TUBE POLLINATION USING STORED POLLEN FOR CREATING *ACACIA AURICULIFORMIS* HYBRIDS

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Received March 2011

KATO K, YAMAGUCHI S, CHIGIRA O, OGAWA Y & ISODA K. 2012. Tube pollination using stored pollen for creating *Acacia auriculiformis* hybrids. Artificial pollination using polyvinyl tubes, a recognised pollination method for the genus *Acacia*, was conducted to determine whether this method was also suitable for the establishment of *Acacia* hybrids (*A. auriculiformis* × *A. mangium*) using stored pollen. This method was used to cross female adults of *A. auriculiformis* with males of *A. mangium*, with intraspecific pollination of *A. auriculiformis* conducted as the control. In this method, pollen was first collected by inserting a tube into spikes of male adults immediately after flowering. The tube was then stored at -18 °C for either one day, one month or two months. The germination rate of *A. mangium* pollen was 17% following storage for one day but increased to greater than 40% following storage for one or two months. In contrast, the germination rate of *A. auriculiformis* pollen exceeded 40% regardless of the storage duration. Artificial pollination was conducted by inserting the stored tube with pollen into flowering spikes of *A. auriculiformis*. Results of interspecific pollination showed that an average of three hybrid seedlings was acquired per pollination using pollen stored for one day, and was greater than five when pollen stored for one or two months was used. However, DNA analysis revealed that 2.6% of seedlings were contaminated by *A. auriculiformis*. In contrast, less than three seedlings were acquired per intraspecific pollination regardless of the duration of pollen storage. These results suggest that this tube method is a suitable procedure for the establishment of *A. auriculiformis* hybrids using stored pollen.

Keywords: *Acacia mangium*, artificial pollination, mature seed, germination, seedling

KATO K, YAMAGUCHI S, CHIGIRA O, OGAWA Y & ISODA K. 2012. Pendebungaan tiub menggunakan debunga yang disimpan untuk menghasilkan hibrid *Acacia auriculiformis*. Pendebungaan buatan menggunakan tiub polivinil merupakan kaedah pendebungaan yang biasa digunakan untuk genus *Acacia*. Kesesuaian kaedah ini dikaji untuk penghasilan hibrid *Acacia* (*A. auriculiformis* × *A. mangium*) menggunakan debunga yang disimpan. Kaedah ini diguna untuk pendebungaan silang *A. auriculiformis* betina dewasa dengan *A. mangium* jantan. Pendebungan intraspesies *A. auriculiformis* dijalankan sebagai kawalan. Dalam kaedah ini, debunga dikumpul dengan memasukkan tiub ke dalam jambak bunga jantan dewasa sebaik sahaja proses pembungaan. Tiub tersebut disimpan pada suhu -18 °C selama satu hari, satu bulan ataupun dua bulan. Kadar percambahan *A. mangium* ialah 17% apabila menggunakan debunga yang disimpan selama satu hari. Bagaimanapun, kadarnya meningkat melebihi 40% apabila debunga disimpan selama satu atau dua bulan. Sebaliknya, kadar percambahan debunga *A. auriculiformis* melebihi 40% tanpa mengira tempoh debunga disimpan. Bagi melaksanakan pendebungaan buatan, tiub yang disimpan bersama-sama debunga di dalamnya dimasukkan ke dalam jambak bunga *A. auriculiformis*. Keputusan pendebungaan antara spesies menunjukkan bahawa secara purata, tiga anak benih hibrid diperoleh bagi setiap pendebungaan yang menggunakan debunga yang disimpan selama satu hari. Jumlah anak benih meningkat melebihi lima apabila debunga yang disimpan selama satu atau dua bulan digunakan. Namun, analisis DNA menunjukkan ketidaktulenan kerana terdapat 2.6% anak benih *A. auriculiformis*. Sebaliknya, kurang daripada tiga anak benih diperoleh bagi setiap pendebungaan intraspesies walau lama mana pun debunga disimpan. Keputusan ini mencadangkan bahawa kaedah tiub sesuai diguna untuk menghasilkan hibrid *A. auriculiformis* menggunakan debunga yang disimpan.

