The effect of temperature on pollen germination, pollen tube growth, and stigmatic receptivity in peach

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Keywords: Pollen germination, pollen tube growth, Prunus persica, stigmatic receptivity, temperature stress.

ABSTRACT

Temperature is a major climatic factor that limits geographical distribution of plant species, and the reproductive phase has proven to be one of the most temperature-vulnerable stages. Here we have used peach to evaluate the effect of temperature on some processes of the progamic phase from pollination to the arrival of pollen tubes to the ovary. Within the range of temperatures studied, 20ºC in the laboratory and, on average, 5.7ºC in the field, the results show an accelerating effect of increasing temperature on pollen germination and pollen tube growth kinetics as well as an increase in the number of pollen tubes that reach the style base. For the last two parameters, although the range of temperature registered in the field was much lower, the results obtained in the laboratory paralleled those obtained in the field. Increasing temperatures drastically reduced stigmatic receptivity. Reduction was sequential, with stigmas first losing the capacity to sustain pollen tube penetration to the transmitting tissue, then their capacity to offer support for pollen germination and, finally, their capacity to support pollen grain adhesion. Within a species-specific range of temperature, this apparent opposite effect of temperature on the male and female side could provide plants plasticity to withstand changing environmental effects, ensuring a good level of fertilization.

INTRODUCTION

Temperature is a major climatic factor that limits geographical distribution of plant species (Saxe et al., 2001). The spread of agriculture has led to the cultivation of species away from their optimum environmental conditions, resulting in the growth of some crops near their temperature limits. Global climatic change (IPCC, 2001), because of the predicted absolute increase in temperature and the frequency and amplitude of temperature variation, could jeopardize plant cultivation in some areas. The consequences of global climate change are already visible in shifting species distributions and phenological traits, such as flowering times (Parmeson and Yohe, 2003; Root et al., 2003; Williams and Abberton, 2004). The reproductive phase has proven to be one of the most vulnerable stages (Hall, 1992), and negative effects on fruit set of increasing temperatures during flowering have been recorded in several species, including peach (Mellenthin et al., 1972; Kuo et al., 1981; Cuevas et al., 1994; Abdulbaki and Stommel, 1995; Mckee and Richards, 1998; Peet et al., 1997, 1998; Sanzol and Herrero, 2001; Kakani et al., 2002; Cross et al., 2003; Prasad et al., 2003, Kozai et al., 2004). It has been shown that increasing temperatures accelerate pollen tube growth (Lewis, 1942, Hedhly et al., 2004) as well as ovule degeneration (Postweiler et al., 1985; Cerovic et al., 2000) and stigma degeneration (Hedhly et al., 2003). This apparently opposite effect of increasing temperatures on the male and female stages could help explain the erratic yields obtained in several fruit tree species depending on the environmental conditions during the blooming season (Sanzol and Herrero, 2001). Besides average temperatures, the range of temperature variation also influences reproduction, but little work has been done on the effects of extreme temperatures and on the integration of the effects of increasing temperatures on the different components of the reproductive process.

Here, we have studied in peach **[***Prunus persica* (L.) Batsch**]** the effect of different temperatures on the progamic phase, which is the phase that elapses from pollination to fertilization (Linskens, 1986). The range studied spans from cool spring temperature (10ºC), through optimum spring temperature (20ºC), to hot spring temperature (30ºC). In the field, we induced an increase in temperature by covering the trees with a polyethylene cage. We evaluated pollen performance through pollen germination *in vitro*, pollen tube growth in the style, and the response of the pistil through the duration of stigmatic receptivity.

MATERIALS AND METHODS

Plant material and experimental conditions

This work was carried out on a peach (*Prunus persica* L. Batsch) collection of cultivars maintained at the CITA-DGA experimental orchard located at the Campus de Aula Dei in Zaragoza, Spain. Four peach cultivars were used in this study: 'Rose Diamond', an early flowering cultivar from USA, and 'Comodín', 'Pavía Amarilla de Tolosa' and 'Moret', which are three late flowering local Spanish cultivars. Evaluations were carried out both in the field, outside and inside a polyethylene cage, and in the laboratory, using detached flowers at balloon stage in controlled temperature chambers at 10ºC, 20ºC, and 30ºC.

