Resilience of rice (*Oryza* spp.) pollen germination and tube growth to temperature stress

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ABSTRACT

Resilience of rice cropping systems to potential global climate change will partly depend on the temperature tolerance of pollen germination (PG) and tube growth (PTG). Pollen germination of high temperature-susceptible *Oryza glaberrima* Steud. (cv. CG14) and *Oryza sativa* L. ssp. indica (cv. IR64) and high temperature-tolerant *O. sativa* ssp. aus (cv. N22), was assessed on a 5.6–45.4 °C temperature gradient system. Mean maximum PG was 85% at 27 °C with 1488 μm PTG at 25 °C. The hypothesis that in each pollen grain, the minimum temperature requirements (T_n) and maximum temperature limits (T_x) for germination operate independently was accepted by comparing multiplicative and subtractive probability models. The maximum temperature limit for PG in 50% of grains ($T_{x(50)}$) was the lowest (29.8 °C) in IR64 compared with CG14 (34.3 °C) and N22 (35.6 °C). Standard deviation (s_x) of T_x was also low in IR64 (2.3 °C) suggesting that the mechanism of IR64's susceptibility to high temperatures may relate to PG. Optimum germination temperatures and thermal times for 1 mm PTG were not linked to tolerating high temperatures at anthesis. However, the parameters $T_{x(50)}$ and s_x in the germination model define new pragmatic criteria for successful and resilient PG, preferable to the more traditional cardinal (maximum and minimum) temperatures.

Key-words: cardinal temperatures; cold; development; germination model; heat; pollen germination.

INTRODUCTION

Rice is grown across wide geographical boundaries from as far north as Manchuria and as far south as Uruguay and New South Wales, and hence, potentially exposed to temperatures ranging between ≤15 °C (Zhang *et al*. 2005) and >40 °C (Wassmann *et al*. 2009). In addition, climate models predict that short-duration high day temperature events, warmer nights, and even extremely cold nights may become more frequent and intense (IPCC 2013), which could reduce the yield of cultivated rice (Peng *et al*. 2004; Wassmann *et al*. 2009; Jena *et al*. 2012; Martínez-Eixarch & Ellis 2015).

Flowering in rice is identified to be the most sensitive stage across both heat and cold stress, with the male reproductive organ determining the level of spikelet sterility (Farrell *et al*. 2006; Jagadish *et al*. 2010). Low or high temperatures at microsporogenesis and anthesis, reduce anther pore size, anther dehiscence, pollen viability, pollen germination (PG) and pollen tube growth (PTG) rate and hence, fertilization and spikelet fertility (Satake 1976; Matsui *et al*. 2001; Andaya & Mackill 2003; Farrell *et al*. 2006; Prasad *et al*. 2006; Jagadish *et al*. 2010, 2014; Martínez-Eixarch & Ellis 2015). With respect to anther pore size, a large basal pore size is positively correlated with high pollen deposition on the stigma (Matsui & Kagata 2003). High germination of deposited pollen and a high tube growth rate characterize rice cultivars, which maintain spikelet fertility and seed set in hot or cold temperature stress (Endo *et al*. 2009; Jagadish *et al*. 2010; Rang *et al*. 2011).

Chen *et al*. (2008) observed that rice pollen germinated on the stigma within 2 min of pollination, and the tube reached the ovule after 40 min although the temperature at which this occurred was not reported. By contrast, in cotton (*Gossypium hirsutum* L.) and maize (*Zea mays* L.), pollen grains do not begin germinating until 10 or 30 min after pollination, respectively (Wedzony & van Lammeren 1996; Kakani *et al*. 2005). Rapid germination and tube growth are necessary in rice because rice pollen dries rapidly (Heslop-Harrison 1979), a consequence of very thin walls that are rich in exinous microchannels (Fu *et al*. 2001). Rapid loss of water from rice pollen leads to a sharp drop in viability, by nearly 50% between 6 and 20 min after anther dehiscence and pollen shedding (Khatun & Flowers 1995; Song *et al*. 2001), compared with 4–6 h for sorghum [*Sorghum bicolor* (L.) Moench; Prasad *et al*. (2011)] and 1–2 d for maize (Fu *et al*. 2008).

Difficulties in achieving rapid germination and maintaining viability have limited systematic *in vitro* research on rice PG and PTG. These problems are exacerbated by cultivar differences in the optimum medium composition to maximize PG (Dai *et al*. 2007; Chen *et al*. 2008). Thus, the medium used by Song *et al*. (2001) gave high germination in one cultivar, but low and variable germination in others.

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Optimizing the germination medium was therefore a prerequisite for this research on PG in relation to temperature stress.

Cardinal temperatures are the critical temperatures that characterize temperature responses for crop growth and development and vary between crops and with developmental stage (Hatfield *et al*. 2008). These temperatures – the minimum below which development does not occur, the optimum at which the rate of development is most rapid and the maximum temperature above which development ceases – have been published for rice developmental stages from seed germination to ripening (Krishnan *et al*. 2011; Shah *et al*. 2011), but not for PG and PTG. In this paper, cardinal temperatures for the quantal response of PG *per se* are distinguished from those for the rate of growth of the pollen tube. By analogy with seed germination, there is no *a priori* reason for the cardinal temperatures to be the same for both characteristics. For example, the optimum temperature for final percentage germination of *Phelipanche aegyptiaca* (Pers.) Pomel was 18–20 °C (Kebreab & Murdoch 1999a) whereas for the rate of germination, a measure of vigour, it was 26–29 °C (Kebreab & Murdoch 1999b). In seeds, cardinal temperatures are typically determined from germination rates rather than percentage germination (Covell *et al*. 1986; Dumur *et al*. 1990; Steinmaus *et al*. 2000; Hardegree & Winstral 2006), but with pollen, responses based on polynomial or split-line models of germination *per se* have been used (Kakani *et al*. 2002; Salem *et al*. 2007; Acar & Kakani 2010) to estimate minimum and maximum temperatures for PG. These theoretical temperature limits are, however, likely to give a misleading measure of tolerance to temperature stress for two main reasons. First of all, spikelet fertility and grain yields are much higher when a large number of pollen grains germinate on each stigma rather than just a few (Rang *et al*. 2011), so that to achieve reliable seed set in rice, 10–20 pollen grains must germinate on the stigma (Matsui *et al*. 2001; Jagadish *et al*. 2010). Secondly, polynomial models ignore the binomial nature of germination (pollen grains either germinate or do not) and parameters have no biological meaning. Other quantal responses in plants, however, have been successfully modelled by probit analysis including dose–response curves of pea (*Pisum sativum* L.) PG to cadmium (Kumar *et al*. 2000), survival over time of air-dry pollen (Hong *et al*. 1999), fungal conidia (Hong *et al*. 1997) and seeds (Ellis & Roberts 1980; Ellis & Hong 2007); minimum and maximum temperature limits for germination of *Orobanche* seeds (Kebreab & Murdoch 1999a, 2000); and seed germination across sub- and supra-optimal temperatures and seedling emergence under seedbed stress (Ellis & Roberts 1981; Hardegree 2006).

