

POLLEN–PISTIL INTERACTIONS BETWEEN AUTOTETRAPLOID AND DIPLOID *ACACIA MANGIUM* AND DIPLOID *A. AURICULIFORMIS*

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NGHIEM QC, HARBARD JL, HARWOOD CE, GRIFFIN AR, HA TH & KOUTOULIS A. 2013. Pollen–pistil interactions between autotetraploid and diploid *Acacia mangium* and diploid *A. auriculiformis*. Development of sterile triploid (3x) planting stock could help manage the risk of invasivity of widely planted *Acacia* species. Triploids may be derived from crosses of diploid and tetraploid plants. This study investigated the effect of cytotype on pollen–pistil interaction following mating between colchicine-induced tetraploid *A. mangium* (AM-4x), diploid *A. mangium* (AM-2x) and *A. auriculiformis* (AA-2x). Following controlled pollinations, pollen tubes grew well in the style, entered the ovary and penetrated ovules within 72 hours, regardless of the mating type. However, mean number of penetrated ovules per ovary was lower for AM-4x than for AM-2x or AA-2x maternal parents for all cross combinations except for self-pollination. Considering crosses between cytotypes, interspecies had significantly greater number of ovules penetrated than intraspecies. However, yields of pods (1.03%) and filled seeds (5.3%) following all inter-cytotype crosses were extremely poor compared with intra-cytotype (7.59 and 76.3% respectively). Thus, there were strong barriers to production of viable 3x seeds, despite the demonstrated absence of pre-zygotic isolation.

Keywords: Tetraploid, triploid, intra- and inter-cytotype hybridisation, pollen tube, style, ovary

NGHIEM QC, HARBARD JL, HARWOOD CE, GRIFFIN AR, HA TH & KOUTOULIS A. 2013. Interaksi debunga pistil antara *Acacia mangium* autotetraploid, *A. mangium* diploid dan *A. auriculiformis* diploid. Pembangunan stok tanaman triploid (3x) yang mandul dapat membantu mengurus risiko perebakan oleh spesies *Acacia* yang ditanam dengan meluas. Triploid boleh diperolehi melalui kacukan antara tumbuhan diploid dengan tumbuhan tetraploid. Kajian ini menyelidik kesan sitotip terhadap interaksi debunga pistil selepas pengawanan di kalangan *A. mangium* tetraploid (AM-4x), *A. mangium* diploid (AM-2x) dan *A. auriculiformis* diploid (AA-2x) yang diaruh dengan kolkisina. Bagi pendebungaan terkawal, tiub debunga tumbuh dengan baik di dalam stil, memasuki ovari dan menembusi ovul dalam masa 72 jam tanpa mengambil kira jenis pengawanan. Namun, purata bilangan ovul yang ditembusi dalam setiap ovari adalah lebih rendah bagi induk ibu AM-4x berbanding dengan induk-induk ibu AM-2x atau AA-2x dalam kesemua kombinasi kacukan kecuali penswadebungaan. Dari segi kacukan antara sitotip, interspesies menunjukkan bilangan ovul yang ditembusi yang lebih tinggi daripada intraspesies. Namun, kesemua kacukan intersitotip menunjukkan hasil lenggai (1.03%) dan biji benih penuh (5.3%) yang amat rendah berbanding dengan kacukan intrasitotip (masing-masing 7.59% dan 76.3%). Kajian menunjukkan bahawa terdapat halangan kepada penghasilan biji benih 3x yang berdaya hidup walaupun prazigot tidak dikesani.

INTRODUCTION

Acacia mangium and *A. auriculiformis* are tropical species in the subgenus *Phyllodineae*, the largest monophyletic group of the genus *Acacia* (Maslin et al. 2003a). They occur naturally in northern Queensland, Australia and adjacent regions of Papua New Guinea

and eastern Indonesia (Maslin & Pedley 1988, Maslin et al. 2003b). Together with their natural hybrid (*A. mangium* × *A. auriculiformis*), they are widely planted in short-rotation plantations in the lowland tropics. They provide an important source of industrial wood

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for products including sawn timber, pulp and fuelwood in South-East Asian countries such as Indonesia and Vietnam (Midgley & Turnbull 2003). However, because of their abundant seed production and propensity to weediness, at least 23 *Acacia* species are currently considered threats to indigenous ecosystems or cultivated areas of other crops (Paula et al. 2010, Griffin et al. 2011). Development of planting stock that is infertile or has low fertility would be useful to reduce the invasive potential of these *Acacia* species.

The development of triploids ($3x$) by crossing colchicine-induced tetraploids ($4x$) with diploids ($2x$) was advocated as one of the potential approaches to manage weediness in *Acacia* (Blakesley et al. 2002, Beck-Pay 2012). In addition to their expected low fertility, viable triploids may display advantageous characters compared with diploids. This is the case with the natural triploids of *Populus tomentosa*, which are superior to diploid clones in stem volume, growth, fibre length, stem form and pest resistance and have become a very important wood source for pulp and timber production in China (Zhu et al. 1998).

Polyploids have been observed in natural populations of some taxa of the genus *Acacia* such as allotetraploid *A. holosericea* and *A. cowleana* and allohexaploid *A. colei* (Maslin & Thomson 1992). Like *A. mangium* and *A. auriculiformis*, they belong to the taxonomic section Juliflorae within the subgenus *Phyllodineae*. However, there are no reports of polyploidy in natural populations of *A. mangium* or *A. auriculiformis*. Attempts to artificially induce polyploidy have been undertaken in *A. mangium* and *A. dealbata* (Blakesley et al. 2002) to support breeding programmes aimed at generating triploids.