INTRODUCTION

Acacia hybrids formed by natural crossing of *Acacia mangium* and *A. auriculiformis* were first discovered in Sabah, Malaysia in the 1970s (Tham 1976, Pedley 1978). Since their morphological and biological traits such as growth rate, stem straightness, wood density and resistance to pests and diseases are equal or superior to those of the respective parental species (Kha 1996), these hybrids have been mass-propagated by rooted cutting. Afforested areas currently occupy greater than 800,000 ha in Vietnam alone. However, because all mother trees originate from open pollination, the parents of specific hybrids cannot be determined.

Inflorescences of *A. mangium* and *A. auriculiformis* are shaped like spikes and are composed of approximately 100 flowers (Sedgley et al. 1992a). Flower buds of *A. mangium* and *A. auriculiformis* change from green to milky white and yellow respectively during anthesis, with flowers on individual spikes beginning to open synchronously around midnight. Pollen grains of *A. mangium* and *A. auriculiformis* are grouped into polyads that consist of 16 pollen grains arranged in a biconvex manner resulting in 1 to 16 pollen tubes extending from a single polyad. The flowers of these two species typically have one pistil surrounded by approximately 100 stamens (Sedgley et al. 1992b).

Artificial pollination using parental species with superior phenotypic characteristics is an effective method for improving the quality of *Acacia* hybrids. However, since the flowering period of each parent may not be well synchronised (Sedgley et al. 1992c, Wickneswari & Norwati 1992), it is often necessary to conduct artificial pollination using the stored pollen of one parent tree. The most successful and convenient method for storage of pollen involves vacuum drying followed by storage at -18 °C (Sedgley & Harbard 1993). These pollen grains successfully produce seeds from the crossing of two *A. auriculiformis* trees by paintbrush pollination method, suggesting that it is possible to establish desired hybrids by artificial pollination using stored pollen. There are a few limitations of this method, however, as not all pollen grains can be used for pollination because many of them do not adhere to the paintbrush. In addition, artificial pollination must be performed efficiently within a restricted time period because pollination only occurs around 9 a.m. (Ogawa et al. 2008).

Another method for artificial pollination of *Acacia* is the tube method. In this method, a polyvinyl chloride tube is inserted into a flowering spike to collect pollen grains (Sedgley et al. 1992a, Ogawa et al. 2008). It was reported that when a tube containing collected pollen was covered with medicine paper and stored at -18 °C in polyethylene bag containing silica gel, pollen germination rate was high even after two months (Yamaguchi & Ogawa 2009). Therefore, pollen grains stored in tubes and used for artificial pollination without modification represent a simple approach for the establishment of hybrids.

To determine whether this method is suitable to produce *Acacia* hybrids, pollen was collected from flowering spikes of *A. mangium* and *A. auriculiformis* using a polyvinyl chloride tube and immediately stored at -18 °C. Germination rates were investigated one day, one month and two months after collection. Artificial pollination was conducted by inserting tubes into flowering *A. auriculiformis* spikes and, subsequently, the percentage of spikes containing mature seeds, number of mature seeds per spike, seed germination rate and seedling survival rate were assessed. To detect the percentage of self-compatibility, the DNA of seedlings harvested by interspecific pollination was analysed. Finally, the number of hybrid seedlings obtained per spike was compared between intraspecific and interspecific pollination. Results of this study will be useful to improve the efficiency of producing artificially pollinated *Acacia* hybrids.

MATERIALS AND METHODS

Study sites and sample trees

The study was conducted at the Iriomote Tropical Forest Tree Breeding Technical Garden, Forest Tree Breeding Center, Forestry and Forest Products Research Institute, Okinawa Prefecture, south-western Japan from August 2008 till March 2010. Four *A. auriculiformis* trees were used as female parents, while three *A. mangium* and two *A. auriculiformis* trees were used as male parents. Trees used as female parents were planted in a mesh house to limit the possibility of unwanted pollination. All trees were raised from seeds bought from the Commonwealth Scientific and Industrial Research Organisation (CSIRO). Two of the four female *A. auriculiformis* trees were

collected from same seedlot while the other, from different seedlots.