In this paper therefore, probit models developed for seed dormancy and germination of the holo-parasitic *Orobanche* and *Phelipanche* spp. (Kebreab & Murdoch 1999a,c, 2000) and for changes in seed dormancy in the hemi-parasitic species, *Striga hermonthica* (Del.) Benth. (Dzomeku & Murdoch 2007), are extended and applied, for the first time, to pollen. This paper is also the first to use probit models to quantify rice PG responses to temperature.

Oryza sativa L. ssp. indica (cv. IR64), *O. glaberrima* Steud. (cv. CG14) and *O. sativa* ssp, aus (cv. N22) were selected on the basis of their contrasting responses to high day temperatures at microsporogenesis and anthesis. The heat tolerance of N22 in terms of spikelet fertility and yield is well established (Yoshida *et al*. 1981; Prasad *et al*. 2006; Jagadish *et al*. 2007, 2008, 2010; Coast *et al*. 2015), while Rang *et al*. (2011) confirmed its 'true tolerance' to high day temperatures (38 °C for 6 h for 4 d at around the time of anthesis) by higher germination of pollen on the stigma and much higher spikelet fertility than for cv. IR64. IR64 is sensitive to high day temperatures at both microsporogenesis and anthesis (Jagadish *et al*. 2008, 2010, 2014; Coast *et al*. 2015). Similarly, CG14 is susceptible to high day temperature stress at microsporogenesis (Jagadish *et al*. 2014) and anthesis (Prasad *et al*. 2006; Jagadish *et al*. 2008), as evidenced by spikelet fertility reductions of 40–60% (at microsporogenesis) and 70% (at anthesis) when exposed to 4–6 consecutive days of 38 compared with 30 °C.

Using these three rice cultivars with contrasting responses to temperature stress, the objectives were (1) to model the effects of temperature on PG and PTG rate; and (2) to investigate if the resilience or susceptibility to temperature stress in these three genotypically diverse rice cultivars could relate to the temperature limits for PG and cardinal temperatures for the rate of PTG. The hypothesis tested is that each individual pollen grain has a minimum temperature requirement and maximum temperature limit, which act independently and control its ability to germinate at any given temperature.

MATERIALS AND METHODS

Field establishment

Seeds of the three rice cultivars originating from different countries and agro-ecologies (Table 1) were utilized. Pregerminated seeds were placed into seed trays filled with natural clay loam soil. Two weeks after sowing, seedlings were transplanted into paddy fields at the International Rice Research Institute (IRRI) in the Philippines (14°11′ N, 121°15′ E). Five or six seedlings per hill were transplanted at a spacing of 0.3×0.2 m into two plots 90×90 m each and 5 m apart. Ten days after transplanting, plants were thinned to three (CG14 and N22) or two (IR64) per hill (IR64 tillers more profusely). Fertilizer was applied according to normal practice at IRRI, that is, basal (30:15:20:2.5 kg N:P:K:Zn ha^{-1}), mid-tillering (20 kg N ha^{-1}), panicle initiation (20 kg N ha^{-1}) and before heading (30 kg N ha^{-1}). Paddy fields were kept continuously flooded. No pest or disease problems were observed. Temperature and relative humidity at panicle height in adjacent rice plots 5 m away were logged every 10 min and mean values recorded every half hour using Hobo Microstation data loggers (Onset Computer Corp., Cape Cod, Massachusetts, USA).

Harvesting panicles and collecting pollen

At 50% anthesis on each of 2 consecutive days for each cultivar, panicles were harvested for pollen between 0700 and

Table 1. Information on cultivars of (*Oryza* spp.) selected for study

Species	Cultivar[a]	Accession number[b]	Origin	Adaptation	Days to 50% anthesis[c]
Oryza glaberrima	CG14	–[d]	Senegal	Upland	57
Oryza sativa ssp. Indica	IR64	IRTP-12158	Philippines	Lowland	60
Oryza sativa ssp. aus	N22	IRTP-03911	India	Upland	50

[a]Germplasm sourced from the International Rice Research Institute (IRRI), Philippines.
[b]IRTP (International Rice Testing Program, now International Network for Genetic Enhancement of Rice).
[c]Days to 50% anthesis from transplanting in the IRRI 2009 dry season breeding experiment.
[d]Sourced from IRRI breeder.

0900 h. The cultivar, N22, was harvested 13 d earlier than the other two cultivars (Table 2) on account of its shorter duration from transplanting to anthesis (Table 1). Panicle stems were bent into test tubes filled with water and cut under water to avoid obstructing transpiration. Harvested panicles were transferred with their stems in the water to the laboratory and kept next to the window (to ensure sufficient light exposure) until spikelets started opening (15–60 min after harvest). For each cultivar, a minimum of 84 panicles were harvested from a mixture of main stems and primary tillers.

PG media

PG media (modified from Dai *et al*. 2007) were freshly prepared with 0.04 g of boric acid and 0.003 g of vitamin B1 and 0.04–0.06 g of calcium nitrate tetrahydrate [$Ca(NO_3)_2.4H_2O$], which were dissolved in 1 L deionized water. Twenty grams of sucrose and 10 g of polyethylene glycol (PEG 4000) were then dissolved in 700 mL of this solution. The solution was thoroughly mixed with a magnetic stirrer at 35 °C before pouring into 30 mm diameter Petri dishes. Chemicals were obtained from Sigma-Aldrich Co. (Buona Vista, Singapore).