A polyploid breeding programme in Vietnam was initiated using colchine-induced autotetraploid clones of *A. mangium* (AM- $4x$). A hybridising orchard designed to promote inter-cytotype pollination was established in south Vietnam with alternate rows of AM- $4x$, diploid *A. mangium* (AM- $2x$) and *A. auriculiformis* (AA- $2x$) (Kha et al. 2009). However, despite heavy flowering and seed

production of all clones in each of the three categories, the orchard has failed to produce viable open-pollinated triploid progenies in significant quantities. Only three stable triploid genotypes, all derived from AA- $2x$ mothers, were identified from screening of 758 open-pollinated seedlings raised from the parent trees in the orchard (JL Harbard, personal observation). Neither the floral phenology and morphology of species/ploidy combinations nor the foraging behaviour of the main insect pollinators (honeybees) appeared to create barriers to inter-cytotype pollination (Nghiem et al. 2011). Thus, barriers to $3x$ seed production appear to be occurring somewhere in the reproductive cycle following pollination.

Acacia mangium and *A. auriculiformis*, like most other Australian acacias, have a complex breeding system influenced by their floral morphology, with andromonoecy, weak protogyny and a degree of self-incompatibility (Kenrick 2003). Earlier studies on natural populations showed high rates of outcrossing in *A. auriculiformis* (Moran et al. 1989) and variable rates of outcrossing in *A. mangium* (Butcher et al. 2004, Harwood et al. 2004). In a study on breeding systems of *A. mangium* in the hybridising orchard in Vietnam, AM- $4x$ trees had extremely high selfing rate (~98%) whereas outcrossing predominated in AM- $2x$ trees, with about 96% of progeny outcrossed (Griffin et al. 2012). Under open pollination, there is evidently a large change in the expressed breeding system of *A. mangium* associated with the change in ploidy. This is consistent with the increase in self fertility of polyploids observed in 235 angiosperm taxa (Mable 2004, Barringer 2007). Nevertheless, we would expect some level of inter-cytotype pollination and production of some triploid seeds in the orchard, unless there are strong barriers to fertilisation and/or successful development of viable seeds.

Previous studies investigating inter- and intraspecific hybridisation in diploid *A. mangium* and *A. auriculiformis* indicated that neither *A. mangium* nor *A. auriculiformis* showed pollen–pistil incompatibility, although the growth of pollen tubes was more disoriented

and there was less penetration of ovules in the cross of *A. mangium* × *A. auriculiformis* than in the reciprocal cross (Sedgley et al. 1992).

In this study, we assessed growth of pollen tubes and the penetration of ovules following different types of mating combinations (e.g. selfing, intra-cyctotype outcrosses and inter-cyctotype outcrosses between the three species/ploidy combinations). Pod and seed yields following controlled and open pollination were also assessed.

MATERIALS AND METHODS

Study site and materials

The orchard was established in 2003 at Bau Bang, Binh Duong province in southern Vietnam (11° 15' N, 106° 38' E and 50 m elevation). It included 33 putative tetraploid *A. mangium* (AM-4x) clones, produced through colchicine induction by Shell Forestry International in England (Blakesley et al. 2002, Harbard et al. 2012), and 10 clones each of diploid *A. mangium* (AM-2x) and diploid *A. auriculiformis* (AA-2x) selected from Vietnam's breeding programmes. The orchard was laid out in four replicates, each with four rows, including two rows of putative AM-4x clones and one row each of AM-2x and AA-2x clones. Positions of individual clones were randomised within rows.

Pollination treatments were applied to one ramet of each of three different clones of AM-2x, AM-4x and AA-2x. The putative AM-4x ramets were confirmed by stomatal counts, measurement of polyad diameters and/or flow cytometry (Harbard et al. 2012). Only 4x ramets with confirmed ploidy level were used in the experiment. Parent trees with easy access to abundant healthy flower spikes, relatively free of insect attack, were selected. Information on flowering time and intensity of individual clones was also used in the selection

process to facilitate cross-pollination among selected trees (Nghiem et al. 2011).

Pollination treatments and five types of mating combinations

Pollination treatments were carried out from October till December 2009. Two treatments were applied on each mother tree (Table 1). Controlled pollination treatment included five different types of mating combinations (Table 2): (i) selfing and (ii) intra-cyctotype outcrosses within each of AM-4x, AM-2x and AA-2x; as well as reciprocal crosses at the species/cyctotype level, including (iii) intra-cyctotype crosses between AM-2x and AA-2x, (iv) inter-cyctotype crosses between AM-2x and AM-4x, and (v) inter-cyctotype crosses between AA-2x and AM-4x. Mating combinations (iii), (iv) and (v) used only two pollen parents because of limited pollen availability at the time these crosses were performed.

Pollen tube growth was studied for the five types of mating produced through controlled-pollination by harvesting some of the flowers shortly (24 and 72 hours) after crossing. Additional flowers from hand controlled pollination treatments, together with those from open pollination were left to develop until pods reached maturity and harvested just before pods opened to study seed yields and seed quality.

Manual controlled pollination techniques

Flower spikes of selected parent trees approaching anthesis but without opened flowers were bagged in the afternoon and spikes were collected early the following morning for pollen extraction. The spikes were dried in desiccators containing silica gel for 3 hours and then sieved through a 63-µm stainless steel sieve. The sieved pollens were collected in cryovial tubes (1.5 mL), each with

Table 1 Pollination treatments applied on each mother tree

Treatment	Technique required	No. of spikes or flowers
Natural open pollination (OP)	No emasculation and unbagged	100 spikes/tree
Manual controlled pollination (CP)	Thinning, emasculating, applying target pollen and bagging	At least 600 flowers/mating

Table 2 Control-pollinated mating matrix

		AM-2x			AM-4x			AA-2x		
		1	2	3	1	2	3	1	2	3
AM-2x	1	S	*		*				*	
	2		S	*		*		*		
	3	*		S	*				*	
AM-4x	1		*		S	*		*		
	2	*				S	*		*	
	3		*		*		S	*		
AA-2x	1	*			*			S	*	
	2		*			*			S	*
	3	*			*			*		S

S = self- and * = cross-pollinations

sufficient amount for one day's pollination and used on the same day.

