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THIS ARTICLE IS PART OF A SPECIAL ISSUE ENTITLED
'POLLEN–PISTIL INTERACTIONS'**The evolution of pollen germination timing in flowering plants: *Austrobaileya scandens* (Austrobaileyaceae)**

Joseph H. Williams*

Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN 37996, USA

Received: 12 December 2011; **Returned for revision:** 24 February 2012; **Accepted:** 28 March 2012; **Published:** 4 April 2012**Citation details:** Williams JH. 2012. The evolution of pollen germination timing in flowering plants: *Austrobaileya scandens* (Austrobaileyaceae). *AoB PLANTS* 2012: pls010; doi:10.1093/aobpla/pls010**Abstract****Background and aims**

The pollination to fertilization process (progamic phase) is thought to have become greatly abbreviated with the origin of flowering plants. In order to understand what developmental mechanisms enabled the speeding of fertilization, comparative data are needed from across the group, especially from early-divergent lineages. I studied the pollen germination process of *Austrobaileya scandens*, a perennial vine endemic to the Wet Tropics area of north-eastern Queensland, Australia, and a member of the ancient angiosperm lineage, Austrobaileyales.

Methodology

I used *in vivo* and *in vitro* hand pollinations and timed collections to study development from late pollen maturation to just after germination. Then I compared the contribution of pollen germination timing to progamic phase duration in 131 angiosperm species (65 families).

Principal findings

Mature pollen of *Austrobaileya* was bicellular, starchless and moderately dehydrated—water content was 31.5 % by weight and volume increased by 57.9 % upon hydration. A callose layer in the inner intine appeared only after pollination. *In vivo* pollen germination followed a logarithmic curve, rising from 28 % at 1 hour after pollination (hap) to 97 % at 12 hap ($R^2 = 0.98$). Sufficient pollen germination to fertilize all ovules was predicted to have occurred within 62 min. Across angiosperms, pollen germination ranged from 1 min to >60 h long and required 8.3 ± 9.8 % of the total duration of the progamic phase.

Significance

Pollen of *Austrobaileya* has many plesiomorphic features that are thought to prolong germination. Yet its germination is quite fast for species with desiccation-tolerant pollen (range: <1 to 60 h). *Austrobaileya* and other early-divergent angiosperms have relatively rapid pollen germination and short progamic phases, comparable to those of many insect-pollinated monocots and eudicots. These results suggest that both the pollen germination and pollen tube growth periods were marked by acceleration of developmental processes early in angiosperm history.

* Corresponding author's e-mail address: joewill@utk.edu

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Introduction

Flowering plants are notable for having evolved rapid reproductive cycles relative to other seed plants (Stebbins 1976, 1992; Takhtajan 1976; Favre-DuChartre 1979; Willson and Burley 1983; Bond 1989; Friedman 1990; Doyle and Donoghue 1993; Taylor and Hickey 1996). An extreme abbreviation of their pollination to fertilization period (progamic phase) is hypothesized to have been the enabling step in the origin of rapid reproduction (Stebbins 1992; Williams 2008, 2009). Male gametophytes interact with maternal tissues and compete for fertilizations during the progamic phase. Hence, the question of how progamic phase duration evolves is intertwined with the question of how male gametophyte developmental rates evolve.

The progamic phase comprises two semi-independent phases of male gametophyte development: pollen germination and pollen tube growth from stigma to egg. The duration of pollen tube growth is most affected by how far and how fast pollen tubes grow. Angiosperm pollen tube pathways have been reconstructed as initially very short (Williams 2008), consistent with their earliest fossil record showing that carpels lacked prominent styles (Friis *et al.* 2010). However, pollen tube growth rates, even among the earliest-divergent angiosperm lineages, are orders of magnitude faster than those of other seed plants (Williams 2009, 2012). Thus, the speeding of pollen tube growth alone explains much about how the angiosperm progamic phase became abbreviated.

To put the role of pollen tube growth into perspective, one must also understand how pollen germination speed evolves and quantify its contribution to the total duration of the progamic phase. Since pollen germination comes before pollen tube growth, selection can act first on pollen germination speed, if there is heritable variation. If pollen tube growth rates were initially slow to evolve, as they seem to be in non-flowering seed plants (Williams 2009, 2012), pollen germination might have been the primary process affected by pollen competition among early angiosperms. Germination competition has been shown to be strong in a number of woody perennials within ancient angiosperm lineages. For example, hand-pollination experiments found high levels of pollen germination and tube growth on the stigma but few pollen tubes within stylar canals of *Amborella trichopoda* (Thien *et al.* 2003; Williams 2009), *Austrobaileya scandens* (Williams 2008) and *Annona cherimola* (Lora *et al.* 2010). In *Amborella*, the probability of reaching an ovule went from <1% on the stigma to 50% at the top of the stylar canal (Williams 2009). If maternal control over the form and/or intensity of competition differs between the stigma

and the stylar canal or ovary, then pollen germination and pollen tube growth should evolve at different rates, especially if the two processes have some degree of modularity.

In this study, I report on the pollen germination process of *Austrobaileya scandens* White (*Austrobaileya*-ceae is monospecific and hereafter I use the genus name for the taxon). *Austrobaileya* is the sister lineage to the rest of *Austrobaileyales*, an order that is itself sister to all angiosperms other than *Nymphaeales* and *Amborella* (Soltis *et al.* 2011 and references therein). It is a perennial vine endemic to the Wet Tropics area of northeastern Queensland, Australia (Endress 1980, 1983a). It flowers in the subcanopy and is thought to be insect pollinated (Endress 1980; Thien *et al.* 2009).

The goal of this study was to understand the contribution of pollen germination speed to the evolution of progamic phase duration in angiosperms. To that end, I first document the timing and duration of pollen germination in *Austrobaileya*, and aspects of pollen structure related to pollen germination speed. Previous studies have described *Austrobaileya* pollen morphology and development (Endress 1980, 1983b; Endress and Honegger 1980; Zavada 1984) and pollen tube growth patterns (Williams 2008). I place these data in context by quantifying the contribution of pollen germination to the total duration of the progamic phase across a broad sample of angiosperms. That analysis highlights some pathways and limitations to the evolution of pollen germination.

