen within the same species from higher elevations (cooler sites) will have higher germination percentages and longer tube lengths at lower temperatures and vice versa. (H_2) The intra- and inter-specific T_{opt_GP} and T_{opt_TL} are correlated to both pollen collection elevations and temperatures when pollen sheds. (H_3) The intera- and inter-specific T_{opt_GP} and T_{opt_TL} are correlated but do not have a one-to-one relationship.

2. MATERIALS AND METHODS

2.1 Species and pollen collection sites

We collected pollen of three conifer species, Lodgepole pine (*Pinus contorta* Dougl. ex. Loud.; PICO), Engelmann spruce (*Picea engelmannii* Parry ex Engelm.; PIEN), and Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.; PIPO), at Tyee Mountain (350 to 2000m; Entiat, Washington, USA) and Slate Peak (1500 to 2200m; Mazama,

Washington, USA) in Washington State (Figure 1). Nine Pendant Temperature/Light Data Loggers UA-002-64 (Onset HOBO, Bourne, Massachusetts, USA) were deployed to record hourly mean air temperatures at Tyee Mountain along the elevational gradient and seven loggers at Slate Peak along the elevational gradient from October 2020 to June 2021 (Figure 1). The recorded spring mean temperature (April, May, and June) ranges at the pollen collection sites for each species are 3.4 to 6.1°C (PICO), 1.7 to 7.4°C (PIEN), and 7.1 to 13.7°C (PIPO) (Table S1). The monthly mean temperature along the elevation and lapse rate in Tyee Mountain and Slate Peak are shown in Figures S1 and S2.

Lodgepole pine is a species with a broad ecological amplitude. It grows under a wide variety of climatic conditions. Engelmann spruce grows in a humid climate with long, cold winters and short, cool summers. It occupies one of the highest and coldest forest environments in the western United States. Ponderosa pine is one of western North America's most widely distributed pines. Ponderosa pine and Engelmann spruce are two species that occupy opposite ecological niches; that is, Ponderosa pine is often found in hot and dry environments at low elevations, and Engelmann spruce is found in cold and wet environments at high elevations on mountains. These three species occupy a wide range of climate niches in Washington State and the western United States (Table S1). Elevation ranges and pollen mature time of these three species in western North America and Washington State, USA (Burns & Honkala, 1990) are shown in Table 1. We collected matured male cones before the scales opened and shed pollen. The collection elevational ranges and dates are also shown in Table 1. The PICO, PIEN, and PIPO pollen were collected from 23, 12, and 30 elevations, respectively. The pollen was collected from multiple cones in the same tree at each elevation. After the pollen

collection, we placed male cones at room temperature drying for five days. We then collected the pollen grains and moved them to the fridge (4°C) prior to the viability tests.

2.2 Pollen germination test

Pollen germination tests were conducted from July to September 2021. A suspension culture method (Shivanna & Rangaswamy, 1992) was used for the pollen germination test. We first cultured pollen with the germination medium of Brewbaker and Kwack (1963), which was autoclaved and supplemented with thiamine, riboflavin, and ascorbic acid (Varis et al., 2011; Table S2). This recipe works well in Scots pine and is close to Tushabe & Rosbakh (2021) suggested for the gymnosperm. The vials were also autoclaved before the test, and 5 ml of culture media and 25 mg of pollen grains from each species and elevation were added to each vial. They were then placed on CO-Z orbital shakers (80 revolutions per minute; Amazon, Seattle, USA) in G-1000 growth chambers (Conviron, Winnipeg, Canada) with temperatures from 5 to 40°C with an interval of 5°C for four days without light (Parantainen & Pasonen, 2004; Parantainen & Pulkkinen, 2002). The pollen grains of each species and elevation were placed in the same chamber at each temperature. Three chambers were used in the test. Two of them were set to two temperature treatments for four days. We then reset the two chambers to another two temperature treatments for another four days. We repeatedly reset temperature treatments until the eight temperature treatments were done. Three test rounds (replicates) of each species, elevation, and temperature treatment were conducted in different chambers. At the end of the four-day period in each round, 3 ml of 0.1% (w/v) aniline blue-lactic acid-glycerol-water solution was added to each vial to arrest

pollen tube growth and stain the tubes blue (Parantainen & Pasonen, 2004; Parantainen & Pulkkinen, 2002). The vials were then moved to a fridge (4°C), waiting for imaging.

In each test round, two subsamples were collected in each vial and considered to be repeat observations. The six subsamples of each species and elevation were used in the statistical analysis and model fitting. The total number of samples in each species is shown in Table S3. Each subsample was placed on a micro slide and covered with a cover glass. We then took images of these samples, as soon as the test was done, under the Trinocular Stereo Microscope SM-2T-LED and 10MP USB 3.0 Color CMOS C-Mount Microscope Camera MU1003 (AmScope, Irvine, California, USA) with the magnification of x16 to x40, depending on the species, to monitor pollen germination and pollen tube growth. The imaging process was done from July 2021 to October 2021. The germination percentage was determined by counting pollen grains from the top left to the bottom right of each image until 200 pollen grains were selected per sample. Pollen grain was considered germinated when the length of its tube was more than the diameter of the pollen grain (Shivanna & Rangaswamy, 1992; Varis et al., 2011). The tube lengths of the first 20 germinated pollen grains from the 200 counts per subsample were selected and measured with the help of AmScope software (version x64, 4.11.21973.20230107, AmScope, Irvine, California, USA). If there were less than 20 pollen grains germinated, we measured all germinated pollen tube lengths. Only the longest branch of the pollen tube was measured if the tube had branches (Parantainen & Pasonen, 2004; Parantainen & Pulkkinen, 2002). For analysis, we used the raw tube length data in each subsample. As for the germination percentage, subsamples were considered repeat observations, and the three test rounds were true replicates.

2.3 Modeling pollen germination percentage and tube length growth

Bell-shaped or peak functions have been widely applied in agricultural science to describe the rate of biological processes as a function of temperature (Archontoulis & Miguez, 2015). We applied both the Gaussian (Eqn. 1) and Gamma function (Eqn. 2) to evaluate their ability to describe the temperature dependence of pollen germination germination percentage (GP) and tube length (TL). The Akaike Information Criterion (AIC) was used to assess the relative quality of Gaussian and Gamma functions in our study.

$$G = G_{max}\, exp\{-0.5[(T - T_o)/b]^2\} \quad \text{Eqn. 1}$$

In Eqn. 1, G is the response variable (pollen germination percentage, unit: %, or tube length, unit: μm), T is the explanatory variable (temperature, unit: °C), G_{max} is the maximum G value, T_o (optimal temperature, unit: °C) is the position of the peak (G_{max}), and b is the coefficient controlling the width of the bell shape.

$$G = \alpha T^{\beta} exp(-\theta T) \quad \text{Eqn. 2}$$

In Eqn. 2, the definitions of G and T are the same as in Eqn. 1, and α (no unit) determines the overall scale or height of the curve, β (no unit) controls the shape of the curve, particularly the steepness of its rise, and θ (no unit) affects the curve's position along the x-axis (horizontal shift) and influences the curve's decay rate.

The Michaelis-Menten equation is well-known and routinely applied to quantify the rate of a process dependent on the substrate (Archontoulis & Miguez, 2015). Since pine pollen grains may accumulate "prepackaged" rRNA that is preprogrammed to synthesize all the proteins needed throughout the entire duration of the germination and tube growth

process (Frankis, 1990) and higher spring mean temperatures at each site speed up the protein-synthetic process in each species, it is reasonable to model the T_{opt_GP} and T_{opt_TL} versus spring mean temperatures across species with a Michaelis-Menten function (Eqn. 3.3) to test H_2.

$$X_o = mT_s/(n + T_s) \quad \text{Eqn. 3}$$

In Eqn. 3, X_o is the response variable (optimal temperatures for each species and elevations, unit: °C), T_s is the explanatory variable (spring temperatures at different sites, unit: °C), m is the higher asymptote of X_o ($T_s \rightarrow X_o$, unit: °C), and n is the T_s value giving a response equal to $m/2$ (unit: °C). We restricted the spring temperature to no greater than the optimal temperature ($T_s \leq X_o$) in this equation. A summary of each variable and parameter in Eqn. 1, Eqn. 2, and Eqn. 3 are shown in Appendix Table A.