Pollen germination *in vitro*

Pollen from 'Rose Diamond', 'Pavía Amarilla de Tolosa' and 'Moret' was obtained from flowers collected randomly one day before anthesis. Anthers were removed and left to dry on a piece of paper for 24-48 hours at room temperature. Pollen was sieved through a 0.26 μm mesh. Immediately after, *in vitro* pollen germination was carried out at the three temperatures in polystyrene Petri dishes (35 by 10 mm) scattering pollen on a solidified germination medium consisting of 0.3 M sucrose, 0.6 mM calcium nitrate, 1.6 mM boric acid, and 0.8% (w/v) agar (Hormaza *et al.*, 1996). Pollen germination was arrested after 0h, 1/2h, 2h, 4h, 8h, and 24 hours by immediate freezing at –20ºC. One day before observation under the microscope, the Petri dishes were thawed at 4ºC. Pollen was scored as germinated when the length of the pollen tube exceeded the diameter of its pollen grain. For each treatment, germination was recorded in five Petri dishes counting complete fields until reaching at least 100 pollen grains in each plate.

For statistical analysis, germination percentage data were subjected to arcsine root square transformation. The experimental design for the dependent variable pollen germination *in vitro* for each time of incubation was double factorial (genotype, temperature). For each time of incubation, an analysis of variance was performed using SAS GLM v. 8.2 (SAS Institute Inc., Cary, N.C.). Means separation was carried out using the lsmeans option ($p<0.05$) in case of significant temperature-genotype interaction and Duncan's multiple range test ($p<0.05$) in case of no significant interaction.

Pollen tube growth *in vivo*

The same pollen of 'Pavía Amarilla de Tolosa' used for pollen germination *in vitro* was used to perform two experiments, one with cut and emasculated flowers in the laboratory and the other with whole trees in the field. For the laboratory experiment, the day prior to anthesis, flowers from the recipient genotype 'Moret' were collected at balloon stage, the anthers and petals were removed, and the emasculated flowers were placed over soaked florist's foam at room temperature (average temperature 20ºC). The following day (anthesis), flowers were pollinated with a paintbrush loaded with pollen, and placed in controlled temperature chambers at 10ºC, 20ºC and 30ºC. Previous work carried out in peach had shown no interfering effect of emasculation (Herrero and Arbeloa, 1989). For the field experiment, we chose two trees of 'Comodín' and flowers were emasculated at balloon stage. On the day of anthesis, flowers were pollinated and one of the two chosen trees (warm treatment) was covered with 0.178-mm-thick polyethylene film with a metallic structure and the other (control) was left uncovered. Such a system has been shown to be a valuable method to increase temperature in the field without negatively affecting other parameters (Rodrigo & Herrero 2001; Hedhly et al., 2003). Temperature inside and outside the plastic cage was monitored throughout the period of sequential fixation. During the maximum lifetime of flowers at each temperature in the laboratory experiment, and during 8 days after anthesis in the field experiment, ten flowers for each treatment were fixed daily in formalin: acetic acid: 70% ethanol (1: 1: 18 v/v; FAA; Johansen, 1940). Microscopic observations were made of squashed pistils washed in water three times, one hour each, autoclaved for 10 min at 1 kg/cm^2 in 5% sodium sulphite (Jefferies and Blecher, 1974), and stained with 0.1% aniline blue in 0.1 N K_3PO_4 (Linskens and Esser, 1957). Preparations were examined under an Ortholux II microscope equipped with UV epifluorescence with a band pass 355-425 exciter filter and an LP 460 barrier filter. We evaluated for each treatment, the percentage of the style traveled by the longest pollen tube, the percentage of flowers with pollen tubes at the base of the style and the number of pollen tubes reaching the base of the style. Using the SAS GLM procedure (v. 8, SAS Institute Inc., Cary, N.C.), an analysis of variance was carried out for pollen tube kinetics in the style and for the number of pollen tubes reaching the base of style (percentage data were arcsine root square transformed) using the lsmean option for means separation ($p<0.05$). For the percentage of flowers with pollen tubes at the base of the style, we performed an analysis of variance using the interaction day-temperature as error and we calculated the LSD.