Pollen collection

Pollen was collected from the male *A. mangium* and *A. auriculiformis* trees using a polyvinyl tube (diameter 0.5 cm, length 7 cm) which was inserted into spikes when nearly all flowers on a spike had bloomed (Figure 1). To attach numerous pollen grains in the tube, the tube was moved up and down in the spikes from five to ten times. A total of 241 and 258 tubes were used for collecting pollen from *A. mangium* and *A. auriculiformis* trees from 1 August 2008 till 8 January 2009 respectively. Following pollen collection, tubes were wrapped in medicine paper and stored at -18 °C in a polyethylene bag containing silica gel until subjected to germination tests or used for artificial pollination.

Pollen germination tests

Germination tests were conducted one day, one month and two months from the time of pollen collection as described by Yamaguchi and Ogawa (2009). Pollen was removed from the stored tubes using a brush and was then dropped on the surface of a medium consisting of 20% sucrose and 1% agar in a petri dish. Following incubation at 23 °C for 48 hours, polyads comprising 16 pollen grains were examined under a microscope. Polyads with at least one extended pollen tube were considered germinated. The number of spikes used for germination ranged from 3 to 35 for each storage period. Mean germination rates of *A. mangium* and *A. auriculiformis* trees were calculated for each investigated storage period.

Figure 1 Pollen collection by inserting a tube into a spike

Pollination

The following four mating schemes were evaluated:

(1) open pollination: non-treated *A. auriculiformis* spike (open pollination or self-pollination)
(2) tube method with no pollination (self-pollination or open pollination): a tube with no pollen was inserted into an *A. auriculiformis* spike
(3) tube method with intraspecific pollination (intraspecific pollination, self-pollination or open pollination): a tube containing *A. auriculiformis* pollen was inserted into an *A. auriculiformis* spike
(4) tube method with interspecific pollination (interspecific pollination, self-pollination, or open pollination): a tube containing *A. mangium* pollen was inserted into an *A. auriculiformis* spike

For intraspecific and interspecific pollination, immediately after nearly all flowers on a spike of a female parent tree had bloomed, a tube containing pollen that had been stored at -18 °C for either one day, one month or two months was inserted into the spike. To attach as many polyads as possible in the tube to the stigma, the tube was gently moved up and down from five to ten times. Pollination was conducted from 6 October 2008 till 9 January 2009. The number of spikes used for pollination in each cross combination ranged from 0 to 68, which corresponded to the relationship between female flowering period and length of pollen storage (Table 1). Due to the difference in the number of flowers on each tree and the time lag of the flowering period among sample trees, some trees (e.g. A1) have no 30 or 60 days intraspecific and interspecific pollination. Although the design of the intraspecific pollination was very unbalanced, these data were sufficient to compare differences between intraspecific and interspecific pollination. All sample spikes were marked using coloured bin-liners for identification of the mating scheme.

Seed collection and propagation

Approximately six months after mating, mature seeds were collected from harvested mature pods. The number of spikes containing mature seeds and the total number of mature seeds were counted for each cross combination. After

Table 1 Number of spikes used for each pollination

Female trees of *Acacia auriculiformis*	Mating scheme							
	1	2	3			4		
			Storage of *Acacia auriculiformis* pollen (days)			Storage of *Acacia mangium* pollen (days)		
			1	30	60	1	30	60
A 1	290	20	35			27	39	
A 2		20	35		19	16	35	16
A 3		15	13		6	15	19	15
A 4	104	15	68	30			33	11
Total	394	70	151	30	20	58	126	42

Mating scheme: 1 = open pollination, 2 = tube method with no pollination, 3 = tube method with intraspecific pollination, 4 = tube method with interspecific pollination

excluding immature seeds, and due to the limited number of seed beds, approximately 25% of mature seeds were sowed in 15 boxes (50 × 30 cm) filled with fertile soil and germination rates of the seeds were calculated. Immediately after germination, the seedlings were transplanted to each pod. The survival rate of seedlings of each pod was then determined six months after germination. In addition, a phyllode from each seedling of interspecific pollination was collected for DNA analysis.