Methods

Pollen hydration

Pollen hydration status was quantified using flowers from a single plant that flowered in the greenhouse in March 2010. Flowers were collected in the morning, placed in a plastic bag and transported to the lab. Flowers were kept in the plastic bag until each was used (within 2 h after flower collection). Pollen from dehiscent anthers was placed in immersion oil for determination of size and shape at presentation. Pollen from the same anther was at the same time placed in B-K medium (Brewbaker and Kwak 1963) with 2.5% sucrose (which gave optimal germination percentage). Pollen on B-K medium reached maximum size within minutes, and was photographed at least 1 h after immersion. Photomicrographs were made using differential interference contrast (DIC) microscopy at $\times 200$ magnification with a Zeiss Axiocam camera at 3000×3900 pixel resolution and analysed using Axiovision, version 4.1 software (Carl Zeiss, Thornwood, NY, USA). Volume was calculated for 30 pollen grains in each treatment using the formula: $V = (\pi \times A \times B^2)/6$, where A is the

length of the major axis and B the minor axis. The volume (V) gain of pollen in its hydrated state relative to its size at anthesis was calculated as $100 \times (V_{\text{hydrated}} - V_{\text{anthesis}}) \div V_{\text{hydrated}}$ (Nepi *et al.* 2001).

Water content was estimated by comparing pollen weight at anthesis and after drying. Fresh pollen was removed from open anthers with a toothpick, placed on weighing paper and weighed within 1–3 min after removal. Weighed pollen was then dried in an oven at 50 °C and reweighed afterwards until near constant weight was reached at 24, 48 or 72 h (Aylor 2002). Weights were determined on a Mettler-Toledo UMX2 (Columbus, OH, USA) microbalance with display resolution of 0.1 µg.

Pollen germination process

The pollen germination study was carried out on private land near the Millaa Millaa lookout, Queensland, Australia (17°31'15"S, 145°33'53"E). The site was clearcut in the 1950s but *Austrobaileya* vines are now very common in the c. 50- to 60-year-old secondary rainforest. Only seven of the many vines had enough flowers to use in a pollination study. The average temperature and relative humidity at this site during the study (13–24 September 2008) were 17.6 ± 4.3 °C and 86.1 ± 16.0 %, as measured hourly in the subcanopy (mean of 2 HOBO Pro series data loggers; Onset, Bourne, MA, USA).

Pollen germination was assessed using hand pollinations of newly receptive flowers on seven vines in the wild or of flowers from cuttings from these vines that were placed in water and kept outdoors, nearby in a similar climate. Cuttings were used because *Austrobaileya* flowers had to be accessed individually in the tree canopy, and each vine produced only a few receptive flowers each day. A comparison of flowers from cuttings vs. wild flowers showed no difference in germination percentage (paired two-tailed t -test, $P = 0.28$, $n = 5$ time classes). Crosses were done among the seven vines, and I attempted to maximize the number of cross combinations per timepoint from among the available male and female phase flowers on each day. Self (geitonogamous) pollinations were also included since self pollen germination was equivalent to outcross germination (J. H. Williams, unpublished data). In both wild flowers and those from cuttings, pollen was transferred directly from living anthers of a single cut flower to a stigma by a toothpick, thus pollen was only exposed to the dispersal environment for a few seconds.

To get a minimum estimate of duration of male phase, six cut flowers were monitored from anther opening until either all anthers or the whole flower abscised. Pollen viability was then assessed under a field microscope at

the end of male phase of each flower as the percentage of pollen germinated 9–12 h after placing pollen on B-K medium as above (4 samples \times 50 pollen grains each per flower).

Gynoecia were fixed in formalin-acetic acid-alcohol (FAA) at 1, 2, 3, 4, 6 and 12 hours after pollination (hap), and either hand sectioned directly or embedded in glycol methacrylate and serial sectioned. Sectioned material was viewed with DIC microscopy or with fluorescence microscopy using aniline blue (AB) or 4',6-diamidino-2-phenylindole (DAPI) stains (methods in Williams 2009). The starch content of pollen was assessed using iodine–potassium iodide (IKI) stain, whereas alcian blue, toluidine blue or ruthenium red were used to detect pectins (Ruzin 1999). Images were processed with Adobe Photoshop version 7 (Adobe, San Jose, CA, USA).

For timing of pollen germination, two carpels from each flower were cut from the apocarpous gynoecium. Since all the stigmas in an *Austrobaileya* gynoecium are united by a massive stigmatic secretion, only a random portion of the secretion and total pollen load was represented in each of these subsamples. All pollen grains were then categorized as either germinated (i.e. with pollen tube longer than pollen grain width) or ungerminated. If no pollen was present, another carpel was taken, until two were found with pollen. The percentage of pollen germinated was averaged from two subsamples of each cross before averaging over crosses at each timepoint (i.e. the cross was the experimental unit). Pollen number did not affect the probability of pollen germination, as indicated by a post hoc linear regression on the data ($P = 0.746$; $R^2 = 0.021$, $n = 244$).

Pollen germination literature survey

A previous literature survey identified data on the time between pollination and fertilization (Williams 2008). I attempted to find data on the *in vivo* time between pollination and pollen germination (emergence of the pollen tube) for as many species in that survey as possible. Data on pollen germination for each taxon were either provided within the same study or in another by the same authors, or in a few cases data on germination from a different author were used. *In vitro* pollen germination data were not used. Few studies provided quantitative data on average time to germination. Most provided data on when germination first began and/or the time when substantial pollen germination had occurred. Thus, I attempted to use values for the earliest time that a substantial (relative to the number of ovules) portion of the pollen load had germinated. The species data were averaged over genera, since multiple species within a genus often had similar

germination times and this also prevented skewing of results towards a few intensively studied genera.