Dai *et al*. (2007) used 0.7 g L^{-1} $Ca(NO_3)_2.4H_2O$, but in preliminary trials for this research, high PG and PTG were obtained at lower concentrations. In IR64, for example, the optimum was 0.06 g L^{-1} of $Ca(NO_3)_2.4H_2O$ (Supporting Information Table S1). Although PG of CG14 was also maximized with 0.06 g L^{-1} $Ca(NO_3)_2.4H_2O$, many pollen grains germinated abnormally, having two pollen tubes, and so a slightly lower concentration (0.055 g L^{-1}) was used, while PG of N22 was better at 0.04 g L^{-1} (O. Coast, unpublished results). Using these media, 80–90% of pollen grains routinely germinated *in vitro* at laboratory temperature (26–28 °C).

Temperature treatments

Temperature treatments were applied using a temperature gradient plate (TGP; Grant Instruments Ltd., Cambridge, UK; Murdoch *et al*. 1989; Kebreab & Murdoch 1999a). The plate was operated in one direction and run twice to provide two sets of 14 constant temperature regimes, one between 5.6 and 27.4 °C and the next day between 24.6 and 45.4 °C. The surface temperature of the TGP was measured underneath a set of Petri dishes placed along the gradient every 5 min for a period of 1 h and a similar set of measurements was recorded immediately afterwards in the germination medium using Campbell dataloggers (CR1000, Campbell Scientific, Inc., Logan, UT, USA). Although some differences were observed, mean differences between the medium and plate temperatures at the same position on the plate were small at +0.2 and –0.7 °C for the 24.6/45.4 °C and 5.6/27.4 °C temperature gradients, respectively (Supporting Information Fig. S1). Analyses were based on temperatures measured on the surface of the TGP.

For each cultivar, there were three replicate Petri dishes for each of the 28 nominal temperature points on the TGP. The dishes were placed on the TGP for about 15 min to equilibrate with the TGP temperature before dusting with pollen.

Using the freshly collected open spikelets, pollen grains from spikelets on one individual panicle at anthesis were dusted onto the germination medium in one Petri dish on the TGP, by gently tapping the panicle. To prevent pollen from getting into other Petri dishes, only one Petri dish was opened at a time. Germination tests were carried out in the dark as is usual for pollen (Kakani *et al*. 2002, 2005). After 4 h on the TGP, the 252 Petri dishes were stored in a refrigerator at 4 °C until assessed. No changes in germination or pollen tube lengths were expected or observed during storage, the storage temperature being well below the minimum temperature for growth.

PG and tube growth rate determination

Germination after 4 h was counted at 100 × magnification using a transmitted light microscope (Primo Star; Carl Zeiss Int., Göttingen, Germany) fitted with a Plan-Achromat lens (Zeiss Int., Jena, Germany). Pollen was considered germinated if the length of the pollen tube was equal to or greater than the diameter of the pollen grain (Kakani *et al*. 2005). PG was calculated as the percentage of germinated pollen grains to the total number of pollen grains averaged across six to ten microscopic field views such that at least 200 pollen grains were assessed to calculate PG in each Petri dish.

To estimate tube growth rate, the lengths of 15–40 pollen tubes were measured in each replicate. Due to the wide range of temperatures tested, at many of which PTG may be slow, a 4 h period was used for this *in vitro* study rather than the 1 or 2 h periods used *in vivo* by Jagadish *et al*. (2010) and Chen *et al*. (2008), respectively. Images of germinated pollen grains were captured using an imaging microscope (Axioplan 2;

Carl Zeiss Int.) at 100 × magnification and a free-hand trace drawn around each pollen tube, its length being recorded automatically using Image Pro-Plus software (Media Cybernetics, Inc., Rockville, MD, USA). The lengths of the three longest pollen tubes from at least two Petri dishes at each temperature were used to calculate mean growth rates per hour, which were used in data analyses.

Model fitting and cardinal temperature determination

GenStat (GenStat® 13th Edition; VSN Intl. Ltd., Hemel Hempstead, UK) was used to fit the various models. After 4 h, PTG data satisfied the assumptions of normality and homogeneity. The mean PTG rates per hour were analysed using a split-line non-linear model in which growth rate was regressed against temperature. The optimum temperature for the rate of PTG was fitted by a standard iterative procedure in GenStat to minimize residual variance. The base and ceiling temperatures are extrapolations to temperatures at which the rate was predicted to be zero. The reciprocal of the slope of the response of PTG rate to mean temperature at suboptimal temperatures estimates the mean thermal time to achieve a tube length of 1 μm from which thermal times for 1 mm tube lengths were calculated.

Non-linear probit models with binomial errors were fitted to the PG data for each cultivar using the FITNONLINEAR function in GenStat. Multiplicative and subtractive models were fitted to compare two alternative hypotheses, namely that the cardinal temperature limits for individual pollen grains are either independent or linked, respectively. According to the probit model, variation in temperature limits is normally distributed within a homogeneous population of pollen grains such that:

$$\Phi_{\min} = [K_n + bT] \quad (1)$$

and

$$\Phi_{\max} = [K_x + cT] \quad (2)$$

where $\Phi_{\min}$ and $\Phi_{\max}$ are the proportions of grains in normal equivalent deviates (n.e.d.) or probits for which the temperature, T (°C), is, respectively, the minimum temperature requirement or the maximum temperature limit for germination; K_n and K_x are intercepts, that is the proportions (n.e.d.) whose requirements/limits are met at 0 °C and b and c are temperature coefficients.

In the multiplicative probability model, the minimum and maximum temperature limits represented by Eqns 1 and 2, are, respectively, positive and negative cumulative normal distributions so that the coefficient, c, is negative. The temperature limits are assumed to be independent so that the proportion of pollen grains germinating, G, is the product of these two functions after back-transformation (Φ^{-1}) of their respective n.e.d. values to probabilities (Eqn 3):

$$G = (\Phi_{\min})^{-1}(\Phi_{\max})^{-1} \quad (3)$$

so that,

$$G = (\Phi^{-1}[K_n + bT])(\Phi^{-1}[K_x - cT]) \quad (4)$$

In the subtractive probability model, the distributions of minimum and maximum temperature limits represented by Eqns 1 and 2, are both positive cumulative normal distributions and hence, c is positive:

$$G = (\Phi^{-1}[K_n + bT]) - (\Phi^{-1}[K_x + cT]) \quad (5)$$

To avoid negative values of G, parameter values are constrained so that $K_n \geq K_x$ and $b \geq c$ so that temperature limits are not assumed to be independent in the subtractive model.