Manual controlled pollination was conducted based on the techniques developed by Sedgley et al. (1992). Spikes were selected in the afternoon the day before carrying out pollination and were bagged using three-dimensional polyester pollination bags in different sizes. Selected spikes were unbagged the following day and unhealthy and/or unopened spikes were removed. The number of flowers on each spike was reduced to 20–30. The remaining flowers were emasculated by removing the stamens using fine forceps. A 20% sucrose solution was added to each stigma tip before transferring sieved pollen to assist polyad adhesion and germination (Griffin et al. 2010) and stigmas were randomly checked following pollination for pollen presence using a handy light scope. Spikes were rebagged for three days and labelled with numbered tags. The bags were temporarily removed for sampling at 24 hours and were not replaced after the 72-hour sample collection.

Study of pollen tube growth

Three to four spikes bearing about 50 pollinated flowers from each of the 36 crosses

(Table 2) were collected at 24 and 72 hours after pollination. These spike samples were fixed in 3:1 methanol:acetic acid solution for a minimum of 3 hours. The solution was replaced with 70% ethanol for transportation.

A clear-squash technique for the study of pollen tube growth was applied (Sedgley et al. 1992). Thirty pistils were dissected from the fixed flowers of each of the 36 crosses. The tissue was then hydrated through an alcohol series, softened by 0.8 N sodium hydroxide and stained with decolorised aniline blue. The styles and corresponding ovaries were observed using fluorescence microscope under UV light at 200× magnification. The images were digitally captured using Axiovision 3.1 software.

The squashed pistils were assessed for the presence of polyad on the stigma, number of styles and ovaries with pollen tubes, number of ovules penetrated per ovary and number of pollen tubes visible at the base of style, where they were most clearly exposed for counting.

Pod and seed harvest and seed categories

The remaining flowers were left for harvesting mature pods. Each pod was examined separately. Seeds extracted from each pod were categorised

into two types: (1) filled seeds of small or normal size and (2) unfilled seeds of small or normal size. Seeds were classified as normal in size if ≥ 4 mm in length and small if < 4 mm (Nghiem et al. 2011). Filled seeds were thought to have some prospect of germinating, while unfilled seeds were clearly lacking a healthy embryo and would not germinate (Figure 1).

Statistical analysis

The observations at 24 and 72 hours after pollination were analysed to study the effects of time and pollination treatment. First, the data for each of the 36 crosses at each sampling time were summarised to produce the following parameters: (1) percentage of stigmas adhering to polyads, (2) percentage of styles and ovaries with pollen tubes, (3) percentage of ovaries with at least one ovule penetrated, (4) mean number of pollen tubes per style and (5) mean number of penetrated ovules per ovary.

The percentages of stigmas adhering to polyads and styles with pollen tubes were calculated on the original 30 pollinated flowers. The other percentages were calculated using the numbers of flowers which carried germinated polyads because ungerminated polyads attached to flowers would not yield useful information on pollen tube development and behaviour. The numbers of pollen tubes per style and ovules penetrated per ovary were mean values for those styles bearing tubes and those ovaries having at least one penetrated ovule.

Univariate analysis of variance was carried out on individual variates using SAS version 9.2. Square root transformations were applied where necessary to achieve normality of residual errors. First, the effect of time (24

vs 72 hours) was examined using one-way analysis of variance (ANOVA). Since the maternal trees of the three different species/ploidy combinations (AM-2*x*, AM-4*x*, AA-2*x*) did not receive exactly the same mating-type treatments (Table 2), a nested treatment structure (i.e. species/crosstype) was not appropriate. Instead, for each of the two times after pollination, all 12 mating types were compared using one-way ANOVA. Multiple mean comparisons were performed using Tukey–Kramer’s range test at $\alpha = 0.05$.

2×2 contingency χ^2 tests were also used to compare the proportions of filled and unfilled seeds for (i) intra- vs inter-cytotype crosses and (ii) intra-cytotype outcrosses of AM-2*x* and AA-2*x* versus pods from open-pollinated parents of AM-2*x* and AA-2*x*.

RESULTS

Stages in the reproductive process, from pollen deposition to ovule penetration are illustrated in Figure 2.

Pollen tube development 24 and 72 hours after pollination

Pollen tube development progressed significantly over 24 and 72 hours after pollination (Table 3). There was significant difference ($p \leq 0.05$) in the percentage of stigmas with adhering polyads at 24 (61.2%) and 72 hours (70.3%). The percentage of styles with pollen tubes did not differ significantly with time. The mean number of pollen tubes counted at the base of the style at 24 hours (8.5 pollen tubes per style) was significantly higher ($p \leq 0.001$) than that at 72 hours (6.2 pollen tubes per style). This indicated that pollen tubes grew down the style within 24 hours and the leading tubes