Stigmatic receptivity

Stigmatic receptivity was analyzed in sequentially pollinated flowers involving the same two peach cultivars described above for pollen tube growth *in vivo* in the laboratory. The duration of stigmatic receptivity over the three temperatures studied was evaluated by the capacity of the stigma to sustain pollen germination. For this purpose, flowers were emasculated at the balloon stage, and placed in trays in the laboratory with soaked florist foam at room temperature. The following day they were placed in the controlled temperature chambers at 10ºC, 20ºC and 30ºC. This day, considered as anthesis, a batch of 10 flowers was pollinated and, subsequently, 10 flowers were pollinated daily during the maximum lifetime of flowers in each controlled temperature chamber. To evaluate the primary effect of temperature on stigmatic receptivity, the pistils were fixed in FAA 24 hours after pollination, a time when the pollen grains have accomplished germination and the pollen tubes have started growing in the transmitting tissue, but well ahead fertilization time. Microscopic observation was carried out on squashed pistils prepared in the same manner as for the study of pollen tube kinetics *in vivo*. The statistical analysis was carried out as described previously for the percentage of flowers with pollen tubes in the base of the style.

RESULTS

Pollen germination *in vitro*

Significant temperature effects on *in vitro* pollen germination kinetics were recorded for all the times of incubation except for 8 hr, and this effect was predominantly genotype-dependent (Table 1). The effect of temperature was more conspicuous in the range 10ºC-20ºC than in the range 20ºC-30ºC. Temperature, while affecting only slightly the final germination percentage, had a clear effect on the rate of germination. Increasing temperature accelerated germination rate in the three cultivars (Fig. 1). Thus, pollen started to germinate 2 hours after sowing at 10ºC, 1 hour at 20ºC and only ½ an hour at 30ºC. Maximum germination rate was attained after 8 hours at 10ºC, 2 hours at 20ºC and just 1 hour after incubation at 30ºC. While at 20ºC and 30ºC pollen behavior was similar for the three cultivars, clear differences were registered at 10ºC. Likewise, there was a clear genotype-temperature interaction in the range 1-4 hours showing that the three genotypes did not respond uniformly to changes in temperature.

Pollen tube growth *in vivo*

The effect of temperature on pollen tube growth was evaluated in the laboratory using cut flowers in controlled temperature chambers at 10ºC, 20ºC and 30ºC, and in the field with the trees outside and inside a polyethylene cage (Fig. 2). The average temperature registered in the field outside the cage (control) was 10.8ºC, and inside the cage (warm treatment) 16.5ºC. Temperature affected pollen tube kinetics in the style both in the laboratory and in the field. This was clear whether evaluated as the length of pollen tubes (Fig. 2a 2b,), as the percentage of flowers with pollen tubes at the base of style (Fig. 2c, d), or as the number of pollen tubes that reach the base of the style (Fig. 2e, f). The effect of temperature was highly significant (Table 2 and 3), and the greatest effect of temperature was registered during the first days after anthesis.

Indeed, for pollen tube kinetics, a significant effect of temperature increasing pollen tube growth rate was registered during the first two days. However, from day 3 and increase in temperature from 20ºC to 30ºC did not affect significantly pollen tube kinetics, and 4 days after pollination the longest tube had reached the base of the style at both 20ºC and 30ºC. This effect of temperature on pollen tube kinetics in the style resulted in a reduction in the time needed to reach the style base (Fig. 2b). Pollen tubes get to the base of the style in all the flowers after 3 days at 30ºC and 4 days at 20ºC. At 10ºC, although pollen tubes started to reach the base of the style 5 days after pollination, they only reach the base of the style in all the flowers five days later. The number of pollen tubes reaching the base of style also increased significantly with temperature: 1.5 at 10ºC, 3 at 20ºC, and 3.4 at 30ºC. In the field, temperature also affected significantly pollen tube growth rate, and the number of pollen tubes that reach the base of the style, and the response obtained paralleled that obtained in the laboratory. Indeed, the response obtained in control conditions in the field (averaging 10.8ºC) was similar to that obtained at 10ºC constant temperature in the laboratory, and the response obtained in the warm treatment (averaging 16.5ºC) was intermediate to that obtained at 10ºC and 20ºC in the laboratory.

Stigmatic receptivity

Stigmatic receptivity was lost gradually in three consecutive steps. Thus, the stigmas appeared to lose first the capacity to offer a support for the penetration of pollen tubes to the transmitting tissue; then, they lost the capacity to sustain germination, and, finally, the capacity to allow the adherence of the pollen grains. The capacity of the stigma to offer a