Following Kebreab & Murdoch's (1999a, 2000) research on seeds, an exponential effect of temperature on the distribution of high-temperature limits (Eqn 2) was also tested as in Eqn 6 for the multiplicative model:

$$G = (\Phi^{-1}[K_n + bT])(\Phi^{-1}[K_x - cr^T]) \quad (6)$$

where r quantifies the rate of exponential decrease in the maximum temperature limit with increase in T. A similar change can be made in the subtractive model.

To provide parameters that might be used to assess the resilience of PG to temperature stress, these equations can be rearranged to model the means and standard deviations of the fitted normal distributions. By definition at the mean minimum and maximum temperature limits, 50% of pollen grains are at the temperature limit, that is $\Phi^{-1} = 0.5$ as a proportion and $\Phi = 0$ n.e.d. The estimated mean limits are hereafter respectively designated $T_{n(50)}$ and $T_{x(50)}$. Moreover, by definition, the reciprocal of the slope of the normal distribution function is the standard deviation (°C) of individual temperature limits within a population of pollen grains, hereafter designated s_n and s_x, for minimum and maximum temperature distributions, respectively. Hence, when $\Phi_{\min} = 0$, Eqn 1 becomes

$$[K_n + T_{n(50)}/s_n] = 0 \quad (7)$$

and

$$K_n = -T_{n(50)}/s_n \quad (8)$$

Substituting for K_n in Eqn 1,

$$\Phi_{\min} = (T - T_{n(50)})/s_n \quad (9)$$

Treating Eqn 2 and the exponential equivalent similarly, Eqns 4, 5 and 6 may, respectively, be rewritten as follows:

$$G = \Phi^{-1}[(T - T_{n(50)})/s_n]\Phi^{-1}[(T_{x(50)} - T)/s_x] \quad (10)$$

$$G = \Phi^{-1}[(T - T_{n(50)})/s_x] - \Phi^{-1}[(T - T_{x(50)})/s_x] \quad (11)$$

$$G = \Phi^{-1}[(T - T_{n(50)})/s_n]\Phi^{-1}[(r^{Tx(50)} - r^T)/s_x]. \quad (12)$$

The optimum temperature (T_o) for germination was estimated as the temperature at which the fitted germination, G, was maximized.

Period	Time of day[a]	Temperature mean (range), °C		Relative humidity mean (range), %	
July	Day	26.7 (23.6 – 28.3)		89.0 (53.7–100.0)	
	Night	25.2 (22.6 – 28.1)		91.7 (47.4–100.0)	
August	Day	28.6 (24.1 – 32.9)		87.7 (69.9–100.0)	
	Night	25.6 (22.8 – 29.2)		94.8 (78.4–100.0)	
		CG14 & IR64	N22	CG14 & IR64	N22
2 dba to anthesis	Day	27.6	26.0	92.1	94.6
	Night	25.5	24.9	96.3	96.1
15 dba to anthesis	Day	28.2	26.8	88.7	89.4
	Night	25.4	25.3	95.7	92.1
At times of panicle harvests		27.8	26.7	92.9	94.4

Table 2. Day and night temperatures and relative humidity of rice paddy plots during the study (July to August 2011), over periods of 2 and 15 days before anthesis (dba) and at times of panicle harvests

Panicles were harvested between 0700 and 0900 h on 2 and 3 August 2011 (cv. N22), and on 15 and 16 August 2011 (cvs CG14 and IR64).
[a]Day = 0600–1800 h EST and Night = 1830–0530 h EST.

RESULTS

Mean day/night temperatures over periods from 2 and 15 days before anthesis (dba) until anthesis were approximately optimal for rice and similar for both the earlier-harvested N22 (26.0/24.9 and 26.8/25.3 °C, respectively) and the later harvested CG14 and IR64 (27.6/25.5 and 28.2/25.4 °C, respectively) (Table 2, Supporting Information Fig. S2). In addition, mean daytime relative humidities over the same periods and at the actual times when the panicles were being harvested were comparable (Table 2, Supporting Information Fig. S2).

PG

Maximum PG was observed at 27 °C for all three cultivars amounting to 86, 77 and 93% for CG14, IR64 and N22, respectively (Fig. 1, Table 3). Although pollen grains germinated over very wide temperature ranges, that is, 12.2–41, 5.7–35 and 5.6–45.4 °C, for CG14, IR64 and N22, respectively, very few pollen grains germinated at low and high temperatures (Fig. 1). So while these temperature ranges are of interest, they reflect extreme individuals in the population and parameters quantifying the performance of the overall population are also needed.

Modelling percentage germination

Residual deviances were significantly lower and adjusted R^2 values were higher for the multiplicative models (Eqns 4 and 6) than for the subtractive model (Eqn 5) in all three cultivars (Suppl. Table S2). A small but significant improvement in the goodness of fit was obtained for CG14 and N22, but not for IR64, when expressing the maximum temperature limit on an exponential scale (Eqn 6). The parameter, r, could not, however, be optimized by non-linear modelling in GenStat and no standard errors could be obtained. Instead, r had to be optimized by varying its value manually to minimize the residual deviance, the optimal value of r varying with cultivar and model (Suppl. Table S2). Visually, the exponential model for maximum temperature limits reduced the highest predicted germination at the optimum temperature but slightly