Results

Pollen ontogeny from flower opening to pollination and germination

Flowers of *Austrobaileya* first opened at any time of day or night, with receptive stigmas. Anthers did not open until 1–2 (up to 3) days after flower opening in the wild. Pollen from newly dehiscent anthers was bright yellow and sticky, tending to clump when handled (Fig. 1A). On cuttings, pollen viability was $75.9 \pm 30.1\%$ initially, and $51.1 \pm 32.1\%$ at the end of male phase (measurements taken 66.1 ± 11.9 h after onset of male phase). Pollen germinates on a massive secretion formed from the many individual stigmas at carpel tips and tubes grow through the secretion to reach open styler canals (Fig. 1B).

In the unopened anther during female phase, pollen was most often in the early two-celled stage (bicellular pollen 1 stage of Blackmore *et al.* 2007), and the vegetative cell cytoplasm stained for abundant starch grains (Fig. 2A). Mature pollen in newly opened anthers did not stain for starch (not shown). In mature pollen, the generative cell was positioned adjacent to the tube nucleus within the centre of the vegetative (tube) cell. At this stage, a remnant callose wall was often seen at the proximal pole where the generative cell had initially formed, sequestered from the microspore wall and vegetative cell cytoplasm (Fig. 2I and J). Upon germination, the generative cell nucleus is seen in close proximity to the tube cell nucleus as they pass into the pollen tube,

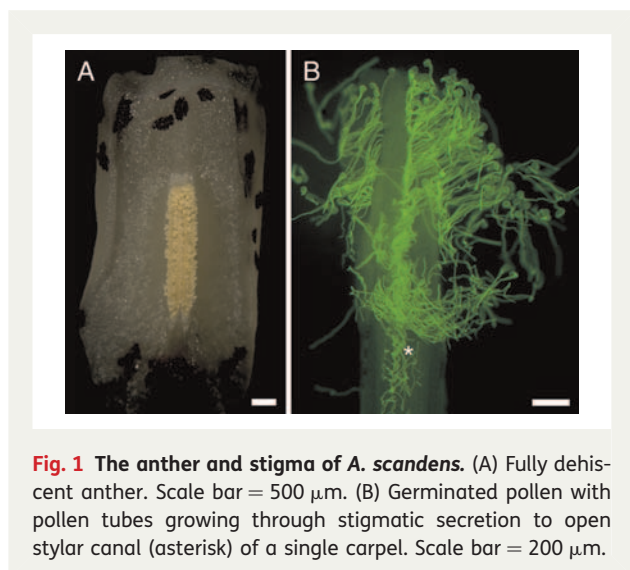


Fig. 1 The anther and stigma of *A. scandens*. (A) Fully dehiscent anther. Scale bar = 500 μm . (B) Germinated pollen with pollen tubes growing through stigmatic secretion to open styler canal (asterisk) of a single carpel. Scale bar = 200 μm .

within $\sim 50 \mu\text{m}$ of the growing tip (Fig. 2K). Sperm were first observed in pollen tubes at 4 hap and most pollen tubes contained sperm at 6 hap.

At maturity, pollen is anasulcate (as observed at the tetrad stage) and the long and wide aperture has prominent margins (Fig. 2B). It has a globose-euprolate shape (Walker and Doyle 1975) at presentation in open anthers (Fig. 2B, see also Fig. 1A). Pollen assumes a nearly spherical shape in aqueous B-K medium (and after fixation in FAA), and abundant hydrophobic droplets are released from the outer wall (Fig. 2C). In unopened anthers, pollen has a very thin AB-staining endexine which is considerably thickened at the aperture margin (Fig. 2D). Mature pollen in newly opened anthers displays the same AB-staining endexine (Fig. 2E). In both immature and mature pollen there is a very thick mass of AB-staining material in the inner aperture wall, isolated from but corresponding in position to the extra-apertural endexine (Fig. 2D and E). Also in both, the extra-apertural intine is very thin, but becomes massive in the apertural area (Fig. 2A and D–F). Strong staining of the intine with ruthenium red indicates that pectins are abundant (as also confirmed with alcian blue and toluidine blue) (Fig. 2F).

Fully hydrated pollen shows a swelling of the vegetative (tube) cell in the distal apertural zone (Fig. 2C), and then a bulging of the tube cell through the sulcus (Fig. 2E–H). Aperture deposits are pushed aside and remain conspicuous long after pollen tube growth commences (Fig. 2E, H and K). The pollen wall sometimes tears as the bulging tube cell emerges (Fig. 2H). The intine of mature pollen lacks AB staining (Fig. 2E), but in germinating pollen it stains brightly with AB and is continuous with the pollen tube wall (Fig. 2G and H). AB is not seen at the tube tip (Fig. 2G). The cylindrical shape of the pollen tube is formed as a small offshoot from the large bulging tube cell outside of the aperture (Fig. 2G, H and K). Note that the reduction to a small and uniform tube diameter can be gradual (Fig. 2K), or quite abrupt in cases where the tube initiates from the side of the bulge (Fig. 2G and H), the more common situation.

Pollen hydration

At presentation, pollen in oil had a mean (\pm s.d.) length \times width of $51.8 \pm 2.8 \times 32.5 \pm 1.6 \mu\text{m}$ (Fig. 2B; Table 1). Hydrated pollen from the same anthers was nearly spherical with a mean length \times width of $53.2 \pm 2.9 \times 49.4 \pm 2.0 \mu\text{m}$ (Fig. 2C). Pollen volume at presentation was 42.1 % of that of hydrated pollen (Table 1). On liquid B-K medium, individual pollen grains reached $\geq 90\%$ of their maximum volume within 3 min (three flowers, mean of 16.5 pollen grains/timepoint).

