Resilience of rice (Oryzaspp.) pollen germination and tube growth to temperature stress


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Short running title: Resilience of rice pollen to temperature stress

Title:

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 temperature tolerance of pollen germination (PG) and tube growth (PTG). Germination of pollen of high temperature susceptible *Oryza glaberrima* Steud. (cv. CG14) and *O. 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, 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 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 1mm 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.

Keywords: heat; cold; development; stress; pollen germination; cardinal temperatures; germination model.
Introduction

Rice is grown across wide geographic 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 yield of cultivated rice (Peng et al. 2004; Wassmann et al. 2009; Jena et al. 2012; Martinez-Eixarch & Ellis 2014).

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 (Jagadish et al. 2010; Farrell et al. 2006). Low or high temperatures at microsporogenesis and anthesis, reduce anther pore size, anther dehiscence, pollen viability, pollen germination (PG) and pollen tube growth rates (PTG) and hence fertilization and spikelet fertility (Satake 1976; Matsui, Omasa & Horie 2001; Andaya & Mackill 2003; Farrell et al. 2006; Prasad et al. 2006; Jagadish et al. 2010, 2013; Martinez-Eixarch & Ellis 2014). 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 characterise 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 two minutes of pollination and the tube reached the ovule after 40 minutes 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, Lu & Chen 2001), compared to 4 to 6 h for sorghum [*Sorghum bicolor* (L.) Moench; Prasad, Boote & Allen (2011)] and 1 to 2 days 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 maximise 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. Optimising the germination medium was therefore a prerequisite for this research on PG in relation to temperature stress.

Cardinal temperatures are the critical temperatures that characterise 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, Pilbeam & Craigon
1990; Steinmaus, Prather & Holt 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). For example, to achieve
reliable seed set in rice, ten to twenty 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 have, however, been successfully modelled by
probit analysis including dose-response curves of pea (*Pisum sativum* L.) pollen germination
to cadmium (Kumar, Dhingra & Rohilla 2000), survival over time of air-dry pollen (Hong *et
al.* 1999), fungal conidia (Hong, Ellis & Moore 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).
Here 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 use of probit models to quantify rice pollen germination 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, Satake & Mackill 1981, Prasad et al. 2006; Jagadish, Craufurd & Wheeler 2007, 2008; Jagadish et al. 2010; Coast et al. 2014), while Rang et al. (2011) confirmed its “true tolerance” to high day temperatures (38 °C for 6 h for 4 days 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, 2013; Coast et al. 2014). Similarly, CG14 is susceptible to high day temperature stress at microsporogenesis (Jagadish et al. 2013) and anthesis (Prasad et al. 2006; Jagadish et al. 2008), as evidenced by spikelet fertility reductions of 40 to 60% (at microsporogenesis) and 70% (at anthesis) when exposed to 4-6 consecutive days of 38 compared to 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. Pre-germinated 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 minutes and mean values recorded every half hour using Hobo Microstation data loggers (Onset Computer Corp., USA).

Harvesting panicles and collecting pollen

At 50% anthesis on each of two consecutive days for each cultivar, panicles were harvested for pollen between 0700 and 0900 h. The cultivar, N22, was harvested 13 days 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 water to the laboratory and kept next to the window (to ensure sufficient light exposure)
until spikelets started opening (15 to 60 min after harvest). For each cultivar, a minimum of 84 panicles were harvested from a mixture of main stems and primary tillers.

**Pollen germination media**

Pollen germination 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 calcium nitrate tetrahydrate \([\text{Ca}(\text{NO}_3)_2\cdot4\text{H}_2\text{O}]\), which were dissolved in 1 l deionized water. Twenty grams of sucrose and 10 g of polyethylene glycol (PEG 4000) were then dissolved in 700ml 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. (Singapore).