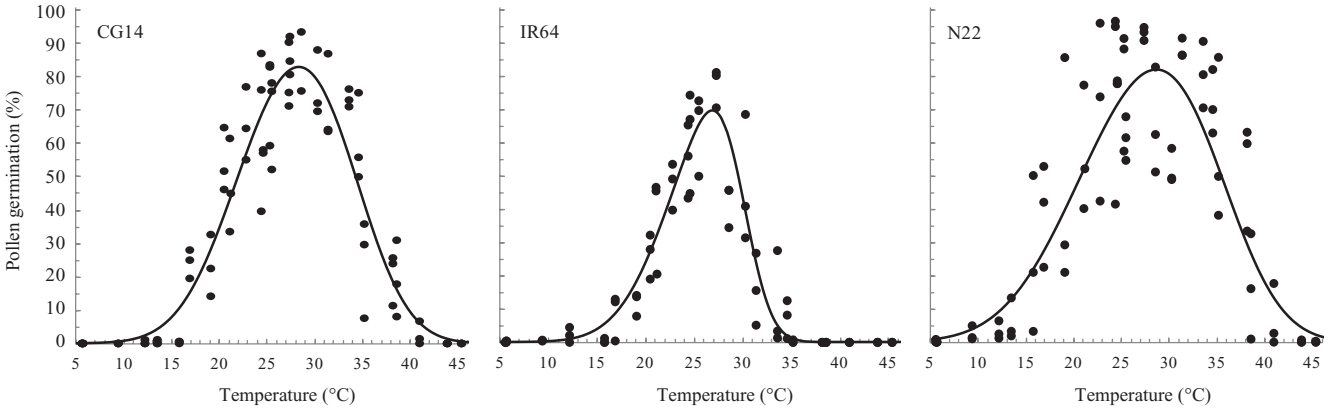

Figure 1. Pollen germination for rice (*Oryza* spp.) cultivars, CG14, IR64 and N22, at different temperatures on a temperature gradient plate. Parameter estimates of the fitted lines according to Eqn 10 are given in Table 3.

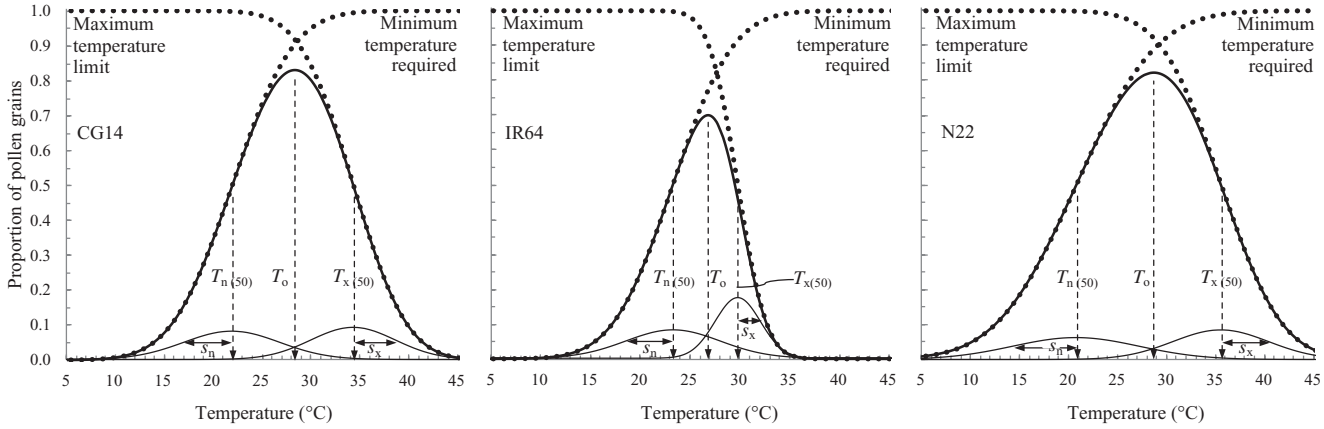

Figure 2. Theoretical underlying distributions and parameters for multiplicative probability model of germination for pollen of rice (*Oryza* spp.) cultivars, CG14, IR64 and N22. Fitted germination curve (thick solid line), the optimum germination temperature (T_o), and the theoretical underlying normal frequency distributions (cumulative (dotted lines) and bell-shaped (thin solid lines)] are shown according to Eqn 10. Respective parameter estimates (Table 3) of these distributions are the mean minimum ($T_{n(50)}$) and maximum ($T_{x(50)}$) temperature limits and standard deviations (s_n and s_x).

improved the goodness of fit for low germination values at high temperatures (cf. Fig. 1). Given the inability to optimize r or determine its standard errors using GenStat together with the principle of minimizing the number of parameters (three extra being needed because r varied with cultivar), the multiplicative probability model with an exponential function for maximum temperature was rejected. Results are therefore presented according to Eqn 10 and the underlying cumulative normal distributions and bell-shaped curves of minimum temperature requirements and maximum temperature limits for germination of each cultivar are shown (Fig. 2). According to the model, germination at the optimum temperature is less than 100%, because of the significant overlap of the two distributions in all three cultivars, but to the greatest extent in IR64 (Fig. 2). This effect in IR64 is partly linked to s_x being about half that for the other two cultivars (Table 3), which also results in PG values decreasing more rapidly in IR64 than CG14 and N22 as temperature increased above T_o (Figs 1 & 2). As a result $T_{x(50)}$ is also much lower in IR64 (Table 3, Fig. 2) the effect of which in combination with the low s_x, is exemplified by no pollen germinating above 35 °C (Fig. 1). Conversely, both N22 and CG14 had higher values for $T_{x(50)}$ and s_x (Table 3, Fig. 2), with some germination above 40 °C in both cultivars (Fig. 1).

PTG rates

The mean maximum pollen tube length across the three rice cultivars after 4 h was 1390 μm at 24.6 °C, but cultivars differed ($P < 0.01$): the mean maximum for N22 (1886 μm at 24.6 °C) was longer ($P < 0.05$), but also more variable than for CG14 (1288 μm at 27.3 °C) and IR64 (1290 μm at 25.5 °C) (Fig. 3 and Table 4 where PTG is shown as a rate per hour). Measurable PTG occurred over a narrower temperature range for IR64 (19.1–35.2 °C) than for CG14 (16.9–38.6 °C) or N22 (13.5–38.2 °C) (Fig. 3).

Modelling the rate of PTG

The estimated optimal and ceiling temperatures for the rate of PTG were higher and the base temperatures lower for CG14 than for IR64 and N22 (Table 4). Thermal times for 1 mm tube lengths were much greater for CG14 at suboptimal temperatures (65.4 °C h) than for the other two cultivars (Table 4). The longer thermal time in CG14 is a reflection of the shallower slope at suboptimal temperatures (Fig. 3, Table 4). The lower base temperature of CG14 (Table 2) compensates partly for its higher thermal time. Nevertheless, assuming both an approximately optimal temperature for rate of PTG (27 °C, Table 4) and also a constant growth rate, the predicted periods to achieve a 1 mm long pollen tube are 3.4, 3.0 and 2.4 h for CG14, IR64 and N22, respectively.