2.4 Statistical analysis

The "AIC" function in the "stats" package (R Core Team, 2021) was used to calculate AIC values. We used simple linear regression to fit (1) the observed GP and TL to different elevations within the same species and tested temperature and (2) the T_{opt_GP} and T_{opt_TL} to different elevations within the same species to test H_1 and H_2. We also used a one-way analysis of variance (ANOVA) and Tukey's honest significance test (HSD) to test the T_{opt_GP} and T_{opt_TL} among species (H_2). A t-test was used to compare the differences between each species' T_{opt_GP} and T_{opt_TL}, and the "emtrends" function in the "emmeans" package (Lenth, 2022) was used to test the one-to-one relationship between T_{opt_GP} and T_{opt_TL} (H_3). A quadratic function was used to fit the coefficient of variation in each species and temperature. The monthly mean temperature along the elevational

gradients in Tyee Mountain and Slake Peak were calculated. We then used simple linear regression to estimate the lapse rate of each month and site (Figures S1 and S2). All analyses were conducted in the R 4.1.2 programming language (R Core Team, 2021).

3. RESULTS

3.1 Modeling pollen GP and TL of each species at different temperatures across elevational gradients with Gaussian and Gamma functions.

The Gamma function effectively described the temperature response curves of GP and TL of all three species, especially in the GP of PICO, along the elevation gradient (Figure 2a). The populations from high-elevation sites tended to show a lower T_{opt_GP} and higher GP when the testing temperatures were below the T_{opt_GP} (Figure 2a). On the contrary, populations from a low elevation exhibited a higher T_{opt_GP} and higher GP when the testing temperatures were above the T_{opt_GP} (Figure 2a). The three elevations in Figure 2 were three representative elevations of low, medium, and high elevations within the elevational range of each species. We will elaborate more about the relationship between T_{opt} and elevations in sections 3.2 and 3.3. The Gaussian function provided similar temperature response curves for GP and TL as the Gamma function (Figure S3). Overall, AIC values indicated that the Gamma function provided a better fit with lower AIC values than the Gaussian function in all species, with the mean ΔAIC of 1.44 for Gamma and 4.80 for Gaussian in PICO; 0.08 for Gamma and 6.13 for Gaussian in PIEN, and 1.95 for Gamma and 1.97 for Gaussianin in PIPO (Tables S4 and S5). Moreover, the Gamma function provides additional flexibility to control the ascending rate before the optimal temperature and the decay rate after the optimal temperature, hence explaining the greater

variability of GP and TL across elevations and species compared to the Gaussian function. Therefore, our study used the Gamma function for subsequent analyses to estimate T_{opt_GP} and T_{opt_TL}. The fitted Gaussian and Gamma curves of GP and TL in all three species at all elevations individually are available in Figures S4 and S5.

3.2 Intraspecific variations along the elevational gradient at different temperatures

Based on our H_1, when temperatures are lower than T_{opt_GP} or T_{opt_TL}, GP or TL increases with elevation (positive slopes; dashed line in Figure 3). Conversely, when temperatures are higher than T_{opt_GP} or T_{opt_TL}, GP or TL decreases with elevation (negative slope; dotted line in Figure 3). In addition, when the temperature is near optimal or extreme, all populations have no differences in GP along the elevational gradient (a slope close to zero; solid line in Figure 3). The slopes (unit: GP % change per meter change in elevation; TL μm change per meter change in elevation) and *p*-values of each GP and TL regression line across elevations in different species and temperatures are shown in Table 2. GP of PICO had a slope that is close to zero at 5°C (p=0.884), positive slopes at 10, 15 (p<0.001) and 20°C (p=0.002), negative slopes at 25 (p=0.082) and 30°C (p=0.005), and slopes that are close to zero at 35 (p=0.108) and 40°C (p=0.114; Table 2 and Figure S6). TL of PICO showed that slopes are close to zero at 5 (p=0.059) and 10°C (p=0.961), positive slopes at 15 and 20°C (p<0.001), and a negative slope at 25°C (p=0.006), and slopes that are close to zero at 30 (p=0.575), 35 (p=0.237), and 40°C (p=0.158; Table 2 and Figure S7). GP and TL of PIEN and PIPO also showed similar trends but different patterns due to their unique niche (Table 2 and Figures S8 to S11).

The variation of GP and TL showed opposite patterns (Figure 4). The coefficients of variation (CV) of GP of each species in all experimental rounds at different temperatures showed that variation increased when the temperatures shifted from optima toward upper or lower limits (Figures 4a, 4c, and 4e). The CV of PICO and PIPO also showed that variation was slightly higher at the lower-limit temperature than at the higher-limit temperature (Figures 4a and 4e). The CV of PIEN was higher at the higher-limit temperature than at the lower-limit temperature (Figure 4c). On the contrary, the CV of TL of each species in all experimental rounds at different temperatures showed that variations were the highest when the temperatures were optimal and decreased when the temperatures shifted from optima toward upper or lower limits (Figures 4b, 4d, and 4f).

3.3 Inter- and intraspecific variations in T_{opt_GP} and T_{opt_TL} are correlated with pollen collection elevations and spring mean temperatures.

T_{opt_GP} and T_{opt_TL} were significantly different between species (Tables 3 and S6), and PIEN (T_{opt_GP}: 16.4°C, T_{opt_TL}: 18.7°C) was the lowest, followed by PICO (T_{opt_GP}: 23.6°C, T_{opt_TL}: 26.5°C) and PIPO (T_{opt_GP}: 26.2°C, T_{opt_TL}: 26.8°C). T_{opt_GP} and T_{opt_TL} in PICO across the elevational gradient showed a significant descending trend ($p<0.001$ and $p=0.004$ in Figures 5a and 5b), but no significant trends were found along the elevational gradient in PIEN ($p=0.553$ and $p=0.886$ in Figures 5c and 5d) and PIPO ($p=0.649$ and $p=0.419$ in Figures 5e and 5f).

The T_{opt_GP} of different species along the elevational gradient was also shown in Figure 6. PICO and PIEN from different sites had overlapping elevations but different T_{opt_GP} ranges (Figure 6a). After converting the elevations to actual spring mean

temperatures, PICO and PIEN were separated farther by spring mean temperatures (Figure 6b). It showed that each species occupies a unique thermal niche related to their natural habitats. The T_{opt_TL} also showed that PICO and PIEN from different sites had overlapping elevations but different T_{opt_TL} ranges (Figures 6c) and fewer overlapping elevations after converting the elevation to spring mean temperatures (Figures 6d). It also showed that T_{opt_TL} was more variable than T_{opt_GP} within each species (Figure 6).

3.4 T_{opt_GP} and T_{opt_TL} are correlated

The populations with higher T_{opt_GP} would also have higher T_{opt_TL}; that is, T_{opt_GP} and T_{opt_TL} had a positive relationship (Figure 7). The T_{opt_TL} was 2.3 to 2.9°C higher than the T_{opt_GP} in PICO and PIEN, but not much difference in PIPO (Tables 3 and S6). We further tested whether the slopes of the three species were significantly different from one, i.e., whether the increments of T_{opt_GP} and T_{opt_TL} were equal. The results showed that they were not (PICO: p=0.012; PIEN: p=0.004; PIPO: p<0.001). The t-tests between T_{opt_GP} and T_{opt_TL} in each species showed that T_{opt_TL} was significantly higher than T_{opt_GP} in PICO (p<0.001) and PIEN (p<0.001), and no difference between T_{opt_GP} and T_{opt_TL} in PIPO (p=0.123, Table 3).

4. DISCUSSIONS

4.1 Intraspecific variations along the elevational gradient at different temperatures

4.1.1 The temperature range of pollen germination and tube growth reflects the population's acclimation or adaptation to the local environment.