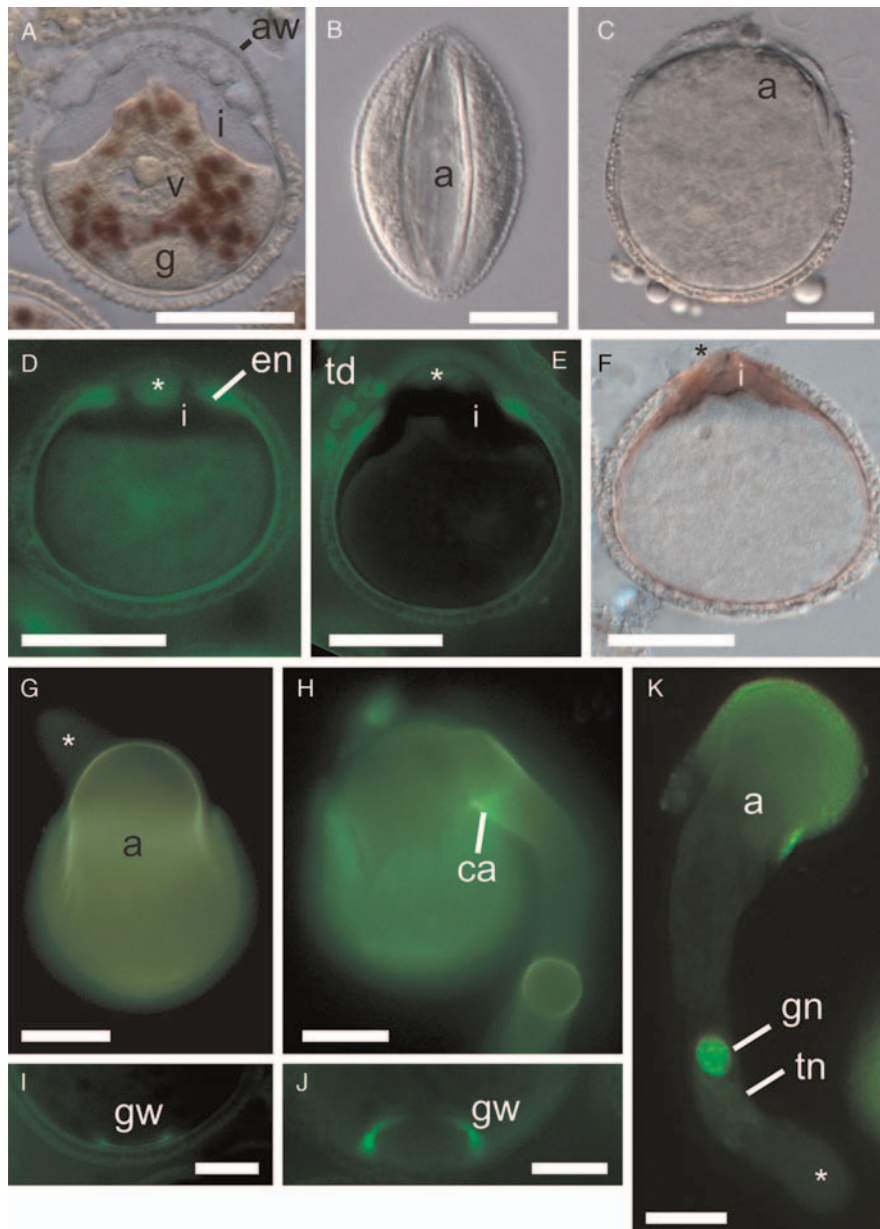


Fig. 2 Pollen germination of *A. scandens*. (A) Two-celled pollen from female phase flower (closed anther) with IKI-stained granules in vegetative (tube) cell cytoplasm. (B) Pollen from open anther in immersion oil, showing dehydrated state at presentation (DIC). (C) *In vitro* hydrated pollen, just before germination (DIC). (D) Immature pollen (female phase of flower) with AB staining of extra-apertural endexine (en) and isolated endexine in aperture wall (asterisk). (E) Mature pollen from open anther (AB). Note AB stain in a thin layer of endexine which is thickened at the aperture edge, absent in its margins and present in the centre of the apertural wall (asterisk). Clumps of AB-stained material (of tapetal origin) are associated with the outer apertural wall. Note also the bulging of intine through aperture, probably caused by partial hydration of pollen during fixation. (F) Pollen from open anther showing intine stained by ruthenium red. (G) *In vitro* germinated pollen showing AB stain in inner pollen wall and its continuity with the emerging inner tube wall. Note that the tube tip (asterisk) in the background lacks AB staining. (H) Emergence of pollen tube has ruptured part of pollen wall and pushed aside aperture covering. Note strong AB staining of a presumably callose annulus (ca) at base of tube. (I, J) A ring of AB-stained material is prominent at the proximal pole of many mature and germinated pollen grains, marking the location of the generative cell wall (gw). Scale bars = 10 μ m. (I) Callose wall of generative cell inside of intine. (J) Remnants of callose wall of generative cell. (K) *In vivo* germinated pollen (3 hap) with faintly stained tube nucleus (tn) in association with the generative cell near the young pollen tube tip (asterisk) (DAPI). Pollen from (G), (H), (J) and (K) was fixed and stained 2 h after inoculation on growth medium. (A), (D–F) and (I) are from methacrylate sections. a, aperture; aw, aperture wall; g, generative cell; gn, generative cell nucleus; i, intine; td, tapetal deposits; v, vegetative cell nucleus. Scale bars = 20 μ m, except where noted.

Table 1 *Austrobaileya* pollen volume changes after hydration. Values are means \pm 1 s.d.