Cardinal germination temperatures

Using the multiplicative probability model, the optimum temperatures, at which fitted PG values were maximized, were similar for CG14 and N22 (28.3–28.7 °C), but slightly cooler for IR64 (26.8 °C) (Fig. 1, Table 3). While there is no *a priori* reason why these optima should be the same as the optima for rate of PTG, it is interesting that these are within 0.6 and 0.1 °C of the estimated optima for rate of PTG in CG14 and IR64, respectively, whereas there is a 2 °C difference for N22 (Tables 3, 4). Overall, however, differences in the optima for PG and PTG were small, being 27–29 °C for each cultivar.

More significantly, the temperature range between which 50% of pollen grains exceeded their minimum temperature requirement ($T_{n(50)}$), but had not exceeded their maximum temperature limit ($T_{x(50)}$) was much wider for CG14 and N22 (c. 21–35 °C) than for IR64 (23–30 °C), the most pertinent observation in terms of resilience perhaps being that IR64 has a 5 °C lower $T_{x(50)}$ than the other two cultivars (Table 3, Fig. 2). Being extrapolations to temperatures at which the

Table 3. Parameter estimates (SEs) and cardinal temperatures of pollen germination of rice (*Oryza* spp.) cultivars

					Fitted values:		Observed values:	
Cultivar	Mean minimum temperature limit $T_{n\ (50)}$, °C	SD of minimum temperature limits s_n, °C (n.e.d.)$^{-1}$	Mean maximum temperature limit $T_{x\ (50)}$, °C	SD of maximum temperature limits s_x, °C (n.e.d.)$^{-1}$	Optimum temperature T_o, °C	Maximum germination (%)	Temperature with highest germination (°C)	Highest germination (%)
CG14	21.9 (0.09)	4.90 (0.12)	34.3 (0.11)	4.32 (0.14)	28.3	83.0	27.4	85.8 (7.4)
IR64	23.3 (0.10)	4.74 (0.13)	29.8 (0.10)	2.28 (0.10)	26.8	69.7	27.2	77.1 (4.8)
N22	20.8 (0.09)	6.45 (0.12)	35.6 (0.08)	4.88 (0.11)	28.7	82.0	27.4	92.7 (9.0)

Estimates are for the multiplicative probability model (Eqn 10) at temperatures between 5.6 and 45.4 °C. The overall normal distribution curves are given in Fig. 2.
n.e.d., normal equivalent deviates (probit-5); s_n and s_x, reciprocals of slopes of fitted lines; $T_{n\ (50)}$, minimum temperature required for germination for 50% of pollen grains; $T_{x\ (50)}$, maximum temperature limit for germination of 50% of pollen grains.

Table 4. Parameter estimates (SEs) of split-line regressions of the rate of pollen tube growth of rice (*Oryza* spp.) cultivars as a function of germination temperature

								Observed optimal values:	
Cultivar	Optimum temperature for PTG rate, T_o (°C)	Fitted mean PTG rate at optimum temperature (μm h^{-1})	Temperature coefficient at suboptimal temperatures, (μm h^{-1}) °C^{-1}	Temperature coefficient at supra-optimal temperatures, (μm h^{-1}) °C^{-1}	Base temperature, T_b (°C)	Ceiling temperature, T_c (°C)	Thermal time above T_b for PTG of 1 mm (°C h)	Temperature with highest mean maximum PTG rate (°C)	Highest mean max. PTG rate (μm h^{-1})
CG14	28.9 (0.55)	319.5 (10.1)	15.3 (1.86)	−24.0 (2.89)	8.02	42.2	65.4	27.3	322.1 (15.7)
IR64	26.9 (0.37)	331.4 (11.1)	21.7 (3.51)	−34.6 (2.67)	11.6	36.4	46.1	25.5	322.4 (46.2)
N22	26.7 (0.74)	415.7 (20.8)	25.2 (4.02)	−31.4 (4.88)	10.2	39.9	39.6	24.6	471.5 (162.5)

Base and ceiling temperatures are calculated from regression parameters. Thermal times are for suboptimal temperatures only. Pollen was germinated for 4 h at temperatures between 5.6 and 45.4 °C. Fitted lines and parameters are shown in Fig. 3.
PTG, pollen tube growth.

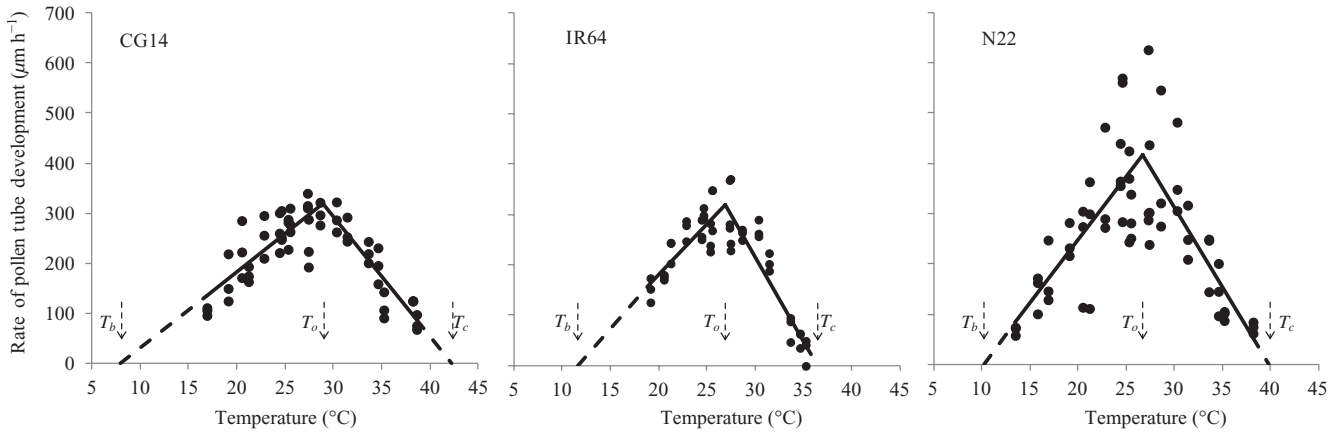

Figure 3. Pollen tube growth rates and cardinal temperatures for rice (*Oryza* spp.) cultivars, CG14, IR64 and N22. Parameter estimates for the fitted lines and optimum (T_o) temperatures are shown in Table 4. Rates were calculated from pollen tube lengths measured after 4 h on a temperature gradient plate at the temperatures shown. Thicker dashed lines are extrapolations to the base (T_b) and ceiling (T_c) temperatures for the rate of tube growth while the optimum temperature (T_o) is the value at which it is maximal.