good support for pollen grain adhesion and germination, and pollen tube penetration to the transmitting tissue decreased significantly as temperature increased from 10ºC to 30ºC (Table 3). The main reduction was registered for the three parameters in the range 10ºC-20ºC. At 20ºC, the stigmas were able to maintain the adhesion of pollen grains in 50% of the flowers during 10 days, the germination during 6 days, and pollen tube penetration during 5 days. A closer examination of the effect of temperature on the capacity of the stigma to offer support for pollen germination (Fig. 3) showed that, while stigmas were able to sustain germination up to 18 days at 10ºC, this capacity was lost 8 days after anthesis at 20ºC, and 3 days at 30ºC. The penetration of pollen tubes to the transmitting tissue closely paralleled the loss of pollen grain germination although decreased faster. Pollen tubes having difficulties to cross the stigmatic area developed a swollen end. Although all flowers belong to the same genotype and were of the same age, clear differences in the duration of stigmatic receptivity were registered among flowers for a given temperature. This is most conspicuous at 10ºC (Table 4) where the whole process slows down. While some flowers loose receptivity after 5 days, others are still receptive up to 14 days after anthesis.

DISCUSSION

Temperature had a clear effect in all the components of the reproductive phase studied. High temperature accelerated both pollen germination *in vitro* and pollen tube growth in the style, but high temperature accelerated also the loss of stigmatic receptivity.

Pollen germination and pollen tube growth as well as the effects of different environmental factors on those processes have been widely documented in the literature (Taylor and Hepler, 1997; Dafni and Firmage, 2000; McKormick, 2004). Among those environmental factors, it is clear that temperature has an effect on pollen germination accelerating pollen aging (Buitink et al., 2000), as well as the rate of germination (Shivanna et al., 1991a; Young et al., 2004). Results herein revealed that, over the three temperatures studied, pollen germination kinetics increased significantly, mainly during the first four hours of incubation and in the lower half of the temperature range studied (10ºC-20ºC). Increasing temperature from 20ºC to 30ºC, although resulted in a faster germination in the three cultivars (1/2h compared to 1h at 20ºC), produced a smaller effect. In spite of the differences in pollen germination, the genotypes studied attained similar germination rates within the range of temperature tested. However, differences between genotypes were significant in the rate of germination at 10ºC. These differences could have a reflection in the different competitive ability of these genotypes at

low temperatures. Previous work in other species has also shown differences in the rate of pollen germination among genotypes both *in vitro* an *in vivo* (Shivanna et al., 1991b, Hormaza and Herrero, 1999).

Increasing temperatures also increased pollen tube growth rate in the style reducing the time needed to reach the base of style. Since the first observation of Lewis (1942) in *Oenothera organensis*, evidences of the effect of increasing temperature accelerating pollen tube growth have been accumulated in a good range of herbaceous and woody species (Mellenthin et al., 1972; Lombard et al., 1972; Thompson and Liu, 1973; Socías i Company et al., 1976; Jefferies et al., 1982; Vasilakakis and Porlingis, 1985; Elgersma et al., 1989; Keulemans and Van Laer, 1989; Cerovic and Ruzic, 1992; Katepa-Mupondwa et al., 1996; Austin et al., 1998; Petropoulou and Alston, 1998; Pasonen et al., 2000; Kakani et al., 2002).

Although the range of temperature obtained in the field (on average 5.7ºC between the average temperatures outside and inside the plastic cage) was narrower than that in the laboratory (20ºC between the minimum and the maximum temperatures studied), it was sufficient to significantly increase pollen tube growth rate and the number of pollen tubes reaching the base of the style. Previous work in sweet cherry (Hedhly et al., 2003) revealed that an increase in the average temperature as low as 2.8ºC was even enough to affect stigmatic receptivity. Larger variations in average temperatures are common in the Mediterranean area between both different years and dates during the blooming season and alerts on the potential effects of global change on reproductive biology of plant species (IPCC, 2001).

The results obtained under field conditions paralleled those obtained in controlled temperature chambers. Thus, for pollen tube kinetics and dynamics, the evaluation carried out in the laboratory seems to be a good predictor of what could happen in nature, and confirms previous results for pollen tube kinetics in plum (Jefferies et al., 1982) and for stigmatic receptivity in cherries (Hedhly et al., 2003). Studying pollen tube growth in detached flowers allows a better control of environmental conditions and offers possibilities for more repetitions in a reduced space.

While much attention has been given to pollen tube growth rate, information is scarcer on the number of pollen tubes that succeed to reach the base of the style. The results obtained in this work show that the number of pollen tubes at the base of the style also increased with temperature. An effect of temperature on the number of pollen tubes that succeed to reach the base of the style has also been observed in sweet cherry. Interestingly, in this species, the

number of pollen tubes reaching the base of style decreased by increasing temperature (Hedhly et al., 2005). These apparently contrasting results in these two species could reflect differential species adaptation to different temperatures, and could play an important role regulating mating in plants if selection under stress for better-adapted genotypes is operating (Mulcahy, 1979; Ottaviano and Mulcahy, 1989).