Flower ID	Volume at anthesis (μm^3)	Volume after hydration (μm^3)	% volume increase
S	24 293 \pm 5216	58 050 \pm 11 902	57.07
T	28 039 \pm 5020	59 695 \pm 21 411	53.03
U	32 213 \pm 8321	75 154 \pm 13 188	57.14
X	28 920 \pm 7167	74 037 \pm 16 432	60.94
AA	29 003 \pm 9198	83 315 \pm 27 022	65.19
BB	28 117 \pm 4416	71 231 \pm 15 887	60.53
CC	31 857 \pm 7796	65 661 \pm 65 661	51.48
Mean			57.91 \pm 4.75

Fresh pollen had a mean (\pm s.d.) weight loss of 31.52 \pm 16.02 % after drying ($n = 13$ flowers). Pollen in most replicates underwent a rapid initial weight loss, within the first 5 min after removal from open anthers (Fig. 3). Since *Austrobaileya* pollen is sticky, some of this rapid weight loss may have been a result of rapid drying of substances in the pollen coating. The mean weight loss of pollen from the four oldest flowers (including U and X in Fig. 3) was 15.71 % and pollen in old flowers was also notably less sticky (and turning light yellow).

In vivo pollen germination timing

Pollen germination began within 1 hap and reached its maximum between 6 and 12 hap (Fig. 4). Pollen germination progression best fit a logarithmic curve: $y = 0.2872 \times \ln(x) + 0.2204$ ($R^2 = 0.981$). The mean (\pm s.d.) number of pollen grains per carpel was 37.8 \pm 26.3, and there was a mean of 8.70 \pm 0.57 ovules per carpel in flowers from these vines ($N = 6$ vines; mean of 7.2 flowers/vine). The predicted time needed for 8.7 of 37.8 pollen grains, or 23 %, of the pollen load to germinate is 62 min (Fig. 4).

Pollen germination literature survey

There were data on both time to pollen germination and the duration of the progamic phase for 131 species in 104 genera (65 families). A pollen germination time of 1.5 h was used to represent *Austrobaileya* (consistent with methodology used in the literature review), which is 8.33 % of its 18-h-long progamic phase (Williams 2008). The time from pollination until pollen germination ranged from <1 min to 60 h, whereas the time between pollination and fertilization in the same set of species ranged from 15 min to 13 months (Fig. 5A). There were 55 genera with ‘fast’ pollen germination of 30 min or less (Nepi *et al.* 2001), all with progamic phases of <60 h. Thirty-six genera had ‘slow’ germination of 1 h

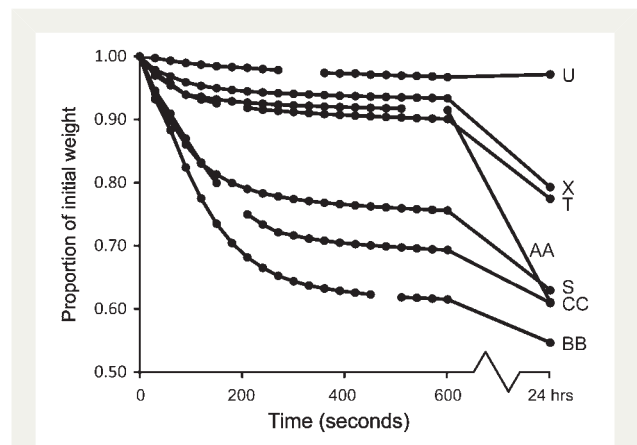


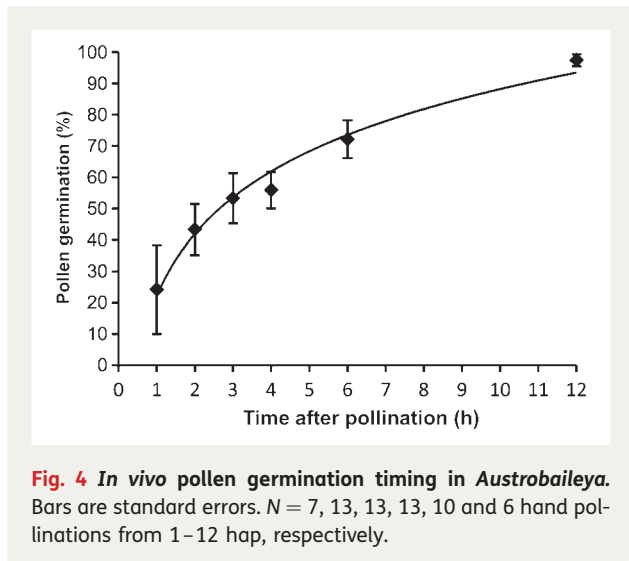
Fig. 3 Timing of initial weight loss from fresh *Austrobaileya* pollen. On the y-axis, 1.00 represents standardized initial weight of pollen just after removal from anthers.

or more. Pollen germination required a mean (\pm s.d.) of 8.3 \pm 9.8 % of the total duration of the progamic phase. However, pollen germination required a significantly greater proportion of time in genera with very short progamic phases (≤ 6 h; 33 genera) than in genera with longer times of >6 h (mean \pm 2s.e. = 14.8 \pm 4.4 vs. 5.3 \pm 1.4 %, respectively; Fig. 5B).