rates of PTG are zero, the temperature differences between the base and ceiling temperatures should be much wider than those between $T_{n\,(50)}$ and $T_{x\,(50)}$. However, while evaluating resilience to both extreme high and low temperatures, it is relevant to note that the range is again widest for CG14 (8 to 42 °C) and narrowest for IR64 (12–36 °C), N22 being intermediate (10–40 °C; Table 4). Although they are extrapolations, the ceiling temperatures (Table 4) estimated from the split-line regressions of PTG rates are fairly realistic, reflecting the highest temperatures at which PG was observed in each cultivar (Fig. 1).

DISCUSSION

High PG and PTG were achieved across diverse rice cultivars by adjusting the concentration of calcium nitrate. The calcium ion is essential for germination and subsequent growth of pollen in many flowering plant species (Brewbaker & Kwack 1963; Ge *et al*. 2007). Extremely high or low calcium ion concentrations *in vitro* affect the cell wall, which may become discontinuous or thickened at the tube tip, respectively, resulting in poor PTG (Ge *et al*. 2007). Nitrate promotes seed germination (Vincent & Roberts 1977; Vandelook *et al*. 2008) including of rice (Roberts 1963), but by terminating seed dormancy rather than initiating germination (Finch-Savage & Leubner-Metzger 2006). Pollen grains are non-dormant, so any role of the nitrate ion is likely to differ from that in seeds.

The 85% PG recorded here is similar to the highest reported previously for *in vitro* research on single rice cultivars (90%: Kariya 1989; 85%: Song *et al*. 2001) and higher than the percentages recorded across multiple cultivars of other crops (36–81%, Table 5). The mean maximum PTG of the three rice cultivars (1390 μm) was also longer than for other crops (437–1020 μm, Table 5). In comparison with PTG through the pistil, Jagadish *et al*. (2010) reported PTG of 1840 and 1350 μm after 1 h for IR64 and N22, respectively, as against *in vitro* values of 1288 and 1886 μm here. The *in vivo* tube lengths reflect the pistil lengths, which were 2340 and 1850 μm, respectively (Jagadish *et al*. 2010), and pollen responses may differ between *in vivo* and *in vitro* conditions (Read *et al*. 1993; Taylor & Hepler 1997; Rosell *et al*. 1999; Poulton *et al*. 2001).

The decrease in PG at high temperature has been linked with alteration in pollen morphology and failure of metabolic processes such as rehydration, reduced sugar activity and utilization, marked by increased sucrose and starch concentrations (Aloni *et al*. 2001; Karni & Aloni 2002). By contrast, at low temperature, the decline in PG has been associated with decreased availability of sucrose and the reducing sugars, fructose and glucose (Shaked *et al*. 2004). Do these changes in physiology, which are associated with low and high temperatures, cause low germination or are they simply secondary effects? If causative, the physiological mechanisms for upper and lower temperature limits are, therefore, quite distinct. That hypothesis was tested and accepted here as a result of the statistical superiority and goodness of fit of the multiplicative probability model to the data. A similar inference was proposed by Kebreab & Murdoch (1999a,b) in discussing primary and secondary dormancy of seeds of *Phelipanche aegyptiaca*. Against this, it could be argued that the variability in temperature limits quantified by fitting probit models might be interpreted probabilistically as pollen grains are genetically similar. Accepting the multiplicative model implies, however, that an individual pollen grain may simultaneously be below its T_n and above its T_x, which could only occur if the mechanisms were different and independent. The biological mechanisms underlying T_n and T_x must, therefore, operate independently.

With respect to achieving high-temperature tolerance, optimum temperatures for both PG and PTG failed to discriminate between the cultivars, being similar among all three tested (Tables 3 and 4). The parameters of the theoretical underlying distributions (Fig. 2) do, however, help in explaining why IR64 cannot tolerate high day temperatures at

Table 5. Comparison of rice pollen performance *in vitro* and cardinal temperatures with some other crops

Species	PG (%)	Optimum temperature (range) for PG, °C	PTG (μm)	Optimum temperature (range) for PTG (C)
Oryza species	85[a]	27.3 (10–42)[a]	1390[a]	25.8 (14–39)[a]
Glycine max (L.)Merr.	81[b]	30.2 (13–47)[b]	437[b]	36.1 (12–47)[b]
Capsicum annuum L.	78[c]	30.7 (15–42)[c]	734[c]	31.2 (12–40)[c]
Arachis hypogaea L.	56[d]	30.1 (14–43)[d]	1020[d]	34.4 (15–44)[d]
Gossypium hirsutum L.	44[e]	27.3 (12–43)[e]	778[j]	27.8 (12–44)[e]
Brassica napus L.	37[f]	23.6 (8–33)[f]	660[f]	25.4 (5–33)[f]
Sorghum bicolor (L.)Moench	36[g]	29.4 (17–42)[h]	–	–

[a]This paper.
[b]Salem *et al*. (2007).
[c]Reddy & Kakani (2007).
[d]Kakani *et al*. (2002).
[e]Liu *et al*. 2006.
[f]Singh *et al*. (2008).
[g]Tuinstra & Wedel (2000).
[h]Prasad *et al*. (2011).
[j]Kakani *et al*. (2005).
—, data not available; PG, pollen germination; PTG, pollen tube growth.

anthesis. Not only was its $T_{x\,(50)}$ relatively low, but its lower s_x also indicates low variability between pollen grains in T_x. Its resilience on exposure to high-temperature stress is therefore limited as very few grains in the population exhibited high-temperature tolerance, none germinating above 35 °C. These results can, therefore, account for the reported decline in spikelet fertility of IR64 when spikelet tissue temperatures exceeded 33.7 °C at anthesis (Jagadish *et al*. 2007; Weerakoon *et al*. 2008).