As described in Figure 3, we expected GP or TL trends along the elevational gradient would have slopes that are positive, negative or close to zero, depending on the temperature because the temperature range of pollen germination and tube growth reflects the population acclimation to the local environment (Hedhly et al., 2004, 2005b; Parantainen & Pulkkinen, 2002; Pasonen et al., 2000; Pham et al., 2015). GP and TL of PICO, PIEN, and PIPO followed the expected trends similarly (Table 2, Figures S6 to S11), but different patterns due to their unique niche. For example, the T_{opt_GP} of PIEN is 16.4°C, but PIEN does not have a positive slope in the tested temperatures lower than T_{opt_GP} (Table 2 and Figure S8), and PIPO has a narrower positive-slope temperature range (20°C) than PICO (10 to 20°C) (Table 2, Figures S10 and S6). There might be a narrow positive-slope temperature range for PIEN between 5 and 10°C that our experiment did not catch. It is also possible that PIEN is one of the species that occupy subalpine regions, and higher elevation means a harsher environment, resulting in lower pollen quality at higher elevations. Overall, our results in GP and TL of PICO and PIPO support our H_1 that the population from higher elevations (cooler sites) will have higher germination percentages and longer tube lengths at lower temperatures and vice versa (Table 2 and Figures S6 to S11).

4.1.2 Intraspecific temperature variations of pollen GP may help species' persistence in the future.

Our results showed that GP has smaller variations near optimal temperatures and larger variations when the temperature moves toward upper or lower limits in each species (Figures 4a, 4c, and 4e). We do not find similar discussions regarding GP variations within the same temperature and across a range of temperatures in the

literature related to pollen, but it has the same results as our study in seed germination (Hsu et al., 2024). In the optimal temperature range, germination is relatively consistent among populations, as the conditions are generally favorable for growth and development. In suboptimal temperature ranges, there is greater variation in germination among populations, as the conditions are less favorable for growth. At temperature limits, such as the minimum or maximum temperature tolerances of species in our study, there is even more significant variation in germination among populations. This is because the conditions are more extreme, and fewer populations can tolerate them. It is also possible that there are many zero germination percentages at extreme temperatures, resulting in a higher coefficient of variation. The zeros at extreme temperatures may increase or decrease the coefficient of variations depending on how many zeros are and what values are not zeros. In our case, zeros increase CV because there are many zeros, and the values that are not zeros are small. This variation is also key to mitigating the species' vulnerability to changing climate and providing species adaptation and conservation opportunities (Chamorro et al., 2018).

4.1.3 Intraspecific temperature variations of pollen TL may contribute to population genetic diversity.

TL has larger variations near optimal temperature and smaller variations when the temperature moves toward upper or lower limits in each species (Figures 4b, 4d, and 4f). Pollen grains have to compete for access to the ovules when there are more pollen grains than there are ovules. In angiosperms, the choosiness from the maternal side is subjective to many factors (e.g., pollen-pollen and pollen-pistil interactions and

resource allocation to specific fertilized ovules) (Ida et al., 2013). Therefore, in vitro pollen tube growth rates do not predict successful fertilization. However, there were more contradicting results in conifer pollen-pollen interactions, e.g., the interaction has no substantial influence on fertilization abilities in vivo of Scots pine (Parantainen & Pasonen, 2004), pollen-tube competition is one of the factors contributing to male fitness in *Picea abies* (Aronen et al., 2002), and the interaction may vary according to genotype and on the combination of genotypes interacting in Scots pine (Varis et al., 2010). Despite the contradiction, they all acknowledged that other elements of the pollination process have to be considered. Overall, only the fastest-growing pollen grains are assumed to achieve successful fertilization, which, consequently, is a determinative factor controlling the paternity of the seeds (Pasonen et al., 1999). Hence, our results suggest that the group of seeds produced will have higher genetic diversity if the pollen germinates at the suboptimal than if the pollen germinates at the optimal temperature. The reason is that the TL variation is smaller at suboptimal, and every pollen has almost equal chances to fertilize ovules. On the other hand, the pollen of the fast-tube-growing genotype will dominate the successful fertilization at the optimal temperature. Therefore, in future climate change scenarios, temperatures may contribute to genetic diversity within populations based on our reasoning above, but we must acknowledge that temperature is not the only factor and must act in conjunction with other factors. When faced with unpredictable environmental conditions, fluctuation in pollen behavior due to genetic variability or phenotypic plasticity is a beneficial trait that allows plant species to fertilize successfully in a

broader range of climates, but it is disadvantageous that plants do not become more adapted to the local environment.

4.1.4 The inclusion of a pollen perspective on intraspecific and temperature variations improves our understanding of range-shift responses to climate change.

With increasing temperatures in the future, depending on what temperature is right now during pollen shedding, GP and TL can either increase or decrease and so can the genetic diversity of seeds. According to our temperature data at the pollen collection sites during pollen shedding (1.7 to 13.7°C; Table S1), they are much lower than T_{opt_GP} and T_{opt_TL} (Table 3), and rising temperatures will increase GP and TL and decrease the genetic diversity of seeds. However, another factor to consider is that the actual fertilization site is inside the female cone (in vivo). Because tree canopy absorbs and retains radiant energy, their temperatures are 15-20°C greater than the air temperature (Flores-Rentería et al., 2018). Therefore, the temperature at the actual fertilization site is much higher than the air temperature. Rising temperatures will likely decrease GP and TL and increase the genetic diversity of seeds if TL is still long enough to fertilize ovules successfully. Either way, the rising temperature will eventually affect pollen performance and change the future population and plant community composition (Rosbakh & Poschlod, 2016). Another consideration is our spring temperature is based on the calendar, whereas plants respond to the temperature. The flowering phenology will also change, but we do not know if the male and female cones will respond to temperatures in the same direction and rate. Overall, the inclusion of a pollen perspective on temperature variation improves our understanding of existing patterns of plant biogeography and range-shift

responses to climate change. Many species distribution models still suffer from an essential lack of temperature- and species-specific ecological data and a mechanistic understanding of how environmental factors shape plant ecophysiology and current species distributions (Mondoni et al., 2015; Parmesan & Hanley, 2015). Furthermore, some studies have shown that pollen germination test in vitro is a good predictor of total seed and percent filled seed in loblolly pine (Moody & Jett, 1990) and Douglas-fir (Webber & Bonnet-Masimbert, 1993). Also, a study in *Picea glauca* showed that pollen germination and the early stages of pollen tube growth were similar in vitro and in vivo, except that germination occurs within hours in vitro but days in vivo (Dawkins & Owens, 1993). We suggest that the temperature requirements of GP and TL in vitro could be integrated into species distribution models as they can estimate reproduction success quantitatively based on the germination percentage and tube length under specific temperature conditions, as Rosbakh et al. (2018) and Rosbakh & Poschlod (2016) proposed, but need further study.

4.2 Intra- and inter-specific variations in T_{opt_GP} and T_{opt_TL} reflect the species' and population's acclimation or adaptation to the spring mean temperatures.

Based on our H_2, we expect the T_{opt_GP} and T_{opt_TL} to follow the trends that the population from higher elevations (cooler sites) should have lower optimal temperatures for pollen germination and tube growth. The T_{opt_GP} and T_{opt_TL} in PICO across the elevational gradient show a significant descending trend (p<0.001 and p=0.004 in Figures 5a and 5b). In other words, the population from higher elevations has lower optimal temperature and vice versa, which suggests PICO populations have acclimated or adapted

to their habitats (Hedhly et al., 2004, 2005b; Parantainen & Pulkkinen, 2002; Pasonen et al., 2000; Pham et al., 2015). However, no significant trends are found in GP and TL along the elevational gradient in PIEN (p=0.553 and p=0.886) and PIPO (p=0.649 and p=0.419) (Figures 5c to 5f). Intraspecifically, our H_2 is supported by PICO but not PIEN and PIPO, suggesting that PICO has a better chance of persisting in future climates. Another explanation is that pollen collection sites in our study came from a relatively narrower geographic range that does not cover the whole range of the species. It is also possible that some other factors (e.g., microenvironment) affect the pollen germination and dilute the effect of elevation. Anderegg (2023) had similar opinions that four reasons may explain inconsistent and weak trait-climate relationships and three of them could be applied to our study, including (1) incomplete sampling niche, (2) confounding factors that are difficult to disentangle geographically, and (3) micro- and macro-climate variation decouples the environment that sampled individual actually experience from environmental predictor used in the analysis.