Dai *et al.* (2007) used 0.7 g l\(^{-1}\) \(\text{Ca}(\text{NO}_3)_2\cdot4\text{H}_2\text{O}\), 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\(^{-1}\) \(\text{Ca}(\text{NO}_3)_2\cdot4\text{H}_2\text{O}\) (Suppl. Table S1). Although PG of CG14 was also maximised with 0.06 g l\(^{-1}\) \(\text{Ca}(\text{NO}_3)_2\cdot4\text{H}_2\text{O}\), 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). Using these media, 80 to 90% of pollen grains routinely germinated *in vitro* at laboratory temperature (26 to 28 °C).

**Temperature treatments**

Temperature treatments were applied using a temperature gradient plate (TGP; Grant Instruments Ltd., Cambridge, UK; Murdoch, Roberts & Goedert 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 five minutes for a period of one hour and a similar set of measurements was recorded immediately afterwards in the germination medium using Campbell data loggers (CR1000, Campbell Scientific Inc., Logan, USA) . Although some differences were observed, on average 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 (Suppl. 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 four hours 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.

**Pollen germination and tube growth rate determination**

Germination after four hours was counted at 100× magnification using a transmitted light microscope (Primo Star; Carl Zeiss Int., Germany) fitted with a Plan-Achromat lens. 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 to 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 four-hour period was used for this in vitro study rather than the one or two hour 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., Germany) 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., 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, UK) was used to fit the various models. Pollen tube growth data after four hours 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 minimise 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 rate of PTG to mean temperature at sub-optimal temperatures estimates the mean thermal time to achieve a tube length of one micron 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:

\[
\Phi_{\text{min}} = [K_n + bT] \quad (1) \\
\Phi_{\text{max}} = [K_x + cT] \quad (2)
\]

where \(\Phi_{\text{min}}\) and \(\Phi_{\text{max}}\) 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 (\(\Phi^{-1}\)) of their respective n.e.d. values to probabilities (Eqn 3):

\[
G = (\Phi_{\text{min}})^{-1} (\Phi_{\text{max}})^{-1} \quad (3)
\]

so that,
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:

$$G = (\Phi^{-1}[K_n + bT]) - (\Phi^{-1}[K_x + cT])$$  \hspace{1cm} (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:

$$G = (\Phi^{-1}[K_n + bT]) (\Phi^{-1}[K_x - c r^T])$$  \hspace{1cm} (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 which might be used to assess the resilience of pollen germination

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 ($^\circ$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 $\Phi_{\text{min}} = 0$, 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),

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

Treating Eqn (2) and the exponential equivalent similarly, Eqns (4), (5) and (6) may respectively be rewritten,

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

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

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

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

Mean day/night temperatures over periods from two 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, Suppl. Fig. 2). 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, Suppl. Fig. 2).

Pollen germination

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, 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 2). 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 optimised by non-linear modelling in GenStat and no standard errors could be obtained. Instead, $r$ had to be optimised by varying its value manually to minimise the residual deviance, the optimal value of $r$ varying with cultivar and model (Suppl. Table 2). Visually,
the exponential model for maximum temperature limits reduced the highest predicted
germination at the optimum temperature but slightly improved the goodness of fit for low
germination values at high temperatures (cf. Fig. 1). Given the inability to optimise \( r \) or
determine its standard errors using GenStat together with the principle of minimising 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. 3). 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.3). 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, 3). As a result \( T_x(50) \) is also much
lower in IR64 (Table 3, Fig. 3) 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. 3), with some germination above 40 °C in
both cultivars (Fig.1).

Pollen tube growth rates

The mean maximum pollen tube length across the three rice cultivars after four hours 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. 2 and Table 4 where PTG is shown as a rate per hour).
Measurable PTG occurred over a narrower temperature range for IR64 (19.1 to 35.2 °C) than for CG14 (16.9 to 38.6 °C) or N22 (13.5 to 38.2 °C) (Fig. 2).