Discussion

Pollen germination process in *Austrobaileya*

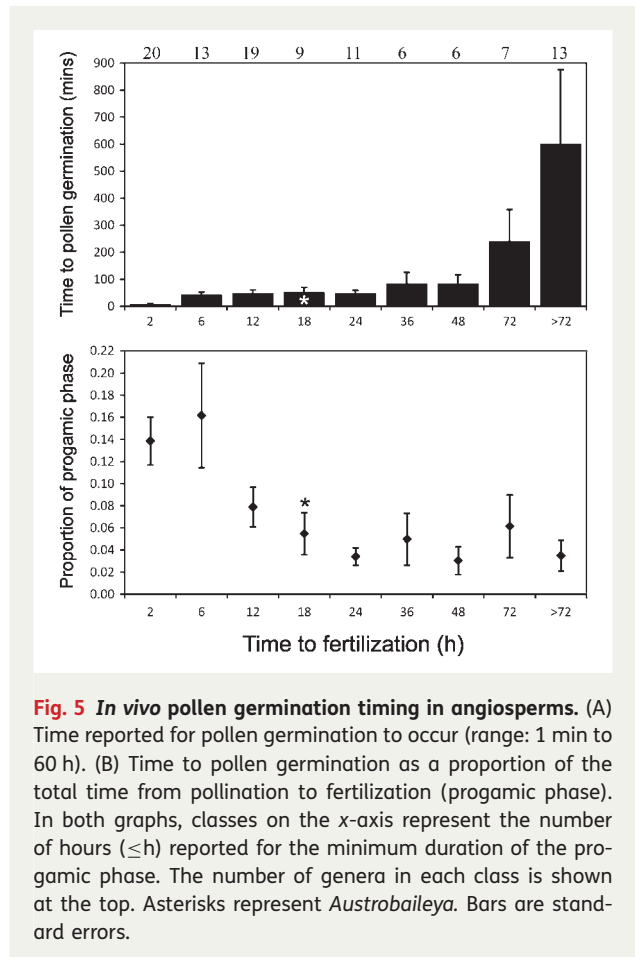
In female phase (within at least a day of anther opening), *Austrobaileya* pollen is at a bicellular stage. At this stage, a conspicuous callose wall was often seen completely surrounding the generative cell just inside of the intine, as also shown by J. Heslop-Harrison and Y. Heslop-Harrison (1997). Some time later in female phase, but before anther opening (onset of male phase), the generative



cell migrates to a central position in the vegetative (tube) cell, in close association with the tube cell nucleus. The callose wall remnants seem to be left in place at the proximal pole, as also shown by [Boavida et al. \(2005\)](#), whereas [Tanaka \(1988\)](#) found that the free generative cell in the cytoplasm may also have a callose wall.

In both immature and mature pollen, there is a conspicuous AB-stained layer beneath the foot layer of the exine, corresponding to the endexine in the transmission electron micrographs of *Austrobaileya* pollen in [Endress and Honegger \(1980\)](#). The extra-apertural endexine is considerably thickened at the edge of the aperture. Endexine is absent from the aperture wall margin, but in the main aperture wall, a very thick but slightly more diffusely stained mass, corresponding in position to the extra-apertural endexine, is present beneath the much reduced exine. Aniline blue staining indicates that there is callose, or perhaps mixed glucans, in the endexine, which is unusual but has previously been reported in *Helianthus* ([Vithanage and Knox 1979](#)).

In immature and mature pollen, the intine stained strongly for pectins in both the thin extra-apertural and the massive apertural areas. Callose was not detected in the inner layer of the intine at these stages, but appeared only later during pollen hydration and germination. [Endress and Honegger \(1980, fig. 4\)](#) showed an inner, electron-translucent intine layer in hydrated *Austrobaileya* pollen, consistent with the occurrence of callose in my material. Thus, the callose wall layer of the intine is secreted after pollination, not before. The pollen tube itself is organized outside of the pollen grain from the wall of the large bulging tube cell, usually at one of the narrow extremes of the sulcus (see also [Y. Heslop-Harrison and J. Heslop-Harrison 1992](#)). The



callose layer of the intine is continuous with the new wall of the cylindrical pollen tube, and a thick callose annulus is present at the base of the tube.

Pollen of *Austrobaileya* undergoes moderate dehydration before anther opening. That an active process of dehydration occurs is indicated by the prolate shape and relatively low water content of mature pollen, and by the $>50\%$ increase in volume to a spheroidal shape upon hydration. Moderately dehydrated pollen, with $>23\%$ water content, is characterized as ‘drought tolerant’ by [Hoekstra et al. \(2001\)](#), but further work would be needed to determine whether *Austrobaileya* pollen is tolerant of more severe desiccation (viable with $\leq 23\%$ water content). The starchless condition of mature pollen suggests some degree of desiccation tolerance, because in pollen with desiccation tolerance starch is converted to sucrose during dehydration ([Speranza et al. 1997](#)), and sucrose is especially important for membrane protection in dehydrated cells ([Hoekstra et al. 1989, 2001](#)).

The starchless condition is associated with insect pollination ([Baker and Baker 1979](#)), and entomophily is also indicated by the stickiness of pollen, which causes

clumping. The abundant oily droplets that emerged from hydrated pollen in aqueous solutions suggest that some form of pollen kitt is the cause of pollen stickiness (Pacini and Hesse 2005), as also suggested by Zavada (1984) from electron microscopy. Pollenkitt has not been reported in pollen of extant non-flowering seed plants (Pacini and Hesse 2005), but has been found in at least one extant early-divergent angiosperm, *Cabomba caroliniana* (Osborn *et al.* 1991). The angiosperm fossil record indicates that pollen clumping was common by the mid-Cretaceous (Hu *et al.* 2008).

Consequences of pollen structure and development for germination speed

Austrobaileya pollen has a number of character states that are thought to be plesiomorphic for angiosperms (Sampson 2000). Most of these have at one time or another been hypothesized to prolong pollen germination. Briefly, germination is said to be slower in: monosulcate vs. multi-aperturate pollen (Dajoz *et al.* 1991; Furness and Rudall 2004); thick- vs. thin-walled pollen (Heslop-Harrison 1979a; Nepi *et al.* 2001); starchless, oil-rich vs. starchy pollen (Baker and Baker 1979; Franchi *et al.* 1996); bicellular vs. tricellular pollen (Torabinejad *et al.* 1998); pollen initiating synthesis of callose intine layer after pollination vs. prior to pollination (Heslop-Harrison 1979b; J. Heslop-Harrison and Y. Heslop-Harrison 1992; Nepi and Pacini 1999; Pacini 2000); dehydrated vs. hydrated pollen (Nepi *et al.* 2001; Franchi *et al.* 2002); pollen with underdeveloped vs. mature mitochondria (Hoekstra and Bruinsma 1978, 1979; Hoekstra 1979; Rounds *et al.* 2011); and pollen with low vs. high metabolic rate (Hoekstra and Bruinsma 1975; Hoekstra 1979). These traits mediate germination speed either passively by affecting the speed of pollen hydration (e.g. aperture and pollen wall composition and sizes) or actively via the metabolic state of mature pollen at pollination. Pollen that does not undergo dehydration prior to dispersal is usually desiccation sensitive (i.e. ‘partially hydrated’, Franchi *et al.* 2011). It is well hydrated and metabolically very active, and hence germination proceeds rapidly (often within minutes) (Franchi *et al.* 2002), but it is generally very short lived (Dafni and Firmage 2000). Active dehydration of pollen before dispersal confers greater longevity but slower germination—developmental and structural characteristics of mature desiccation-tolerant pollen, as well as the lower metabolic rate of germinating pollen, result in great variation in the duration of the pollen germination process.