PIEN occupies one of the highest and coldest forest environments in the western United States. Our results show that PIEN also has the lowest GP and TL optimal temperature, followed by PICO and PIPO (Table 3). The PIEN has a distinct elevation, and PICO and PIPO have overlapping ranges (Burns & Honkala, 1990), and the T_{opt_GP} and T_{opt_TL} of each species are similar to their bioclimatic ranges. (Figure 6). PICO and PIEN from different sites had overlapping elevations but different T_{opt_GP} and T_{opt_TL} ranges (Figures 6a and 6c). After converting the elevations to actual spring mean temperatures, there is no overlap between PICO and PIEN in T_{opt_GP} and T_{opt_TL} (Figures 6b and 6d). These results suggest that species have acclimated or adapted to the

environment they occupy (Hedhly et al., 2004, 2005b; Parantainen & Pulkkinen, 2002; Pasonen et al., 2000). Pham et al. (2015) had similar findings that species' origin can explain the temperature range for pollen germination. Our results show that each species occupies a unique thermal niche related to its natural habitats. Interspecifically, our H_2 is supported.

Varis et al. (2011) stated that in the species or populations from colder regions, where the growing season is relatively short, pollen must either begin germinating at a lower temperature or grow pollen tubes faster than pollen from the warmer region. Our results support these explanations that PIEN has the lowest T_{opt_GP} and T_{opt_TL} (Table 3) and longer tube length (approx.180-260μm, peak values in Figure S5d) than PICO (approx.100-160μm, peak values in Figure S5b) and PIPO (approx. 90-170μm, peak values in Figure S5f), but we do not know if it is due to germinated earlier or grew faster. A study of different herbaceous species in the Bavarian Alps along an elevational gradient also found a strong positive relationship between temperature conditions at pollen collection sites and the minimum temperature for both pollen germination and pollen tube growth and a significant correlation between the maximum temperature of pollen tube growth and temperature of flowering month (Rosbakh & Poschlod, 2016).

4.3 Correlation between T_{opt_GP} and T_{opt_TL}.

The populations with higher T_{opt_GP} also have higher T_{opt_TL} (Figure 7). Kakani et al. (2002) found that minimum and maximum temperatures for pollen germination and pollen tube length were correlated, reflecting the overall adaptation of plants to extreme temperatures; however, the optimal temperatures were not correlated. The *t*-tests between

T_{opt_GP} and T_{opt_TL} in each species show that T_{opt_TL} is significantly higher than T_{opt_GP} in PICO (p<0.001) and PIEN (p<0.001), but no differences between T_{opt_GP} and T_{opt_TL} in PIPO (p=0.123; Table 3). Our H_3 is mostly supported, except the T_{opt_GP} and T_{opt_TL} have no difference in PIPO. Some studies also found that T_{opt_TL} is higher than T_{opt_GP} (Rosbakh & Poschlod, 2016; Steinacher & Wagner, 2012). We found that T_{opt_GP} and T_{opt_TL} had a positive relationship, and T_{opt_TL} was significantly higher than T_{opt_GP} in PICO and PIEN, and no difference between T_{opt_GP} and T_{opt_TL} in PIPO (Table 3). Therefore, we further tested whether the slopes of the three species were significantly different from one, i.e., whether the increments of T_{opt_GP} and T_{opt_TL} were equal. The results showed that they are not (PICO p=0.012; PIEN p=0.004; PIPO p<0.001), suggesting that pollen germination and tube growth evolved to different optimal temperatures independently and hinting an independent genetic control (Hedhly et al., 2004).

Pollen of *Pinus* may germinate soon after pollination, and pollen tubes penetrate the nucellus (Breygina et al., 2021; Fernando et al., 2005). After that, the seed cone and pollen tubes become dormant by midsummer and resume growth the following spring (Fernando et al., 2005). Therefore, our observations might be related to the fact that pollen tubes grow after pollen germination, resulting in pollen tube growth acclimating or adapting to a higher temperature in the later season. Temperature strongly affects the tube growth rate in the conifer species in our study. Many studies have reported that pollen tubes grow faster with rising temperatures for various tree species (Hedhly et al., 2005a, 2005b; Pasonen et al., 2000) because pollen tubes are the fastest-growing plant cells known, and their growth is highly dependent on energy production and biosynthetic

capacity (Gass et al., 2005). These metabolic processes are related to proteins whose rates are highly influenced by temperature. That is, pollen tube grows fast with rising temperatures within the optimal temperature range, and growth rates decrease at low and high temperatures. However, the reasons for decreasing rates at low and high temperatures are different. At cold but non-freezing temperatures, slow tube growth primarily results from the delay of metabolic process, and the tubes promptly resume growth and fertilization occurs as temperatures increase; conversely, high temperatures lead to irreversible functional disruptions, resulting in abnormal tube shapes or polarity issues (Steinacher & Wagner, 2012).

5. CONCLUSIONS

Pollen germination and tube growth are highly sensitive to climatic conditions and may vary among species and populations. Our results illustrate the temperature dependence of pollen germination and tube growth across a wide range of temperatures of three conifer species along an elevational gradient in the eastern Cascades of Washington. Our understanding of pollination in conifers has advanced rapidly in the past few decades, but it still falls behind our knowledge of this process in flowering plants. To be able to explain better how temperature responses of pollen performance vary among conifer species and within species under climate change, we suggest future studies in the pollination mechanism of conifers, the influence of pollen-pollen interaction on fertilization abilities, other environmental factors (e.g., relative humidity) related to pollination mechanism in different conifer genera, and the plasticity of response on the individual level. Determining the temperature dependence of conifer pollen germination and pollen tube growth from different

conifer species and populations is essential to evaluating species' response to temperature for predicting and mitigating the impacts of climate change on plant populations and ecosystems. Nevertheless, temperature-induced reproductive disorder and failure, e.g., mismatches with seasonality and inability of pollen to germinate, etc., are rarely considered in species distribution models because this species-specific information is hard to obtain. Our study on the temperature requirements of pollen germination and tube growth in vitro could be integrated into species distribution models as they can estimate reproduction success quantitatively under specific temperature conditions. We anticipate these results will provide the information needed to improve current conifer species distribution models and help researchers, policymakers, and stakeholders develop climate adaptation strategies.

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SUPPLEMENTARY INFORMATION

Supplementary Tables S1-S6 and Supplementary Figures S1-S11.

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APPENDIX

Table A. Variables and parameters used in the Gaussian, Gamma, and Michaelis-Menten functions and their descriptions.

Symbol	Unit	Description
		Gaussian function [Eqn. 1]
G	%	The response variable: germination percentage.
	μm	The response variable: tube length.
T	°C	The explanatory variable: temperature.
G_{max}	%	Germination percentage: the maximum value for the expected response of G.
	μm	Tube length: the maximum value for the expected response of G.
T_o	°C	The temperature at G_{max} (optimal temperature).
b	-	Shape parameter controlling the width of the bell shape.
		Gamma function [Eqn. 2]
G	%	The response variable: germination percentage.
	μm	The response variable: tube length.
T	°C	The explanatory variable: temperature.
α	-	Shape parameter controlling the overall scale or height of the curve.
β	-	Shape parameter controlling the steepness of the curve's rise.
θ	-	Shape parameter controlling the curve's position along the x-axis (horizontal shift) and its decay rate.
		Michaelis-Menten function [Eqn. 3]
X_o	°C	The response variable: optimal temperatures for different species and elevations.
T_s	°C	The explanatory variable: spring temperature at different sites.
m	°C	The higher asymptote of X_o ($T_s \rightarrow X_o$).
n	°C	The T_s value giving a response equal to $m/2$