Modelling the rate of pollen tube growth

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 sub-optimal 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 sub-optimal temperatures (Fig. 2, 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 maximised, 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. 3). Being extrapolations to temperatures at
which the rates of PTG are zero, the temperature differences between the base and ceiling
temperatures should be much wider than those between $T_{n(50)}$. 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 to 36 °C), N22 being
intermediate (10 to 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, Tian & Russell
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, de
Moer & van Assche 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 one hour 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, Clarke & Bacic 1993;
Taylor & Hepler 1997; Rosell, Herrero & Galán Saúco 1999; Poulton, Koide & Stephenson
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,
Rosenfeld & Pressman 2004). Do these changes in physiology, which are associated with low
and high temperatures, cause low germination or they are 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 since 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 the cultivars, being similar amongst all three tested (Tables 3-4). The parameters of the theoretical underlying distributions (Figure 3) do, however, help in explaining why IR64 cannot tolerate high day temperatures at 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, Maruyama & Ohba 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 compared to 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 fertilisation 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 fertilisation 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
minimise the risk of yield loss in rice due to 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 fertilisation 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 which 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 since 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.
Acknowledgements

Our thanks to the Felix Trust, which supported OC, the USAID-BMGF Cereal Systems Initiative for South Asia (CSISA) programme through IRRI for financial support, Cheryl Quiñones for technical assistance, Patria Gonzalez and Rowena Oane for help with microscopy and Bill Hardy and Michael Shaw, who commented on the manuscript.
References


chickpea, lentil, soybean and cowpea at constant temperatures. *Journal of Experimental Botany* 37, 705-715.


U.S. Climate Change Science Program and the Subcommittee on Global Change Research. [Backlund P., Janetos A., Schimel D, et al. (eds)] Washington, DC., USA, 362 pp


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

<table>
<thead>
<tr>
<th>Species</th>
<th>Cultivar(^a)</th>
<th>Accession number(^b)</th>
<th>Origin</th>
<th>Adaptation</th>
<th>Days to 50% anthesis(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. glaberrima</em></td>
<td>CG14</td>
<td>---(^d)</td>
<td>Senegal</td>
<td>Upland</td>
<td>57</td>
</tr>
<tr>
<td><em>O. sativa ssp. Indica</em></td>
<td>IR64</td>
<td>IRTP-12158</td>
<td>Philippines</td>
<td>Lowland</td>
<td>60</td>
</tr>
<tr>
<td><em>O. sativa ssp. aus</em></td>
<td>N22</td>
<td>IRTP-03911</td>
<td>India</td>
<td>Upland</td>
<td>50</td>
</tr>
</tbody>
</table>

\(^a\)=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

Preferred position in text: between sections ‘Field Establishment’ and ‘Harvesting Panicles and Collecting Pollen’
Table 2. Day and night temperatures and relative humidity of rice paddy plots during the study (July to August 2011), over periods of two 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).

<table>
<thead>
<tr>
<th>Period</th>
<th>Time of day(^a)</th>
<th>Temperature mean (range), °C</th>
<th>Relative humidity mean (range), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>July</td>
<td>Day</td>
<td>26.7 (23.6 – 28.3)</td>
<td>89.0 (53.7 – 100)</td>
</tr>
<tr>
<td></td>
<td>Night</td>
<td>25.2 (22.6 – 28.1)</td>
<td>91.7 (47.4 – 100)</td>
</tr>
<tr>
<td>August</td>
<td>Day</td>
<td>28.6 (24.1 – 32.9)</td>
<td>87.7 (69.9 – 100)</td>
</tr>
<tr>
<td></td>
<td>Night</td>
<td>25.6 (22.8 – 29.2)</td>
<td>94.8 (78.4 – 100)</td>
</tr>
<tr>
<td>2 dba to anthesis</td>
<td>Day</td>
<td>27.6</td>
<td>92.1</td>
</tr>
<tr>
<td></td>
<td>Night</td>
<td>25.5</td>
<td>96.3</td>
</tr>
<tr>
<td>15 dba to anthesis</td>
<td>Day</td>
<td>28.2</td>
<td>88.7</td>
</tr>
<tr>
<td></td>
<td>Night</td>
<td>25.4</td>
<td>95.7</td>
</tr>
<tr>
<td>At times of panicle harvests</td>
<td>Day</td>
<td>27.8</td>
<td>92.9</td>
</tr>
<tr>
<td></td>
<td>Night</td>
<td>26.7</td>
<td>94.4</td>
</tr>
</tbody>
</table>