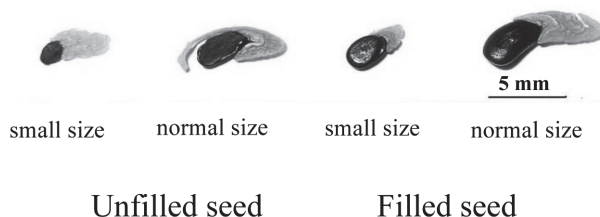


Figure 1 Seed category

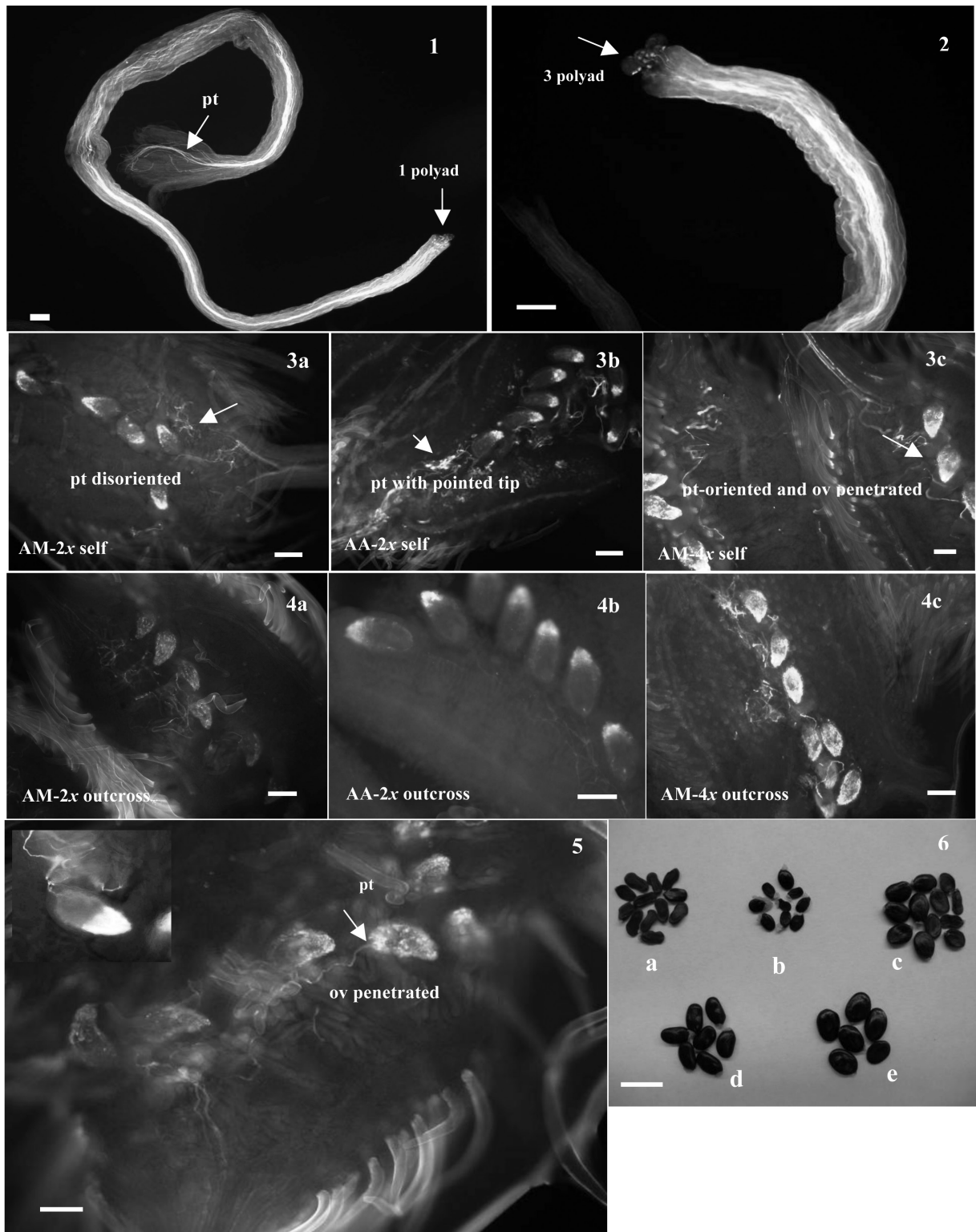


Figure 2 Figures show pollen tube (pt) growth and ovule (ov) penetration at different stages: (1 + 2) on the stigma and down the style (scale bar = 45 μ m); (3: a,b,c) self-pollination (scale bar = 50 μ m); (4: a,b,c) outcross-pollination (scale bar = 50 μ m); (5) inter-cyctotype pollination (scale bar = 30 μ m); (6) seed size: (a) inter-cyctotype AM; (b) inter-cyctotype AA; and (c–d–e) diploid outcrosses (AA-2x \times AM-2x, AM-2x \times AM-2x and AA-2x \times AA-2x respectively) (scale bar = 5 mm)

Table 3 Effect of time after pollination on pollen tube growth

Time after pollination (hours)	Stigma with one or more polyads (%)	Style with pollen tube (%)	Mean no. of pollen tube per style	Ovary with pollen tube (%)	Ovary with at least one ovule penetrated (%)	Mean no. of ovules penetrated per ovary
24	61.2	81.2	8.5	51.6	6.1	1.25
72	70.3	82.1	6.2	90.3	29.3	1.58
Significance of differences between time	*	ns	***	***	**	*

ns = Not significant, * = $p \leq 0.05$, ** = $0.001 \leq p \leq 0.01$, *** = $p \leq 0.001$

had entered the ovaries at 72 hours, resulting in lower counts as they were not easily visible at the base of the style.

Fewer pollen tubes were observed growing down the ovaries at 24 hours than at 72 hours. This led to a significantly lower ($p \leq 0.001$) percentage of ovaries with pollen tubes (51.6%) and with at least one ovule penetrated (6.1%) as well as mean number of penetrated ovules per ovary (1.25) compared with the corresponding values at 72 hours (90.3%, 29.3% and 1.58 respectively).

The trend of an increasing percentage of ovaries with pollen tubes and with ovules penetrated and greater number of ovules penetrated at 72 hours compared with 24 hours after pollination was observed for all 12 cross combinations (Table 4).

Pollen tube growth and ovule penetration between 12 mating types

Percentage of stigma with adhered polyad and percentage of style with pollen tube differed significantly ($p \leq 0.05$) at 24 hours (Table 4). Outcrosses among AM-4x trees had the lowest values for both variables, with only 27.8% of stigmas having adhered polyads and 47.8% of styles having pollen tubes.

Mean number of pollen tube counted at the base of the style did not differ significantly at 24 hours, However, there were differences ($p \leq 0.01$) at 72 hours. AA-2x pistils showed higher numbers, 7.0–8.9 tubes per style compared with 4.2–6.7 tubes for AM-2x and AM-4x pistils. The inter-cytotype crosses in

A. mangium did not differ significantly with regard to stigma with polyad, style with pollen tube and pollen tube per style.