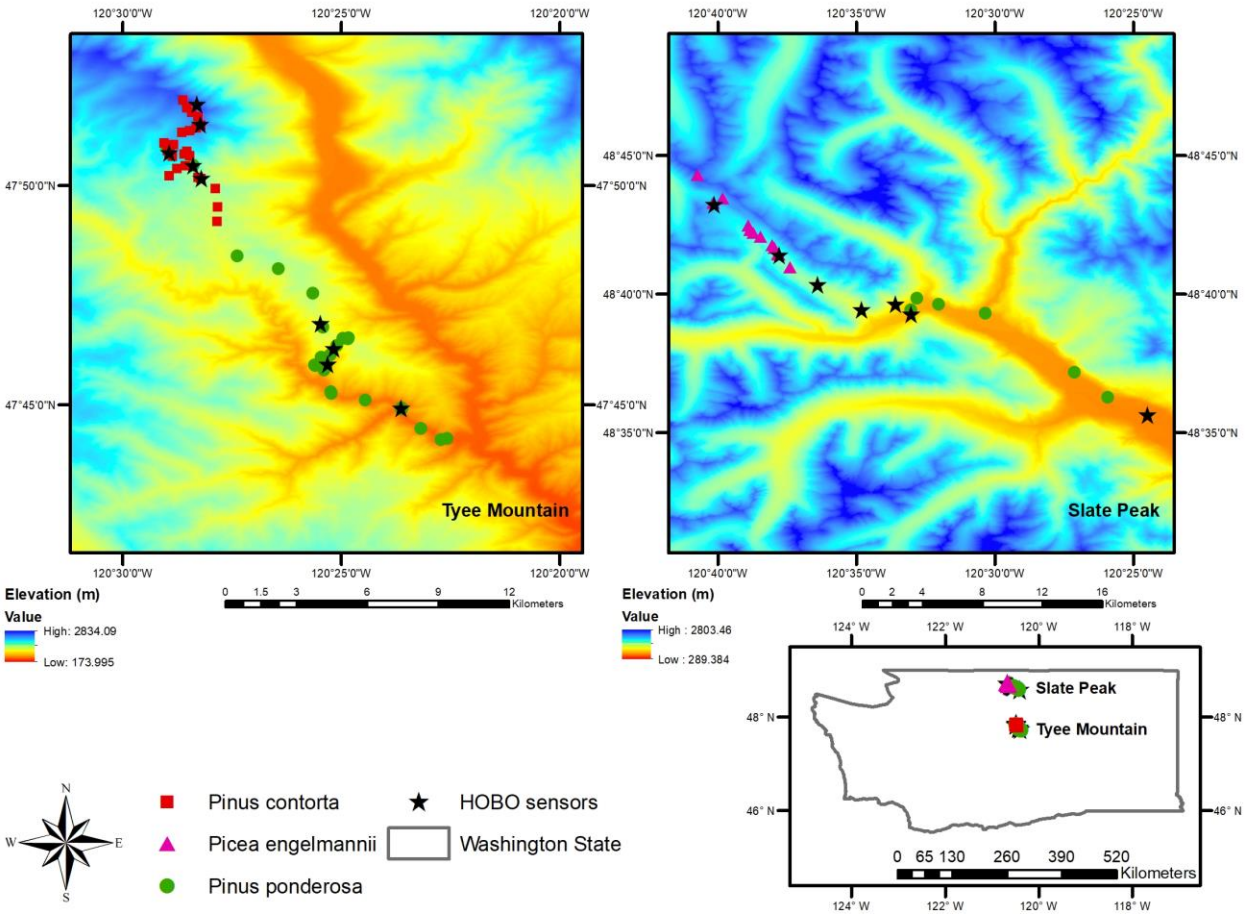

Figure 1. Temperature data and pollen of *Pinus contorta*, *Picea engelmannii*, and *Pinus ponderosa* were collected from the elevational gradients at Tyee Mountain and Slate Peak in Washington State, USA.

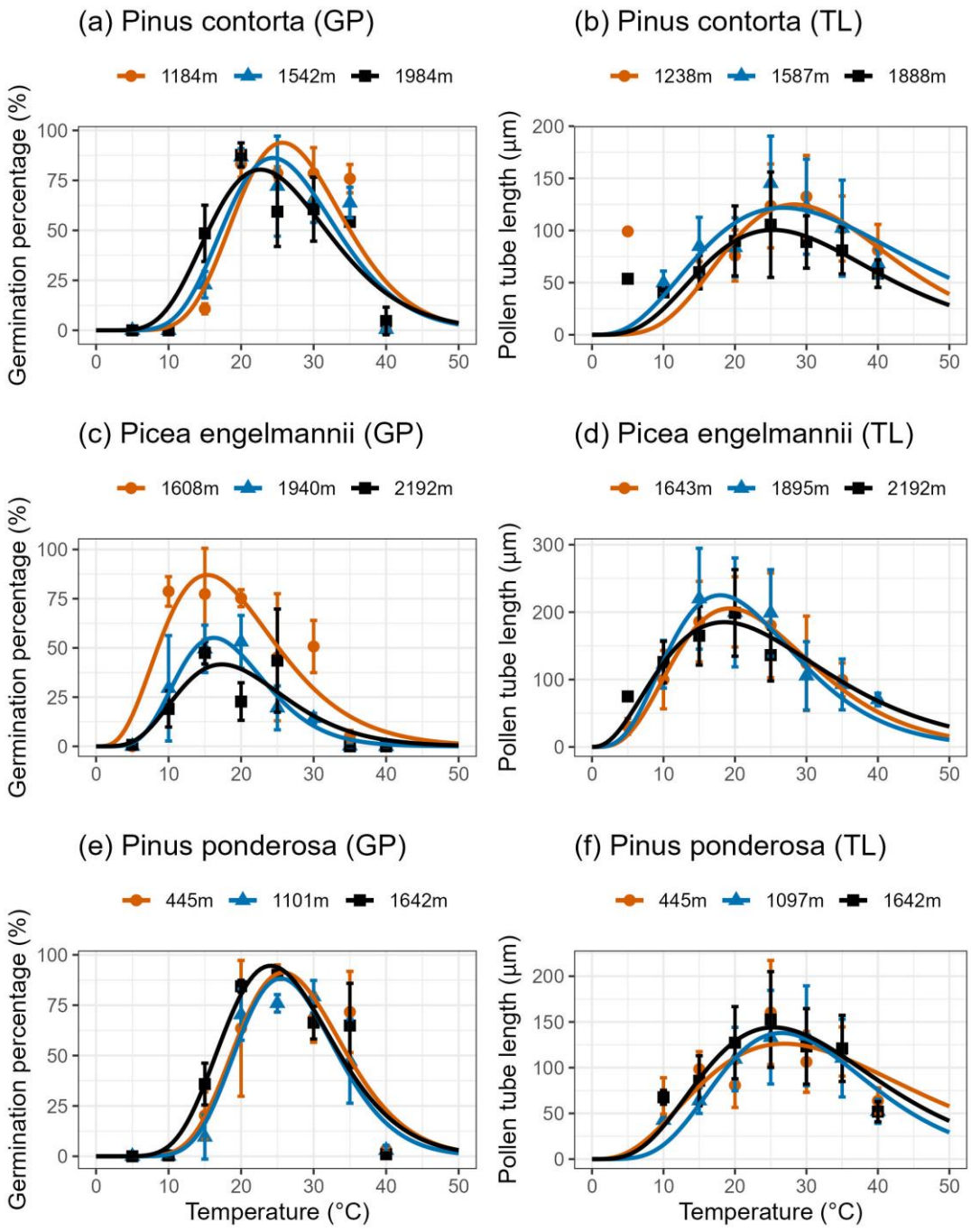

Figure 2. Gamma temperature response curves of pollen germination percentages (GP) and tube lengths (TL) in *Pinus contorta* (PICO; a and b), *Picea engelmannii* (PIEN; c and d), and *Pinus ponderosa* (PIPO; e and f) from three elevations (low, medium, and high) of each species that are close to the regression lines in Figure 4.