Austrobaileya pollen germinated within 1 h, but full germination was not reached until after 6 h. Both the delay in germination and the variation are consistent with its moderately dehydrated status at maturity, as well as

with its single aperture and the need to initiate synthesis of the callose layer in the inner wall after pollination (Nepi and Pacini 1999; Nepi *et al.* 2001). Among angiosperm pollens with some degree of desiccation tolerance, however, germination within 1 h is considered fairly rapid (Franchi *et al.* 2002). In *Austrobaileya*, relatively rapid hydration of pollen occurs in part because of the humid environment and the aperture structure. The likely route for initial hydration of the pollen grain is along the edge of the aperture, where the pectinaceous intine is most exposed to stigmatic secretions due to the highly reduced apertural exine and lack of an endexine layer (J. Heslop-Harrison and Y. Heslop-Harrison 1991).

Pollen longevity was not measured in this study, but it is likely to be at least 3 days, since pollen from late male-phase flowers still had >50 % *in vitro* germination. *Austrobaileya* vines grow in a wet tropical rainforest environment, and though they flower in the subcanopy during the driest month of the year (September), the relative humidity during this study averaged 86 %. Since pollen dispersal via insects may take only a few minutes and occurs in humid conditions, one might wonder why *Austrobaileya* pollen undergoes dehydration at all. In the populations I studied most flowers did not set fruit in each of three years, and most flowers that abscised received no pollen (Thien *et al.* 2009). If insect pollination is rare, then pollen limitation may explain why a moderately long period of pollen presentation has been maintained in *Austrobaileya* (Ashman 2004; Harder and Aizen 2010). Because pollen presentation lasts 3 days or more, individual pollens must maintain viability for at least a few days, and *Austrobaileya* pollen from old flowers was viable. Thus, the pollination ecology of *Austrobaileya* favours some degree of developmental arrest via dehydration in the face of pollinator uncertainty; and the humid environment allows weak desiccation protection mechanisms and hence fairly rapid germination.

Evolution of pollen germination timing and the origin of angiosperms

Among seed plants, the intensity of pollen competition is thought to have become greatly magnified in angiosperms (Mulcahy 1979). If so, then one prediction is that extant angiosperms should have faster pollen germination and faster pollen tube growth rates than other seed plants. Hoekstra (1983) used *in vitro* experiments to show that a broad sample of phylogenetically derived angiosperms had faster pollen germination and faster pollen tube growth rates than a number of species of conifers and Gnetales. Williams (2008, 2009) showed that *in vivo* pollen tube growth rates within some early-divergent lineages of angiosperms were faster than *in vivo* rates of representatives from all

major non-flowering seed plant groups, but slower than or comparable to those of some monocots and eudicots. However, patterns of *in vivo* pollen germination timing in angiosperms are less well known.

A review of studies of *in vivo* pollen germination supports the prediction that both early-divergent and derived angiosperms should have shorter pollen germination times than non-flowering seed plants. In *Austrobaileya*, substantial *in vivo* pollen germination occurred within about an hour and a half. This is considered quite rapid relative to that of non-flowering seed plants, in which *in vivo* germination times range from 1 to 72 h in Gnetales, from 0.5 to 9 months in conifers (Williams 2009) and several days or more among cycads (Pettitt 1982; Choi and Friedman 1991). Nor is *Austrobaileya* unusual: among early-divergent angiosperms pollen germination occurs within 1–3 h in the woody perennials *A. trichopoda* (Williams 2009) and *Illicium floridanum* (Koehl et al. 2004; Williams 2009), and in 15 min or less in aquatics of the order Nymphaeales, such as *C. caroliniana* and *Brasenia schreberi* (Taylor and Williams 2009), *Nymphaea odorata* (Williams et al. 2010) and *Trithuria austinensis* and *T. submersa* (Taylor and Williams 2012). Many aspects of pollen structure of extant early-divergent angiosperms are similar to those of early Cretaceous fossil pollens (Endress and Honegger 1980; Zavada 1984; Hughes 1994; Sampson 2000; Hesse 2001; Doyle 2005), and furthermore, because of habitat tracking, many of these species still occupy environments similar to those present early in angiosperm history (Feild et al. 2009). Seen in this light, pollen germination timing of extant early-divergent angiosperms is a reasonable model for that of an ancestor of extant angiosperms.

Among monocots and eudicots, *in vivo* pollen germination in less than 30 min is considered rapid (Nepi et al. 2001; Franchi et al. 2002) and very rapid germinators (1–15 min) almost always have desiccation-sensitive pollen at maturity (Nepi et al. 2001). As noted above, all Nymphaeales studied to date are rapid germinators. Because desiccation-sensitive pollen has a very short lifespan (Dafni and Firmage 2000), pollen that germinates in >1 h is increasingly likely to have undergone some degree of dehydration before maturity. Among angiosperm pollens with some degree of desiccation tolerance, germination can last up to 60 h (Fig. 5A) or even 3 days (references in Nepi et al. 2001). Thus, pollen germination of *Austrobaileya* and other early-divergent woody angiosperms with desiccation-tolerant pollen is near the fastest possible (Nepi et al. 2001). These data from extant early-divergent lineages suggest that an acceleration of pollen germination occurred prior to or during the origin of extant angiosperms. As such, an evolutionary transition to a very short progamic phase in an ancestor of extant

flowering plants (as reconstructed in Williams 2008) involved accelerations of both the pollen germination and pollen tube growth processes.