The evaluation of the effect of temperature on the first female structure the pollen tubes have to face in their way towards the female gametophyte, revealed a clear reduction in the duration of stigmatic receptivity as temperature increases. While stigmas were able to sustain the penetration of pollen tubes to the transmitting tissue in 50% of the flowers up to 14 days at 10ºC, they retained this capacity for 5 days at 20ºC and just for 2 days at 30ºC. Interestingly, although the experiment started with synchronized flowers, clear differences were recorded among flowers in the duration of stigmatic receptivity. This may be related to flower quality that appears to be associated to the starch content of the flower at anthesis (Rodrigo et al., 2000).

The capacity of the stigma to offer support for pollen germination and adhesion followed a similar pattern. The loss of stigmatic receptivity was sequential and the stigma loses first its capacity to sustain the penetration of pollen tubes to the transmitting tissue, then the germination, and finally the adhesion of pollen grains. The results obtained here confirm previous findings in pear (Sanzol *et al*., 2003) and sweet cherry (Hedhly *et al*., 2003). Likewise, a reduction of stigmatic receptivity with increasing temperatures has been observed in cherry (Hedhly et al., 2003). This observation could be extended to overall female receptivity since, in other works, it has also been shown that temperature reduces ovule longevity in cherry (Postweiler et al., 1985) and plum (Cerovic et al., 2000).

Consequently, the effect of the range of temperatures studied in this work seems to have two opposite consequences on the reproductive process in peach. An increase in temperature accelerates pollen grain germination rate and pollen tube growth, and increases the number of pollen tubes that reach the base of the style. However, it also decreases the duration of stigmatic receptivity. Thus, high temperature appears to accelerate development both in the male and female phases, while low temperature reduces development rate in both partners. It has been suggested that male-female synchrony is required for successful mating (Herrero, 2003), and this parallel response of both partners in response to temperature would contribute to maintain synchrony. Indeed, within a species-specific range of temperature variation, this effect on the male and female stages could give plants plasticity to withstand changing

environmental conditions, ensuring the arrival of the pollen tubes at the right time to the ovary. Outside this range, the effect of temperature would result in the breakdown of the equilibrium. Thus, despite the apparent negative effects of temperature variations in the reproductive process, plants have developed strategies to cope with changing environmental conditions presenting plasticity in their reproductive response to ensure fertilization.

ACKNOWLEDGMENTS

The authors thank A. Escota and R. Lopez for technical assistance. A. H. was supported by an AECI, SIA-DGA, and a CSIC fellowship. Financial support for this work was provided by project grants INIA-RTA 01-103, CICYT AGL2003-05318-C02-01 and by the "Grupo de Investigación Consolidado de Aragón: A-02, A-43".

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Figure Legends:

Figure 1 Effect of temperature on pollen germination kinetics *in vitro* at 10ºC, 20ºC, and 30ºC (mean ± s.e.) for three cultivars.

Figure 2 Pollen tube growth in the style in controlled temperature chambers at 10ºC, 20ºC and 30ºC, and in the field outside and inside a plastic cage, expressed as (a, b) % of style traveled by the longest pollen tube (mean ± s.e.), (c, d) % of flowers with pollen tubes at the base of the style, and (e, f) number of pollen tubes at style base (mean ± s.e.).

Figure 3 Effect of temperature (10ºC, 20ºC y 30ºC) on stigmatic receptivity, expressed as the percentage of flowers with germinated pollen grains on the stigmas.