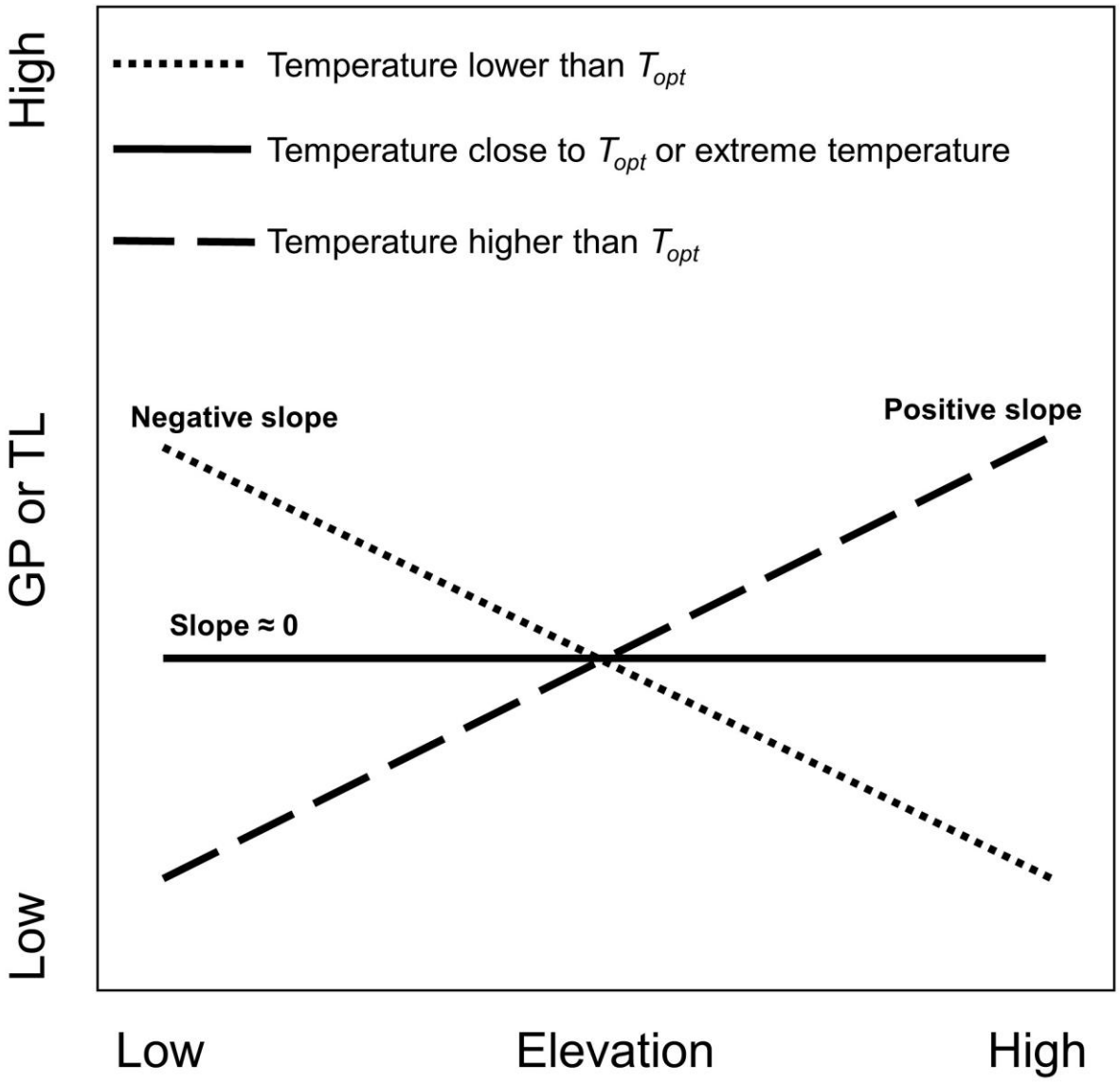

Figure 3. A conceptual diagram illustrates the slope of pollen germination percentage (GP) or tube length (TL) along the elevational gradient when the temperature is lower (dotted line) or higher (dashed line) than optimal temperatures or close to optimal temperatures (T_{opt}) and temperature extremes (solid line).

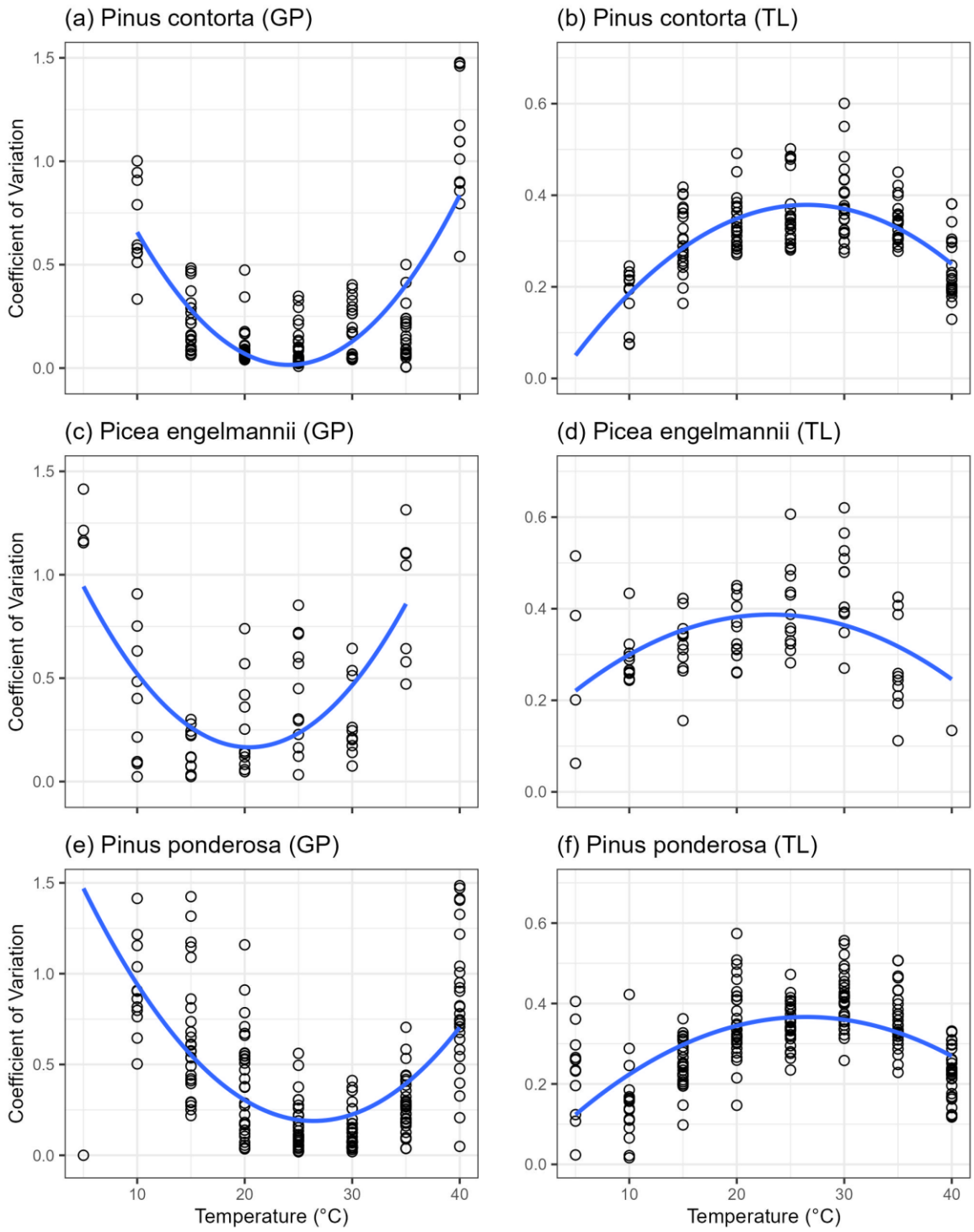

Figure 4. The coefficient of variations of pollen germination percentages (GP) and tube lengths (TL) in *Pinus contorta* (PICO; a and b), *Picea engelmannii* (PIEN; c and d), and *Pinus ponderosa* (PIPO; e and f) from different elevations at each temperature.