Mating type affected the subsequent behaviour of pollen tubes. The percentage of ovary with pollen tube visible did not differ between the 12 mating types at 24 hours. Therefore, only the 72-hour data are presented in Table 4. There was significant ($p \leq 0.001$) variation in the percentage of ovary with one or more ovules penetrated and in the mean number of penetrated ovules per ovary 72 hours after pollination (Table 4). AA-2x ovary showed the highest ovule penetration following intra- or inter-cytotype crosses, whereas the penetration of AM-4x outcrossed ovules was poor, with only 1.0 ovule penetrated per ovary.

Self- and outcross-pollen tube growth and ovule penetration of individual genotypes

The response of tetraploid parent trees to self- and outcross-pollination appeared to differ from that observed on diploid parents. On average, AM-4x selfs had higher average percentage of ovaries with at least one ovule penetrated (33.2%) as well as larger number of penetrated ovule per ovary (1.39) than AM-4x outcrosses (20.6% and 1.00 respectively), in contrast to diploids of both *A. mangium* (35.9% and 1.29) and *A. auriculiformis* (35.6% and 1.33), for which outcrossing resulted in better ovule penetration (Table 4). However, there was variation between individual crosses within treatments. For instance, clone 51 of

Table 4 Pollen tube growth, ovule penetration and pod/seed set from different mating types within each species/ploidy combination

Maternal parent	Type of mating	24 hours post-pollination			72 hours post-pollination			Pod set ¹ (%)	Filled seeds/total seeds per pod
		Stigma with polyad (%)	Style with pollen tube (%)	Pollen tube per style (no.)	Ovary with at least one ovule penetrated (%)	Ovule penetrated per ovary (no.)	Pollen tube per style (no.)		
AM-2x	Open pollination						0.41+	7.2/ 7.9	
	Self	60.0 ab	79.2 ab	7.4	26.4 bc	1.20 bc	0.00	0.0	
	AM-2x × AM-2x	70.0 ab	82.4 ab	9.9	29.7 bc	1.36 bc	7.02	9.4/ 9.7	
	AM-2x × AA-2x	60.0 ab	86.7 a	8.8	17.6 c	1.64 bc	–	–	
	AM-2x × AM-4x	70.0 ab	74.2 ab	7.1	14.3 c	1.33 bc	1.04	0.8/ 3.5	
AA-2x	Open pollination						0.38+	6.7/ 7.1	
	Self	66.7 ab	78.3 ab	10.0	12.2 c	1.58 bc	0.00	0.0	
	AA-2x × AA-2x	70.0 ab	96.4 a	9.7	46.0 ab	1.85 bc	7.25	5.5/ 6.9	
	AA-2x × AM-2x	82.2 a	93.2 a	10.1	59.9 a	2.77 a	8.49	8.9/ 9.8	
	AA-2x × AM-4x	53.3 ab	87.5 a	7.7	37.7 b	1.92 b	1.02	0.3/ 5.9	
AM-4x	Open pollination						0.09+	2.7/ 3.7	
	Self	43.3 ab	79.6 ab	6.6	33.2 b	1.39 bc	0.08	2.3/ 3.7	
	AM-4x × AM-4x	27.8 b	47.8 b	6.4	20.6 bc	1.00 c	–	–	
	AM-4x × AM-2x	71.1 a	90.5 a	9.2	35.9 b	1.29 bc	0.00	0.0	
	AM-4x × AA-2x	58.9 ab	78.5 ab	9.3	35.6 b	1.33 bc	0.00	0.0	

Significance differences between taxa-by-mating combinations

**

ns

*

*

ns = Not significant, * = $p \leq 0.05$, ** = $0.001 \leq p \leq 0.01$ and *** = $p \leq 0.001$; letters show significant differences of the 12 mating types using Tukey–Kramer test at $p < 0.05$; ¹ = (total no. of pods set/ total no. flowers pollinated) × 100; – = few pollinated flowers were left after collecting 72 hours samples, so they failed to develop pod; + = open-pollinated pod set (%) estimated based on mean no. of hermaphrodite flowers on the spike × no. of hermaphrodite flowers on the spike × no. of hermaphrodite flowers on the spike × no. of hermaphrodite flowers on the spike (Nghiem et al. 2011)

AM-4x had slightly higher percentage of ovary with ovule penetrated following outcrossing (18.3%) than following selfing (17.3%). On the other hand, the reverse was true for clone 14 of AM-2x (26.4 and 14.8% for selfing and outcrossing respectively) (Table 5).

Further crosses were, therefore, made to better understand the variation in AM-2x and AM-4x pollen tube behaviour. Additional

outcrosses of AM-2x and AM-4x were conducted in 2011 and the flower samples were collected a day later than in the earlier study (96 hours post-pollination) to allow more opportunity for ovule penetration (Table 5). A higher percentage of ovary with at least one ovule penetrated and more penetrated ovules per ovary were observed in AM-2x and AM-4x crosses at 96 hours.

Table 5 Ovule penetration following self- and outcross-pollination of individual crosses

Maternal parent	Type of cross	Subsample collected in 2009 72 hours post-pollination			Subsamples collected in 2011 96 hours post-pollination		
		Cross ID	Ovary with at least one ovule penetrated (%)	Ovule penetrated (no.)	Cross ID	Ovary with at least one ovule penetrated (%)	Ovule penetrated (no.)
AM-4x	Self	36 × 36	46.6	1.29		–	–
		51 × 51	17.3	1.50		–	–
		66 × 66	34.8	1.38		–	–
		Mean	33.2	1.39			
	Outcross	36 × 51	24.8	1.00	44 × 40	23.1	1.67
		51 × 36	18.3	1.00	40 × 10	14.3	1.00
		66 × 36	19.8	1.00	10 × 36	38.9	1.71
					36 × 60	18.2	1.50
					60 × 11	25.0	1.33
					11 × 44	28.6	1.00
	Mean	20.6	1.00		24.7 b	1.43 b	
AM-2x	Self	30 × 30	0.0	0.00		–	–
		14 × 14	26.4	1.20		–	–
		68 × 68	0.0	0.00		–	–
		Mean	26.4	1.20			
	Outcross	30 × 68	40.9	1.89	82 × 63	36.4	1.88
		14 × 68	14.8	1.00	63 × 30	40.0	2.13
		68 × 14	33.4	1.20	30 × 63	38.1	2.25
		Mean	29.7	1.36		38.2 a	2.09 a