An interesting observation brought up by the literature review is that pollen germination requires such a consistent proportion of a progamic phase that varies from 30 min to over a year in duration (Fig. 5). This implies an evolutionary association between pollen germination speed and the duration of pollen tube growth, even though germination and tube growth are physiologically semi-autonomous (Addicott 1943). Furthermore, pollen hydration is a largely passive process and its duration is completely independent of one aspect of pollen tube growth duration: the distance tubes have to grow. The apparent correlated evolution of these traits is also surprising given that causes of evolution might be quite different on the stigma than within the reproductive tract.

There have been previous studies supporting the correlated evolution of germination speed and pollen tube growth rate: for example, it has often been noted that species with tricellular pollen have faster pollen germination and faster initial pollen tube growth rates than species with bicellular pollen (Brewbaker and Majumder 1961; Hoekstra and Bruinsma 1979; Hoekstra 1983; Mulcahy and Mulcahy 1983). An underlying cause of that observation is that tricellular pollen is usually desiccation sensitive and metabolically active at dispersal, whereas bicellular pollen is often desiccation tolerant and developmentally delayed. Thus, a more relevant test for evolutionary dissociation would be among pollens with some degree of desiccation tolerance and among those that are desiccation sensitive. Outside of model system taxa, few studies of pollen tube growth provide data on pollen hydration status, and virtually none on either water potential or metabolic rates of mature pollen (Heslop-Harrison 1979c; Taylor and Hepler 1997; Aylor et al. 2005; Rounds et al. 2011). Clearly, some aspects of pollen germination speed and pollen tube growth rate should be similar due to their shared metabolic machinery. However, if pollen competition has been important in a lineage over a long period, one might expect both processes to have approached their limits of acceleration, erasing indicators of evolutionary dissociation.

Despite these caveats, there are indications from the literature survey that pollen tube growth rates and pollen germination speed have evolved independently (future work will consider correlations in light of phylogenetic relationships). First, there are cases of long germination/short pollen tube growth periods and vice versa. In *Talinum teretifolium* pollen germination takes up 67 % of its 3-h-long progamic phase, whereas pollen tubes grow for only an hour at a very fast rate of 2–4 mm h⁻¹ (Dubay 1981). Conversely, some species with very rapid germination of

desiccation-tolerant pollen (e.g. *Erythronium grandiflorum*) or desiccation-sensitive pollen (e.g. *A. cherimola*) have very slow tube growth rates ($<0.300 \text{ mm h}^{-1}$) (Thomson 1989; Cruzan 1990; Lora *et al.* 2009, 2010).

Secondly, in groups with very short progamic phases ($\leq 6 \text{ h}$), pollen germination required an almost 3-fold higher percentage of the progamic phase (14.8 %) than it did in all other groups (5.3 %) (Fig. 5). The species in the former group include grasses and asterids, which have some of the fastest known pollen tube growth rates in angiosperms. This suggests that in the group most likely to have been selected for rapid reproductive cycles, pollen tubes have had a great capacity to evolve ever faster growth rates even after pollen germination speed has approached a limit.

Finally, as noted earlier, pollen germination speeds in early-divergent angiosperms are not only faster than those of almost all non-flowering seed plants, but are already near the maxima reported for both desiccation-sensitive and desiccation-tolerant pollens of monocots and eudicots. Pollen tube growth rates of these same species are also much faster than those of any non-flowering seed plant, but such rates are near the minima, not the maxima, reported for monocots and eudicots (Williams 2009). These observations suggest that with the evolution of novel features of angiosperm pollen, germination speed evolved rapidly, approaching its maximum limit early in history, whereas pollen tube growth rates have evolved slowly, relative to their maximum potential. Nevertheless, pollen tube growth rates in Nymphaeales are 2–10 times faster than those of *Amborella*, *Austrobaileya* and other ancient woody perennials. Together, these observations suggest that the rapid progamic phases of early angiosperms evolved via accelerations of both germination and tube growth simultaneously but independently, as shown by patterns of dissociation that are present even among extant early lineages.

Conclusions and forward look

The progamic phase is a life history period whose duration is determined by interactions between male and female tissues. Flowering plants, from *Amborella*, Nymphaeales and *Austrobaileya* to monocots and eudicots have remarkably short progamic phases, implying that the evolution of novel pollen–carpel interactions was involved in the speeding of fertilization. This study indicates that the pollen germination process of *Austrobaileya* is relatively rapid, and comparable in duration to those of other insect-pollinated early-divergent angiosperms as well as monocots and eudicots. Because pollen germination precedes pollen tube growth, pollen germination speed may have responded rapidly to the

stronger pollen competition regimes brought on by the early origin(s) of insect pollination and larger pollen loads. We still know little about the evolutionary developmental relationship between germination speed and early and late pollen tube growth rates.

Sources of funding

This work was supported by National Science Foundation award IOS 1052291 to J.H.W.

Contributions by the authors

J.H.W. conceived of and carried out the work, and authored the article.

Acknowledgements

I am grateful to Andrew Moffatt for sectioning pollen and taking several photographs (Fig. 2D–F), and to Matthew Lettre for lab assistance. I thank Taylor Feild for providing flowers from his greenhouse-grown *Austrobaileya* plant and the owner of private land near McHugh lookout, Millaa Millaa, for access to the population. I especially thank Bruce Sampson, Brian Murray and two anonymous reviewers for insightful comments. I greatly appreciate the academic support provided by Anne Gaskett and the School of Biological Sciences, University of Auckland, during the writing of this manuscript.

Conflict of interest statement

None declared.

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