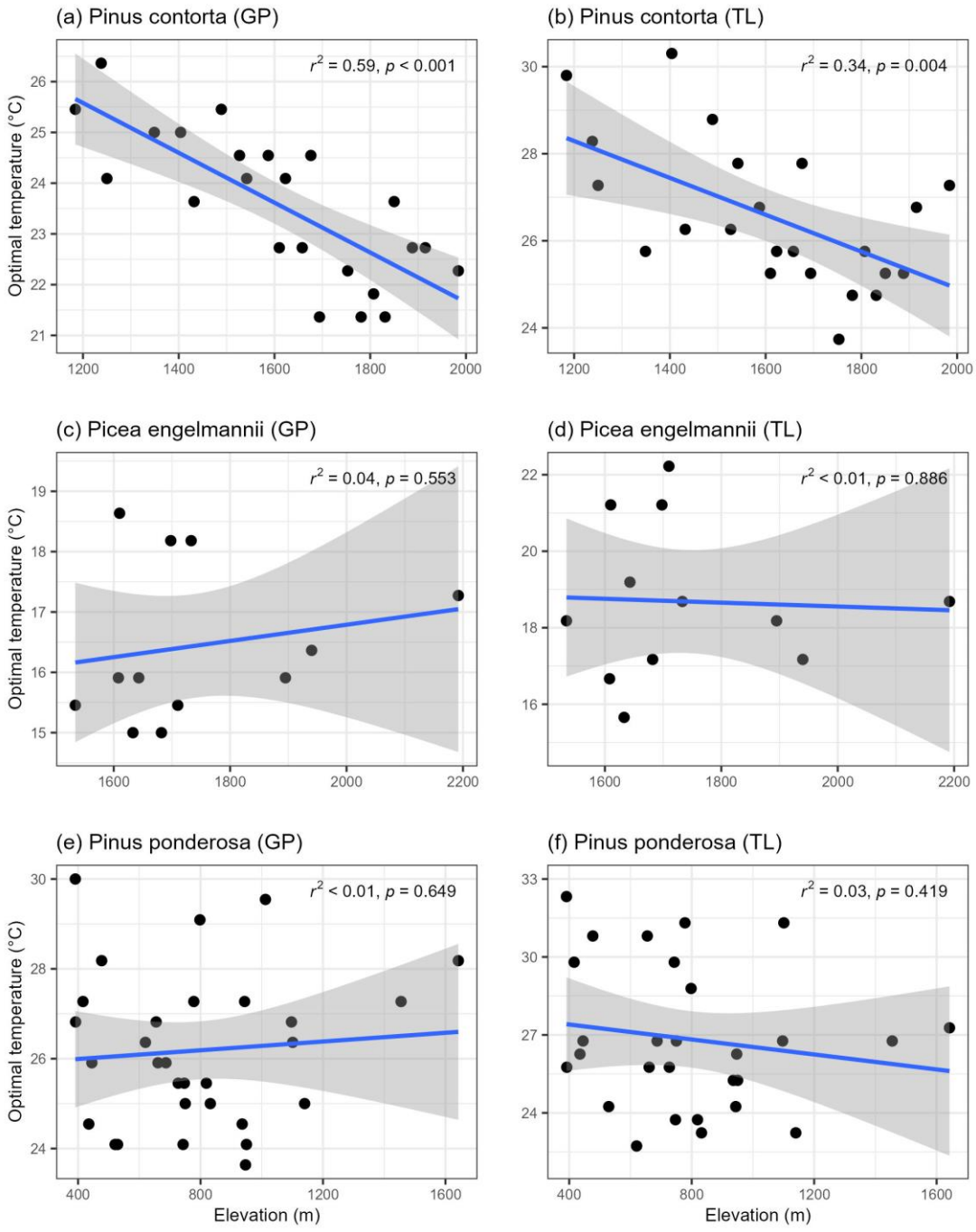

Figure 5. The regression lines of optimal temperatures of pollen germination percentages (GP) and tube lengths (TL) in *Pinus contorta* (PICO; a and b), *Picea engelmannii* (PIEN; c and d), and *Pinus ponderosa* (PIPO; e and f) across the elevational gradients. The shaded areas are 95% confidence intervals.

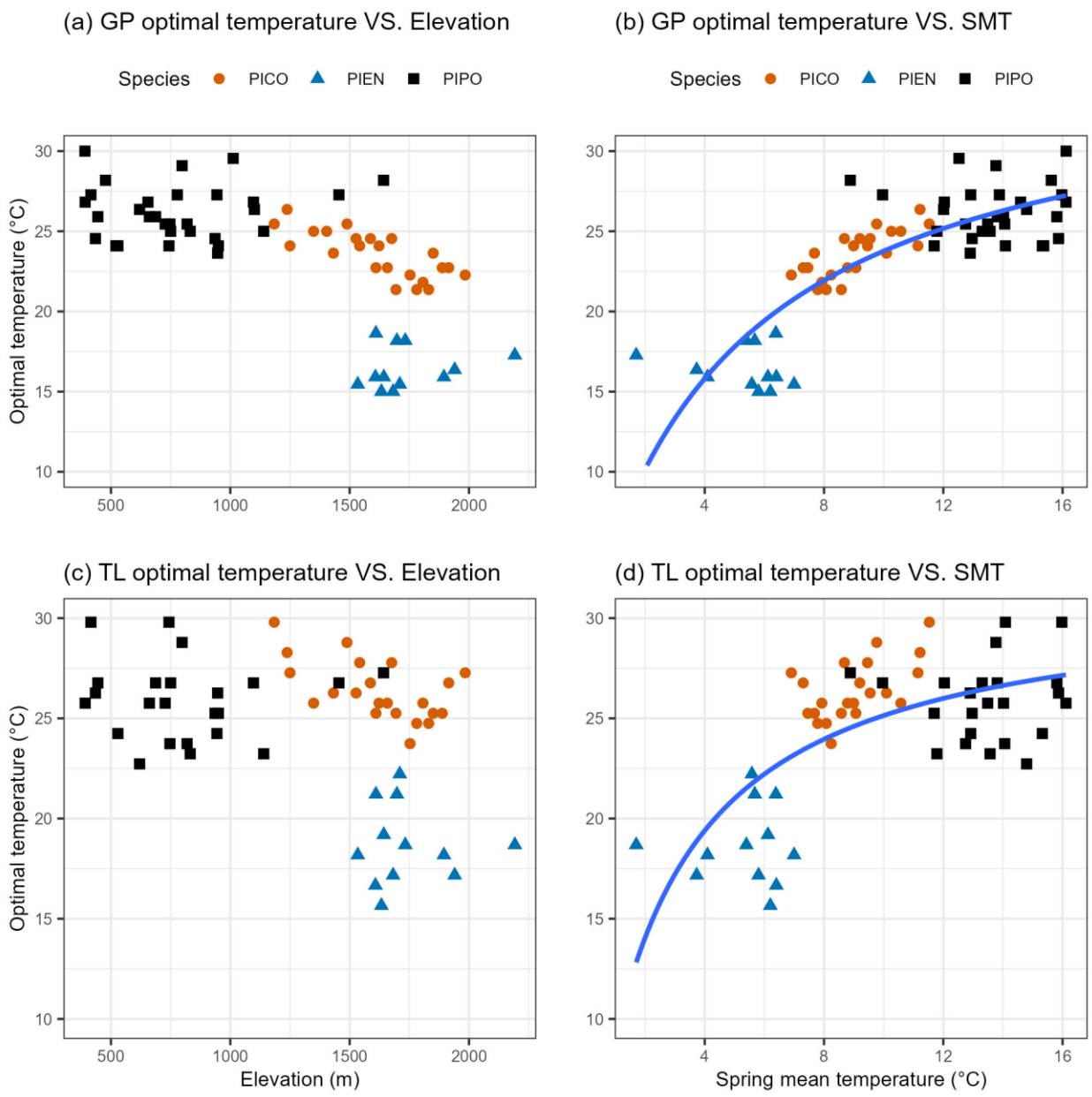

Figure 6. The germination percentages (GP) and tube length (TL) optimal temperatures of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), and *Pinus ponderosa* (PIPO) along the elevational gradient (a and c) and the spring mean temperature (SMT) gradient (b and d). The blue lines are Michaelis-Menten equations.