– = Self-pollinated flowers not collected in 2011; letter indicates significant difference using Tukey–Kramer at $p < 0.05$

Neither of the two additional AM-2*x* maternal genotypes (clones 63 and 82) had as low a percentage of ovule penetration following outcrossing as did tree 14 (14.8%) in the previous year. In the follow-up crossing conducted in 2011, percentage of ovary with at least one ovule penetrated (38.2%) and mean number of ovule penetrated (2.09) for AM-2*x* outcrosses were significantly ($p \leq 0.05$) higher than those of AM-4*x* (24.7% and 1.43). Despite some variations between crosses, the six additional AM-4*x* outcrosses conducted in 2011 all displayed penetration of some ovules by pollen tubes for at least 14.3% of flowers (Table 5).

The growth of AM-4*x* self pollen tubes in the ovary was more targeted towards ovules than that of tubes in AM-2*x* selfcrosses and AM-4*x* outcrosses, which displayed either a pointed tip or disorientation (Figure 2: 3 and 4: a, b, c). This difference was, however, not quantified.

Pollen tube growth and ovule penetration at 72 hours following different types of species/cyotype reciprocal crosses

*Intra-cyotype interspecies (AA-2*x* × AM-2*x* vs AM-2*x* × AA-2*x*)*

The crosses with AA-2*x* as mother had 59.9% of ovary penetrated with an average of 2.77 penetrated ovules per ovary compared with 17.6% and 1.64 respectively when AM-2*x* was used as mother (Table 4).

*Inter-cyotype intraspecies (AM-2*x* × AM-4*x* vs AM-4*x* × AM-2*x*)*

There was significantly lower percentage of AM-2*x* ovaries with at least one ovule penetrated (14.3%) than the AM-4*x* ovaries (35.9%). However, the mean number of penetrated ovule per ovary was similar, with an average of 1.29 AM-4*x* ovules compared with 1.33 AM-2*x* ovules penetrated per ovary (Table 4).

*Inter-cyotype interspecies (AA-2*x* × AM-4*x* vs AM-4*x* × AA-2*x*)*

When AA-2*x* was the mother, mean number of ovule penetrated per ovary was greater

with 1.92 AA-2*x* ovules penetrated compared with only 1.33 ovules for the AM-4*x* mother, even though the percentage of ovary with at least one ovule penetrated was similar, approximately 38% (Table 4).

Pod and seed set

Self-pollinated flowers of AM-2*x* and AA-2*x* trees failed to produce pods while 0.08% (Table 4) of the self-pollinated AM-4*x* flowers developed three pods (Table 6). These three pods displayed similar mean numbers of filled and total seeds per pod (2.3/ 3.7) as were observed in open-pollinated AM-4*x* pods (2.7/ 3.7).

There was a wide range in pod and seed set success among the other crosses. The diploid hybrid between AA-2*x* and AM-2*x* was most successful in pod/seed set, with 8.49% of pollination yielding pods and a mean of 8.9 filled seeds out of 9.8 total seeds per pod. Next in terms of success were intraspecies outcrosses within diploids of AM and AA, which were similar in pod set (~7%) but differed in seed set per pod. AM-2*x* produced more seeds per pod (9.4 filled seeds and 9.7 total seeds per pod) than AA-2*x* (5.5 filled seeds and 6.9 total seeds per pod). Finally, pod and seed set from inter-cyotype crosses were similar in quantity within and between species and extremely poor in quality, with only ~1% pod set and low ratios of 0.8 filled/3.5 total seeds for AM-2*x* × AM-4*x* and 0.3 filled/5.9 total seeds for AA-2*x* × AM-4*x*. No pods were obtained from inter-cyotype crosses when AM-4*x* was used as maternal parent (Table 4).

Percentages of open-pollinated pods set from the parent trees (0.41% for AM-2*x*; 0.38% for AA-2*x* and 0.09% for AM-4*x*) were very low (Table 4).

Seed categories following types of cross combination

Intra-cyotype outcrosses produced significantly ($p < 0.001$) greater ratio of filled to total seeds than that of inter-cyotype crosses (446 filled of 493 total seeds harvested compared with only 55 of 607) (Table 6). For filled seeds, the ratio of small seeds was significantly higher in inter-cyotype crosses (23 small filled seeds out of 55 total filled seeds) than in intra-cyotype crosses

Table 6 Pods harvested and number of filled and unfilled seeds harvested

Pollination treatment	No. of flowers pollinated	No. of pods harvested	No. of filled seeds		No. of unfilled seeds		Total filled seeds to total seeds harvested
			Small size	Normal size	Small size	Normal size	
Controlled pollination (CP)							
Selfing							
AM-4x	3655	3	2	5	1	3	7/11
Intra-cyctotype crosses							
AM-2x × AM-2x	242	17	7	152	0	5	159/164
AA-2x × AA-2x	207	15	21	62	1	19	83/103
AA-2x × AM-2x	259	23	42	162	5	17	204/226
Inter-cyctotype crosses							
AM-2x × AM-4x	3087	32	18	8	67	19	26/112
AA-2x × AM-4x	8064	82	5	24	180	286	29/495
Open pollination (OP) of parent trees							
AM-2x	36,432*	146	75	975	34	63	1050/1147
AA-2x	24,700*	94	19	611	7	31	630/668
AM-4x	49,536*	47	41	88	14	30	129/173

Significance of difference in ratio of filled to unfilled seeds per pod

(i) between intra-cyctotype and inter-cyctotype crosses: significant ($\chi^2_{df=1} = 726.9$, $p < 0.001$)

(ii) between intra-cyctotype crosses and OP: not significant ($\chi^2_{df=1} = 1.22$)

* No. of flowers pollinated calculated as no. of spikes tagged × mean no. of hermaphrodite flowers per spike (AM-2x = 176.3, AA-2x = 76 and AM-4x = 144; Nghiem et al. 2011)

(70 small filled seeds out of 446). The quality of seeds of inter-cyctotype crosses was extremely poor with only 9% of total seeds being filled compared with controlled intra-cyctotype (~90.5%) and open-pollinated crosses (~91%).