Figure 1

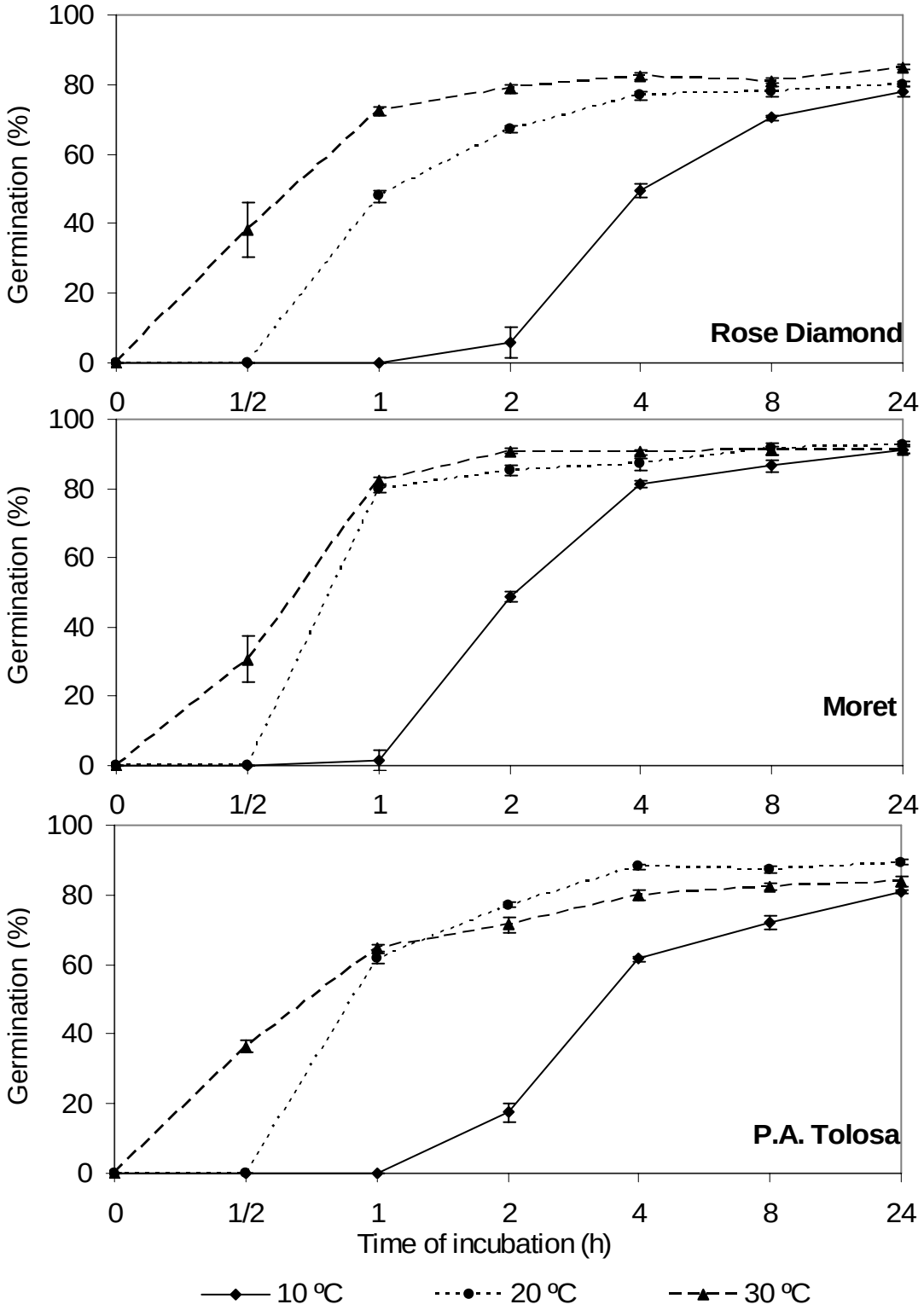
Germination (%)
100
80
60
40
20
0
Rose Diamond
Moret
P.A. Tolosa
0
1/2
1
2
4
8
24
Time of incubation (h)
10 ºC
20 ºC
30 ºC