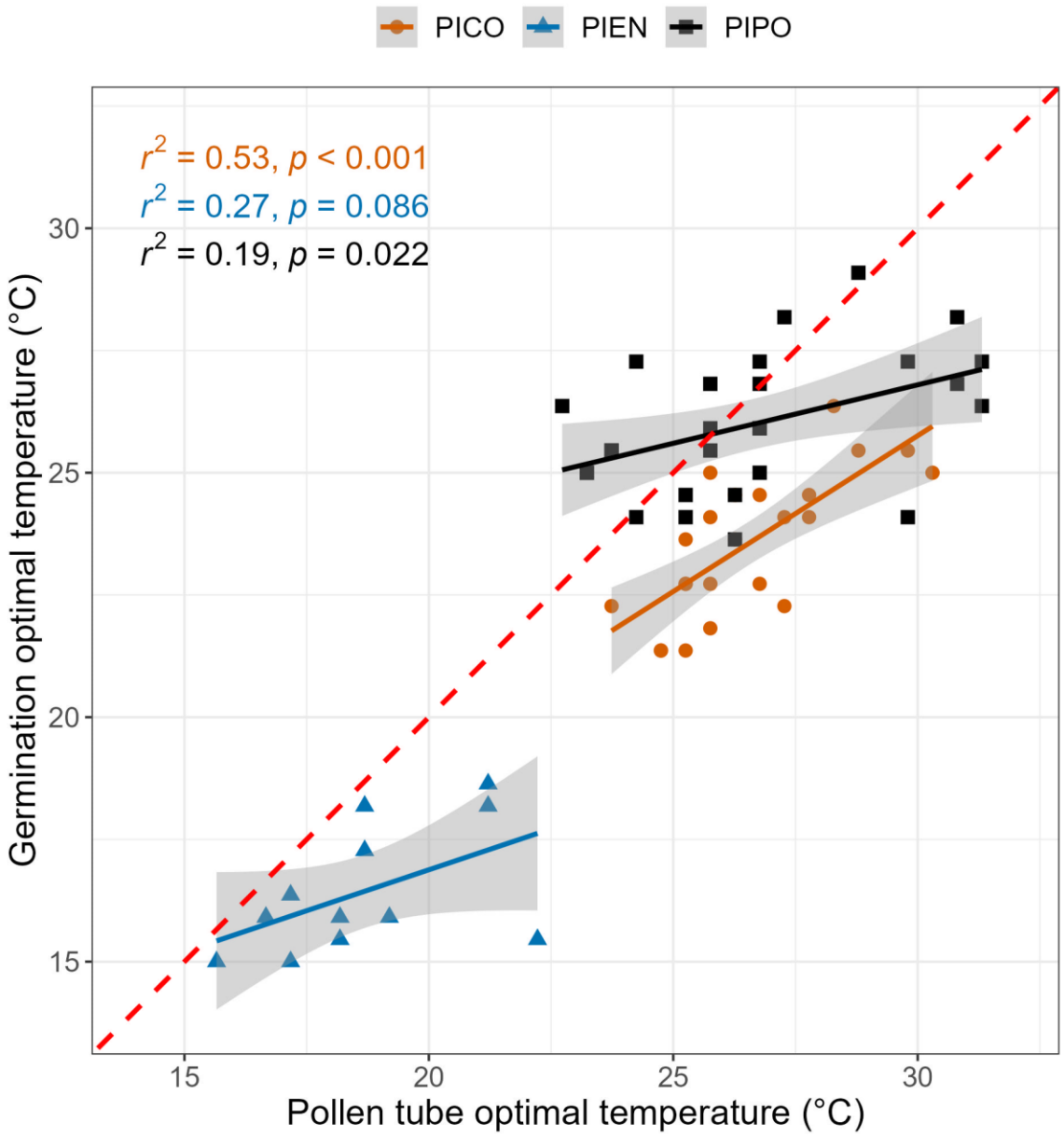

Figure 7. Simple linear regressions between the optimal temperatures of pollen germination percentage and tube lengths in *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), and *Pinus ponderosa* (PIPO) across the elevational gradients. The red dashed line is the 1:1 temperature ratio. The shaded areas are 95% confidence intervals.

Table 1. Elevation ranges and pollen mature time of *Pinus contorta*, *Picea engelmannii*, and *Pinus ponderosa* in western North America and Washington State, USA (Burns and Honkala, 1990). The pollen collection elevations and dates in Washington state, USA, are also shown here.

	Species		
	Pinus contorta	*Picea engelmannii*	*Pinus ponderosa*
Natural habitats in western North America			
Elevation range	490 – 3660 m	762 – 3353 m	0 – 3050 m
Pollen mature time	Mid-May to mid-July	1. Low elevations: late May and early June 2. High elevations: mid-June to early July	Mid-April to late June
Natural habitats in the Washington State, USA			
Elevation range	790 – 1300 m	1219 – 1829 m	0 – 1220 m
Pollen mature time	Mid-June	Early June to late June	Late May to mid-June
Pollen collection sites in Washington State, USA			
Elevation range	1184 – 1984 m	1534 – 2192 m	391 – 1642 m
Pollen collection dates	June 5 to 21	June 6 to 30	May 7 to June 5

Table 2. The slopes (unit: GP % change per meter change in elevation; TL μm change per meter change in elevation) and *p*-values of each regression line in pollen germination percentages (GP) and tube lengths (TL) of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), and *Pinus ponderosa* (PIPO) in each temperature along the elevational gradient. Asterisks represented whether the slope is statistically different from zero at each temperature. ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$.

Temperature (°C)	**Species**					
	PICO		**PIEN**		**PIPO**	
	Pollen germination percentage (GP)					
	Slope	*p*-value	Slope	*p*-value	Slope	*p*-value
5	0.000	0.884	-0.000	0.9	-0.000	0.499
10	0.003	<0.001***	-0.045	0.118	-0.0004	0.05*
15	0.080	<0.001***	-0.055	<0.001***	-0.001	0.849
20	0.016	0.002**	-0.050	0.015*	0.015	0.049*
25	-0.011	0.082	-0.041	0.04*	0.007	0.123
30	-0.021	0.005**	-0.008	0.772	-0.003	0.453
35	-0.013	0.108	-0.003	0.303	-0.010	0.112
40	0.003	0.114	-0.000	0.817	0.000	0.981
	Pollen tube length (TL)					
	Slope	*p*-value	Slope	*p*-value	Slope	*p*-value
5	-0.068	0.059	0.051	0.086	-0.055	0.006**
10	-0.000	0.961	0.009	0.273	-0.002	0.707
15	0.025	<0.001***	-0.041	0.009**	0.001	0.418
20	0.053	<0.001***	0.008	0.599	0.023	<0.001***
25	-0.014	0.006**	-0.084	<0.001***	-0.003	0.38
30	-0.003	0.575	-0.024	0.221	-0.021	<0.001***
35	-0.004	0.237	-0.012	0.696	-0.019	<0.001***
40	0.008	0.158	-	-	0.011	<0.001***

Table 3. The optimal temperature for pollen germination percentages (T_{opt_GP}) and tube lengths (T_{opt_TL}) of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), and *Pinus ponderosa* (PIPO). Different letters (a, b, c) indicate significant differences between species (row-wise comparisons) by using Tukey's honest significance test (HSD). Different italic letters (*a* and *b*) indicate significant differences between T_{opt_GP} and T_{opt_TL} within each species (column-wise comparisons) by using Tukey's honest significance test (HSD). ***: $p<0.001$.

T_{opt_GP} (°C)			
PICO	PIEN	PIPO	*p*-value
$23.6 \pm 1.5^{b, b}$	$16.4 \pm 1.3^{c, b}$	$26.2 \pm 1.7^{a, a}$	<0.001***
T_{opt_TL} (°C)			
$26.5 \pm 1.7^{a, a}$	$18.7 \pm 2.0^{b, a}$	$26.8 \pm 2.8^{a, a}$	<0.001***
p-value			
<0.001***	<0.001***	0.123	