The ratio of filled seeds to total seeds harvested from intra-type outcrosses of AM-2x and AA-2x (242 filled/267 total) was not significantly different from the corresponding ratio in the open-pollinated pods from the same female parents (1680 filled/ 1815 total) (Table 6).

Three mature pods of AM-4x self displayed a proportion of filled seeds to total seeds (7 filled/11 total) that was similar to that of the AM-4x open-pollinated pods (129 filled/173 total).

DISCUSSION

In angiosperms, the ovules are enclosed within the carpel; thus the male gametes are transferred to the female organs via a number of sequential steps involving pollen–pistil interaction. These steps include recognition and match between pollen and receptive stigma, pollen germination and tube growth down the style and into the ovary, and ovule penetration (Sedgley & Griffin 1989).

Our study examines the effects of mating type across this phase of the reproductive process in *A. mangium* and *A. auriculiformis*. We assessed the extent to which these pre-zygotic factors could contribute to observed variation in mature seed production between selfs

and outcrosses for the three species/ploidy combinations studied, including differences between intra- and inter-cytotype crosses within and between species. For practical and logistical reasons, most experiments were limited to three female parents of each of AM-2 x , AM-4 x and AA-2 x . Thus, we were unable to robustly quantify the degree of variation between crosses within mating types.

The percentage of stigma with polyad attached exceeded 53% > 24 hours after pollination regardless of species or cytotype (Table 4). This was in accordance with earlier findings that pollination stimulated the stigmatic secretion process in *Acacia* even with inter-generic crosses (Kenrick & Knox 1981, Marginson et al. 1985) and with the conclusion that variation in stigma and polyad size between cytotypes would not adversely affect inter-cytotype pollination (Nghiem et al. 2011). The percentage was at the higher end of the range reported in other pollination studies of *Acacia* (Sedgley et al. 1992, Tandon et al. 2001), suggesting that our pollination protocol was working well. We observed that a significantly greater percentage of flowers collected after 72 hours had adhering polyads (71.2%) compared with that of the 24 hours collection (61.3%) (Table 3). We suggest that it may be an artefact of the handling procedure. Harvested flowers were placed in vials of 70% ethanol for transport. The polyads collected at 24 hours might have been less securely anchored by pollen tubes and might have been washed off more easily in transit.

The mean number of pollen tubes at the style base at 24 hours was not significantly different between mating types in spite of a slightly lower AM-4 x pollen tube number (6.9 tubes on average) compared with AM-2 x and AA-2 x (9.3 tubes). This was in agreement with results from *in vitro* pollen tests where AM-4 x had a lower proportion of germinated polyads with four or more pollen tubes than did AM-2 x and AA-2 x (Nghiem et al. 2011). The reduced mean number of pollen tubes counted at 72 hours compared with 24 hours was presumed due to the inability to visualise the leading pollen tubes since they had already entered the ovary 24 hours after pollination. Kendrick (2003) and Tandon et al. (2001) also reported that *Acacia* pollen tubes reached the ovary within 24 hours. This was a good time

to sample for comparative studies of tube growth and the 72-hour sample data were more conclusive regarding the later stage of development within the ovule.

Regardless of mating type, the percentages of ovaries with pollen tubes and with at least one ovule penetrated as well as mean number of penetrated ovules per ovary were significantly higher at 72 hours after pollination than at 24 hours. At 24 hours, pollen tubes had penetrated one or more ovules in very few ovaries. However, the penetration of ovules in *Acacia* hybrid (*A. mangium* \times *A. auriculiformis*) appeared to be more rapid, with the micropylar nucellus of the ovule being penetrated by a pollen tube approximately 12 hours after open-pollination of hybrid flowers (Sornsathapornkul & Owens 1999).

At 72 hours, the percentage of ovary with penetrated ovule and mean number of penetrated ovule per ovary varied significantly between the 12 mating types in our study (Table 4). It is clear from the literature that there are complex and long distance exchanges of signals between developing pollen tubes and ovules (Gauze & McCormick 1999, Higashiyama 2010).

Self- and outcross matings and open pollination

Pollen tubes were present in self-styles and ovaries of AM-2 x and AA-2 x and mean numbers of ovules penetrated per ovary in selfs (1.20 for AM-2 x and 1.59 for AA-2 x at 72 hours; Table 4) were similar to those obtained for the corresponding selfs by Sedgley et al. (1992) (1.59 and 0.98 respectively). Although there was no significant difference in ovule penetration between selfs and outcrosses, no pods or seed were harvested from AM-2 x and AA-2 x selfs. In intra-type outcross, 7% of the flowers produced pods and displayed a high yield of filled seeds per pod. Thus, it appears that the effects of mating type on mature seed yields are attributable to post-zygotic factors rather than pollen–pistil interaction. As in most species of Mimosoideae, competition for maternal resource occurs between the individual pods in an infructescence rather than between individual embryos within a pod (Kenrick 2003, Butcher et al. 2004). In diploid *A. mangium*, selfed pods appear to compete poorly with

outcrossed pods for resources, as outcrossed seeds predominate in the open pollinated seed crop in this seed orchard (Griffin et al. 2012). Failure of a high proportion of selfed flowers following open pollination probably contributed to low mature pod set in AM-2x (0.41%) and AA-2x (0.38%). The proportion of selfed to outcrossed flowers in open-pollinated *Acacia* inflorescences remains unknown. A similar mating system favouring the maturation of outcrossed seeds appears to operate in Australian natural populations of *A. mearnsii*, which displays 94% outcrossing in the seed resulting from open pollination. The proportion of functionally female flowers that yielded pods was very low. Variation in pod yields from year to year (0.04 to 0.10%) appeared to be associated with variation in flowering intensity in the stand, which might have influenced the rate of self-pollination (Grant et al. 1994). However, in two other *A. mangium* seed orchards in Vietnam where inter-tree pollination was considered to be poor, high proportions of selfed seeds were obtained at harvest (Butcher et al. 2004). This shows that there is no absolute barrier to setting selfed seed in diploid *A. mangium*. A high proportion of selfed pollen could have contributed to the low observed pod set in open-pollinated AM-4x (0.09%) because controlled outcrossing failed to produce any AM-4x pods and seeds (Table 4). It is very likely that not all flowers in the open-pollinated spikes received polyads and this would contribute to the low estimates of percentage pod set under open pollination.