\(^a\)Day = 0600 – 1800 h EST and Night = 1830 – 0530 h EST

Preferred position in text: at end of Materials and Methods.
Table 3. Parameter estimates (standard errors) and cardinal temperatures of pollen germination of rice (*Oryza* spp.) cultivars. Estimates are for the multiplicative probability model (Eqn 10) at temperatures between 5 and 45 °C. The overall normal distribution curves are given in Fig. 3.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Mean minimum temperature limit</th>
<th>Standard deviation of min. temp. limits*</th>
<th>Mean maximum temperature limit</th>
<th>Standard deviation of max. temp. limits*</th>
<th>Fitted values:</th>
<th>Observed values:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{n(50)}$, °C</td>
<td>$s_n$, °C (n.e.d.)^−1</td>
<td>$T_{x(50)}$, °C</td>
<td>$s_x$, °C (n.e.d.)^−1</td>
<td>Optimum temperature</td>
<td>Maximum germination</td>
</tr>
<tr>
<td>CG14</td>
<td>21.9 (0.09)</td>
<td>4.90 (0.12)</td>
<td>34.3 (0.11)</td>
<td>4.32 (0.14)</td>
<td>28.3</td>
<td>83.0</td>
</tr>
<tr>
<td>IR64</td>
<td>23.3 (0.10)</td>
<td>4.74 (0.13)</td>
<td>29.8 (0.10)</td>
<td>2.28 (0.10)</td>
<td>26.8</td>
<td>69.7</td>
</tr>
<tr>
<td>N22</td>
<td>20.8 (0.09)</td>
<td>6.45 (0.12)</td>
<td>35.6 (0.08)</td>
<td>4.88 (0.11)</td>
<td>28.7</td>
<td>82.0</td>
</tr>
</tbody>
</table>

* $s_n$ and $s_x$ are reciprocals of slopes of fitted lines; n.e.d.: normal equivalent deviates (probit-5)

$T_{n(50)} =$ minimum temperature required for germination for 50% of pollen grains;

$T_{x(50)} =$ maximum temperature limit for germination of 50% of pollen grains.

Preferred position in text: between sections ‘Pollen Germination’ and ‘Pollen Tube Growth Rate’
Table 4. Parameter estimates (standard errors) of split-line regressions of the rate of pollen tube growth of rice (*Oryza* spp.) cultivars as a function of germination temperature. Base and ceiling temperatures are calculated from regression parameters. Thermal times are for sub-optimal temperatures only. Pollen was germinated for four hours at temperatures between 5 and 45 °C.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Optimum temperature for PTG rate, $T_o$</th>
<th>Fitted mean PTG rate at optimum temperature, $\mu m \cdot h^{-1}$</th>
<th>Temperature coefficient at supra-optimal temperatures, $\beta_s$</th>
<th>Temperature coefficient at sub-optimal temperatures, $\beta_s$</th>
<th>Base temperature, $T_b$</th>
<th>Ceiling temperature, $T_c$</th>
<th>Thermal time above $T_b$ for PTG of 1 mm*, °C h</th>
<th>Observed optimal values:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG14</td>
<td>28.9 (0.55)</td>
<td>319.5 (10.1)</td>
<td>15.3 (1.86)</td>
<td>-24.0 (2.89)</td>
<td>8.02</td>
<td>42.2°C</td>
<td>65.4</td>
<td>27.3</td>
</tr>
<tr>
<td>IR64</td>
<td>26.9 (0.37)</td>
<td>331.4 (11.1)</td>
<td>21.7 (3.51)</td>
<td>-34.6 (2.67)</td>
<td>11.6</td>
<td>36.4°C</td>
<td>46.1</td>
<td>25.5</td>
</tr>
<tr>
<td>N22</td>
<td>26.7 (0.74)</td>
<td>415.7 (20.8)</td>
<td>25.2 (4.02)</td>
<td>-31.4 (4.88)</td>
<td>10.2</td>
<td>39.9°C</td>
<td>39.6</td>
<td>24.6</td>
</tr>
</tbody>
</table>