Figure 2

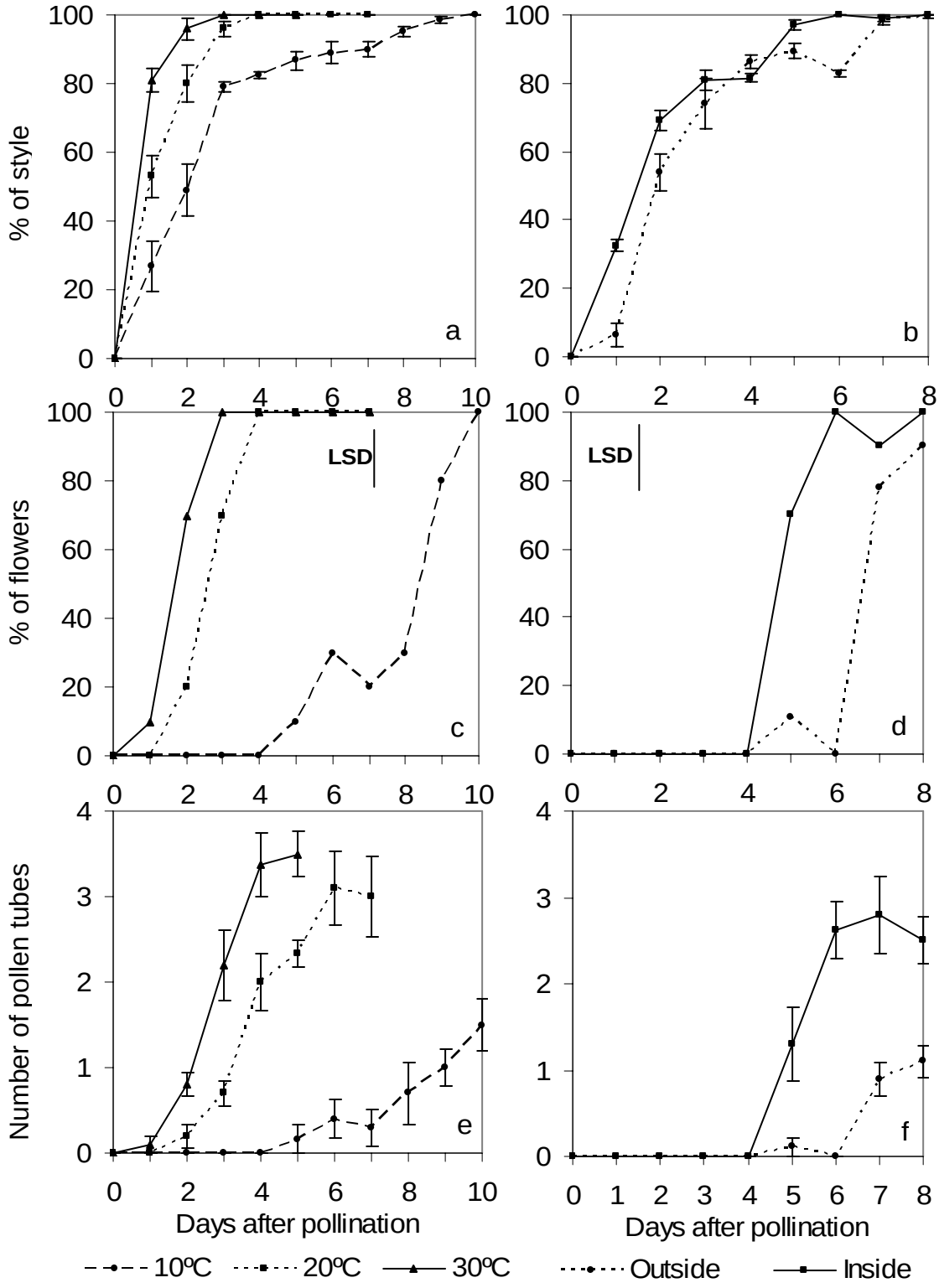
% of style
% of flowers
Number of pollen tubes
Days after pollination
Days after pollination
LSD
LSD
a
b
c
d
e
f
10ºC
20ºC
30ºC
Outside
Inside

Figure 3

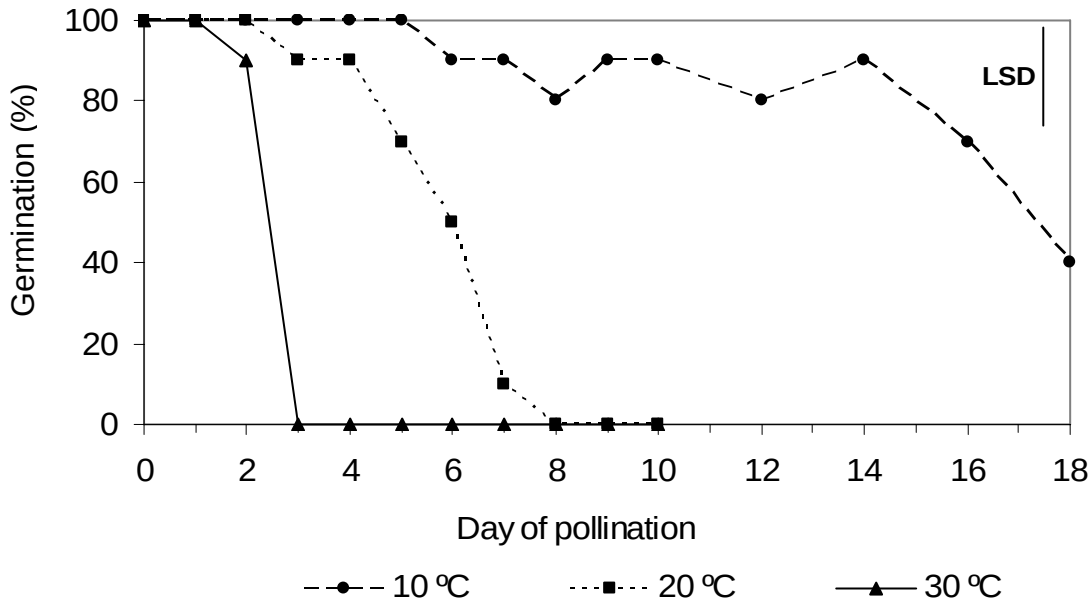
Germination (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
Day of pollination
LSD
10 ºC
20 ºC
30 ºC