The selfed AM-4x pollen tubes displayed more targeted growth towards the ovules than outcrossed pollen tubes (Figure 2: 3c). In the initial experiment, a significantly higher number of AM-4x-selfed ovules were penetrated than AM-4x-outcrosses (Table 4). Unlike the diploid trees, 0.08% of the AM-4x selfed flowers produced mature pods. The numbers of filled seeds and total seed per pod were similar to those in the pods from open-pollinated AM-4x. This supported the finding of Griffin et al. (2012) that AM-4x trees were more self-fertile than AM-2x. However, it was difficult to explain the apparent superiority of ovule penetration of selfs so we conducted more pollination in 2011 and found that the

average ovule penetration of AM-4x selfs in 2010 and outcrosses in 2011 was effectively the same (Table 5).

Reciprocal intra-cytype crosses between AM-2x and AA-2x

The reciprocal cross-pollinations of AA-2x and AM-2x were successful as judged by pollen tube growth in both AM-2x and AA-2x styles and ovule penetration (Table 4). In accordance with the literature, the growth of AM-2x pollen tubes in AA-2x styles was more oriented and resulted in more AA-2x-penetrated ovules (2.77) than the reciprocal (1.64). Sedgley et al. (1992) also found that *A. auriculiformis* used as female parent was more fertile than *A. mangium* and suggested there might be weaker attraction of *A. mangium* ovules to *A. auriculiformis* pollen tubes leading to disoriented pollen tube growth and fewer ovule penetrations. No pods matured from the AM-2x × AA-2x cross harvested but the AA-2x × AM-2x had higher pod set (8.49%) and filled seeds yield (8.9/9.8) than the intra-specific AA-2x outcross (7.25% and 5.5/6.9 respectively). It is clear that male and female roles regulate post-pollination events to ensure successful fertilisation and pod production.

Inter-cytype crosses

Since we had no *A. auriculiformis* tetraploids we were not able to examine reciprocal inter-cytype crosses between the two species, only the reciprocal AM-2x × AM-4x and AM-4x × AM-2x combinations (Table 4). Across these inter-cytype crosses, an average of 82.7% of styles contained pollen tubes at 24 hours. No barrier to mating at this stage was, therefore, detected. However, there were differences in ovule penetration. Within *A. mangium*, the direction of the inter-cytype cross did not affect the number of pollen tubes per style or the number of ovules penetrated. However, despite having lower ovary penetration rates, only crosses with AM-2x as the mother set pods and few seeds were produced per pod with a small proportion of filled to total seeds (0.8 filled to 3.5 total). In a similar study with *A. mearnsii*, Beck-Pay (2012) indicated that using

the tetraploid as the maternal parent was more successful, attributing this to the better quality of the diploid pollen and therefore higher fertilisation of tetraploid ovaries.

In the AA-2x × AM-4x cross we also found that the AM-4x pollen performed well with respect to pod set. There were as many pollen tubes per style and ovules penetrated per ovary as in the diploid outcrosses of *A. auriculiformis*. Once again the difference lies in what happens in the course of embryo and pod development after fertilisation. The pod set of the inter-cytotype cross was 1% compared with 7% for the diploid outcrosses and the filled to total seed ratio was much lower (0.3/5.9 compared with 5.5/6.9). These results suggest that, in the hybridising orchard, we are more likely to find open pollinated hybrid triploid seeds from AA-2x mothers than from inter-cytotype crosses of *A. mangium* clones. A comprehensive review has documented that allopolyploids of plant species are much more frequent than autopolyploids in nature (Ramsey & Schemske 1998).

Seed quality

Inter-cytotype crosses, which were expected to yield triploid seeds, displayed very low ratios of filled to total seeds and high ratios of small to large filled seeds in comparison with intra-cytotype crosses of AM-2x and AA-2x (Table 6). This poor seed development is presumed to be an effect of genetic abnormalities resulting from meiotic complexity (e.g. multivalent pairing, imbalanced gametes) occurring in either male or female gametophytes at various development stages in inter-cytotype crosses (Ramsey & Schemske 2002). However, we were able to obtain some seeds from inter-cytotype crosses within and between *A. mangium* and *A. auriculiformis*. Evidence of germination and viability as well as ploidy level of these controlled pollination seeds will be reported separately. Evidently, production of triploids is not easy. However, it has been observed in breeding and natural population evolution that the reproductive barriers of neo-polyploids can be reduced through a few generations of selection (Ramsey & Schemske 2002, Ranney 2006). This offers some encouragement that we will be able to achieve the practical aim of producing viable

and useful triploid progenies if we work with larger populations of polyploids and select those which are capable of producing some triploids for further breeding and selection.

In summary, no reproductive barriers arose from pollen–pistil interactions between male and female reproductive tissues in crosses between the two cytotypes. Triploid offspring were expected to be produced in both directions of the diploid (2x) by tetraploid (4x) cross, especially when AA-2x was the female parent. However, pods and seed yields produced from the inter-cytotype crosses in this controlled pollination experiment were extremely low and seeds were poor in quality, with most seeds unfilled. Hence, it was apparent that post-zygotic effects caused embryo abortion at different stages of seed development.

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