The above provides an example of within-cultivar uniformity being disadvantageous to resilience. By contrast, an important potential contribution of PG to N22's resilience to high-temperature stress has been quantified here by its higher $T_{x\,(50)}$ and wider s_x so that unlike IR64, over 50% of N22's pollen grains could germinate at 35 °C.

Interestingly however, these two parameters were only slightly lower in the high temperature-susceptible CG14 than in N22. The wide PG and PTG temperature range displayed by CG14 is perhaps not surprising as it is an *O. glaberrima* with traits that have been employed in the development of other abiotic stress-tolerant cultivars (Jones *et al*. 1997; Agnoun *et al*. 2012). Clearly, other factors must override the relatively high $T_{x\,(50)}$ and wide s_x in CG14. The longer thermal time required for CG14 to achieve 1 mm pollen tube length could contribute to its susceptibility if the effect of that were that fertilization took place at the hottest time of the day. However, CG14 tends to flower earlier in the morning than many cultivars including N22 (Prasad *et al*. 2006; Jagadish *et al*. 2008) and so even if the process of fertilization took longer in CG14 compared with N22, its earlier flowering could compensate for potential heat damage. It is therefore suggested that the resilience of N22 and the susceptibility of IR64 to high-temperature stress at anthesis can be explained in terms of their respective values of $T_{x\,(50)}$ and s_x. In CG14, however, the dynamics of flowering patterns in the panicle during the course of the day and other physiological processes occurring after germination such as pollen tube–ovary signalling prior to and during fertilization and early embryo development, may need to be invoked to account for its susceptibility.

In order to compare the results obtained here with cardinal PG temperatures quantified by polynomial regression, the lower and upper temperatures for 1% germination were predicted for PG by Eqn 10. Averaged across the three cultivars, the predicted values were 10 and 42 °C, respectively, which are similar to cardinal temperatures for PG of certain other crops (Table 5). Although a wide s_x may mean 1% PG at 42 °C in CG14 and N22, this low PG and the very low rate of PTG by these extreme individuals in the population is unlikely to give an agriculturally acceptable level of spikelet fertility (compare Rang *et al*. 2011). The use of these cardinal temperatures to assess resilience to high-temperature stress may therefore be misleading. By contrast, the mean limits (i.e. $T_{n\,(50)}$ and $T_{x\,(50)}$) used in this paper will probably allow greater than the minimum germination required to achieve spikelet fertility, and thus arguably provide a 'fail-safe' estimate of the temperature range required to minimize the risk of yield loss in rice because of either low PG or low PTG when assessing cultivars.

The previous discussion has focussed on upper temperature limits. The data on low-temperature limits is also interesting as global change scenarios may also include extreme low-temperature events or breeders may consider adapting cultivars for other environments where temperatures are lower. Pollen of both CG14 and N22 germinated at or below 13 °C, which is considered a critical threshold for cold tolerance in rice (Farrell *et al*. 2006), but as noted already, the performance of extreme individuals can be misleading. Based on the $T_{n(50)}$ values, adequate germination for good fertilization would need a temperature of approximately 20 °C, N22 being slightly more tolerant of low temperatures according to this criterion, although rates of PTG would, however, be

slower at low temperatures. Further research is needed to test the hypotheses relating to the pollen traits of most significance for conferring low-temperature tolerance at anthesis.

The large temperature range that exists naturally with rice cultivation across tropical and temperate regions highlights the agronomic relevance of pollen performance in the tested range of temperatures. In addition, with a changing climate, identifying and utilizing genetic diversity in PG and PTG is a reliable approach towards developing tolerant rice cultivars to sustain future rice production. Subject to the caveat that the applicability of the models developed here needs to be confirmed on a larger set of genotypes, it can be concluded that optimal temperatures for *in vitro* rice PG and PTG do not discriminate between rice genotypes that were either susceptible or tolerant of high temperatures at anthesis. Moreover, the traditional use of base and ceiling temperatures gives a misleading impression of resilience as PG at temperatures close to these extrapolated limits was very low and PTG was slow. While further research is required to confirm that the responses of *in vitro* PG and PTG to temperature reflect *in vivo* performance on the stigma, it is clear that parameters derived from modelling variation in temperature limits for PG (specifically, s_x and $T_{x(50)}$) can together be applied to identify those cultivars where PG is likely to improve resilience to high day temperature stress.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Temperatures on the temperature gradient plate, measured with thermocouples placed directly on the surface of the plate (dashed line, triangles) underneath the Petri dishes containing pollen germination medium or in the pollen medium itself (solid line, squares). The plate was run twice for each pollen type; once with a 5.6–27.4 °C gradient and once with a 24.6–45.4 °C gradient. Linear regressions for these two gradients were $y = -1.67x + 29.68$ ($R^2 = 0.98$) and $y = -1.58x+46.04$ ($R^2 = 0.99$) on the plate surface and $y = -1.65x + 30.21$ ($R^2 = 0.98$) and $y = -1.30x + 43.75$ ($R^2 = 0.96$) in the pollen media, respectively.

Figure S2. Diurnal air temperature (a) and relative humidity (b) of field environment from 2 d before anthesis to anthesis for rice cultivar, N22 (dotted line; on 2–3 August 2011), and for cultivars CG14 and IR64 (solid line; on 15–16 August 2011).

Table S1. *In vitro* germination of pollen of rice cultivar IR64 at different concentrations of $Ca(NO_3)_2.4H_2O$ in pollen germination medium. Values are means of four replicates. (SED = 12.5%, 15 degrees of freedom).

Table S2. Comparisons of models of pollen germination of three rice cultivars at temperatures between 5.6 and 45.4 °C. Models compared are multiplicative (Mult.) or subtractive (Subt.) probability models with either a linear (Lin.) or an exponential (Exp.) effect of temperature (T) on the variation in maximum temperature limits (s_x). The value of the parameter, r, is shown for exponential models. Increases in residual deviances (RD) are shown relative to model (1).