Preferred position in text: between sections ‘Pollen Tube Growth Rate’ and ‘Cardinal Temperature Determination’
Table 5. Comparison of rice pollen performance *in vitro* and cardinal temperatures with some other crops.

<table>
<thead>
<tr>
<th>Species</th>
<th>Pollen germination (PG), %</th>
<th>Optimum temperature (range) for PG, °C</th>
<th>Pollen tube growth (PTG), µm</th>
<th>Optimum temperature (range) for PTG, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Oryza</em> species</td>
<td>85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.3 (10-42)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1390&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.8 (14-39)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Glycine max</em> (L.) Merr.</td>
<td>81&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.2 (13-47)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>437&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.1 (12-47)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Capsicum annuum</em> L.</td>
<td>78&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.7 (15-42)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>734&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.2 (12-40)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Arachis hypogaea</em> L.</td>
<td>56&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30.1 (14-43)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1020&lt;sup&gt;d&lt;/sup&gt;</td>
<td>34.4 (15-44)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Gossypium hirsutum</em> L.</td>
<td>44&lt;sup&gt;e&lt;/sup&gt;</td>
<td>27.3 (12-43)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>778&lt;sup&gt;j&lt;/sup&gt;</td>
<td>27.8(12-44)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Brassica napus</em> L.</td>
<td>37&lt;sup&gt;f&lt;/sup&gt;</td>
<td>23.6 (8-33)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>660&lt;sup&gt;f&lt;/sup&gt;</td>
<td>25.04 (5-33)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Sorghum bicolor</em> (L.) Moench</td>
<td>36&lt;sup&gt;g&lt;/sup&gt;</td>
<td>29.4 (17-42)&lt;sup&gt;h&lt;/sup&gt;</td>
<td>…</td>
<td>…</td>
</tr>
</tbody>
</table>

<sup>a</sup> this paper; <sup>b</sup> Salem *et al.* (2007); <sup>c</sup> Reddy & Kakani (2007); <sup>d</sup> Kakani *et al.* (2002); <sup>e</sup> Liu *et al.* 2006; <sup>f</sup> Singh *et al.* (2008); <sup>g</sup> Tuinstra & Wedel (2000); <sup>h</sup> Prasad *et al.* (2011); <sup>j</sup> Kakani *et al.* (2005); … data not available.

Preferred position in text: near start of Discussion
**Figure legends**

Fig. 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.

Fig. 2. 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*<sub>o</sub>) temperatures are shown in Table 4. Rates were calculated from pollen tube lengths measured after four hours on a temperature gradient plate at the temperatures shown. Thicker dashed lines are extrapolations to the base (*T*<sub>b</sub>) and ceiling (*T*<sub>c</sub>) temperatures for the rate of tube growth whilst the optimum temperature (*T*<sub>o</sub>) is the value at which it is maximal.

Fig. 3. 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) and the optimum germination temperature (*T*<sub>o</sub>), 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*<sub>n(50)</sub>) and maximum (*T*<sub>x(50)</sub>) temperature limits and standard deviations (*s*<sub>n</sub> and *s*<sub>x</sub>).
Fig. 1.
Fig. 2.
Fig. 3

Proportion of pollen grains

Maximum temperature limit
Minimum temperature required

Temperature (°C)

CG14

IR64

N22

$T_{m(50)}$, $T_{o}$, $T_{s(50)}$