Table 1 Pollen germination *in vitro***.** Analysis of variance by General Linear Model (GLM) procedure at each time of incubation and means separation for the independent variables (temperature and genotype) and the dependent variable (percentage of pollen germination).

Incubation time (h)		1/2	1	2	4	8	24
Temperature (df[1] 2)	MS[2]	4953.06	14986.30	6613.78	877.00	137.25	44.18
	Pr>F[3,4]	<0.0001	<0.0001	<0.0001	<0.0001	0.24	0.0015
Genotype (df 2)	MS	44.67	480.53	1370.00	504.35	793.73	330.22
	Pr>F	0.49	<0.0001	<0.0001	<0.0001	0.001	<0.0001
Temperature* Genotype (df 4)	MS	44.67	104.20	252.37	106.21	158.04	28.47
	Pr>F	0.57	<0.0001	<0.0001	<0.0001	0.17	0.0024
Error (df 36)	MS	60.83	10.75	22.33	7.12	93.73	5.63

[1] df, degrees of freedom; [2]MS, mean square; [3]F, F-statistic calculated, [4]Pr>F, the probability that the table value for F is greater than the calculated value.

Table 2 Pollen tube growth *in vivo* in the laboratory at 10ºC, 20ºC and 30ºC. Analysis of variance by GLM for the independent variables temperature and day, and the dependent variables kinetics (pollen tube length expressed as percentage of the style length), and tube nº (number of pollen tubes reaching the base of the style).

		MS[2]		F[3]		Pr > F[4]	
Source of variation	df[1]	Kinetics	Tube nº	Kinetics	Tube nº	Kinetics	Tube nº
Day	5	29836.04	22.01	491.36	74.56	<.0001	<.0001
Temperature	2	8716.50	35.91	143.55	121.62	<.0001	<.0001
Day*temperature	10	581.0452	5.91	9.57	20.02	<.0001	<.0001
Error	153	60.72	0.30				

[1] df, degrees of freedom; [2]MS, mean square; [3]F, F-statistic calculated, [4]Pr>F, the probability that the table value for F is greater than the calculated value.

Table 3 Pollen tube growth *in vivo* in the field outside and inside the plastic cage. Analysis of variance by GLM for the independent variables temperature and day, and the dependent variables kinetics (pollen tube length expressed as the percentage of the style), and tube nº (number of pollen tubes reaching the base of the style).

		MS[2]		F[3]		Pr > F[4]	
Source of variation	df[1]	Kinetics	Tube nº	Kinetics	Tube nº	Kinetics	Tube nº
Day	8	17965.19	12.45	339.29	33.88	<.0001	<.0001
Temperature	1	2819.48	25.91	53.25	70.50	<.0001	<.0001
Day*temperature	8	513.97	4.64	9.71	12.62	<.0001	<.0001
Error	149	52.95	0.37				

[1] df, degrees of freedom; [2]MS, mean square; [3]F, F-statistic calculated, [4]Pr>F, the probability that the table value for F is greater than the calculated value.

Table 3 Stigmatic receptivity. Number of days during which pollen adhesion, germination, and pollen tube penetration to the transmitting tissue are maintained in 100% and in 50% of the flowers at 10ºC, 20ºC, and 30ºC.

	Adhesion		Germination		Penetration	
Temperature	100%	50%	100%	50%	100%	50%
10ºC	16	18	5	16	5	14
20ºC	3	10	2	6	2	5
30ºC	2	8	1	2	1	2