DNA analysis

The phyllodes collected from seedlings produced by interspecific pollination were desiccated at 100 °C for 24 hours. DNA was extracted from the treated phyllodes using cetyltrimethylammonium bromide (CTAB) method. The DNA was then used as a template for polymerase chain reactions (PCRs) designed to amplify the sequence characterised amplified region (SCAR) marker (Huang et al. 2005). The amplified PCR products were analysed by electrophoresis in 1.3% agarose gels containing SYBR Safe stain. From the visualised DNA bands, the percentage of hybrid seedlings generated from each cross combination was calculated.

Statistical analysis

Germination rates of pollen per spike for each pollen storage period, and mean percentage of spikes containing mature seeds, mean number of mature seeds harvested per spike, mean germination rate of harvested seeds, mean survival of seedlings six months after germination, and mean number of hybrid seedlings acquired per spike per cross combination were calculated for each pollination condition divided by the number of pollen storage days in each mating scheme. The rates, percentages and numbers for each cross combination were compared between tree species or mating schemes using the t-test. All statistical analyses were performed using STATISTICA version 6 (2009).

RESULTS

Mean germination rates of pollen grains following storage at -18 °C for one day were 17.8% for *A. mangium* and 60.8% for *A. auriculiformis* (Figure 2). However, after one month of storage, the mean germination rate increased significantly to 52.3% for *A. mangium* ($t = 7.1$, $p < 0.001$) but decreased for *A. auriculiformis*, falling to 38.3% ($t = 3.4$, $p < 0.01$). After two months of storage, the mean germination rates of pollen were nearly 40% for both species.

No mature seeds were formed using open and no-pollination mating schemes, whereas a considerable number of mature seeds were harvested from pods by both intraspecific and interspecific pollination (Table 2). For intraspecific pollination, the mean percentage of spikes containing mature seeds was 76.7% when pollen stored for one month was used compared with only 36.7 and 32.2% when using pollen stored for one day and two months respectively (Figure 3). For interspecific pollination, the mean

percentage of spikes containing mature seeds was 41.4% when crosses were performed with pollen stored for one day. However, the value increased to more than 80% for pollen stored for either one or two months ($t = 2.2$ and 2.9 respectively, $p < 0.10$). Notably, the value for pollen stored for two months was significantly higher in interspecific than intraspecific pollination ($t = 4.3$, $p < 0.05$).

A total of 753 and 3750 mature seeds were harvested from pods subjected to intraspecific and interspecific pollination respectively (Table 2). For intraspecific pollination, a mean number of 19 mature seeds were harvested per spike when pollen stored for one day was used for mating but decreased to 6.8 and 5.1 for pollen stored for one and two months respectively, although this difference was not significant ($t = 0.5$ and 0.9 respectively, $p > 0.05$) (Figure 4). The mean number of harvested mature seeds per spike was approximately 20 for pollen stored for one day and one month in interspecific pollination, but increased to 27.4 for pollen stored for two months. This value was significantly higher than that of intraspecific pollination for pollen stored for one month ($t = 3.1$, $p < 0.01$).

For intraspecific pollination, the mean germination rate of harvested seeds exceeded 80% regardless of the duration of pollen storage (Figure 5). Although the germination rate of harvested seeds in interspecific pollination was also higher than 80% for pollen stored for one day, the rate gradually decreased with increasing length of storage although not significant ($r = -0.09$, $p > 0.05$). For intraspecific pollination, the survival of seedlings six months after germination was greater than 50% for pollen stored for one day and 32% for one month (Figure 6). A similar seedling survival rate was observed for interspecific pollination, with rates of 47 and 51% for pollen stored for one day and one month respectively, and a slightly decreased rate of 37% for pollen stored for two months.

Phyllodes used for DNA analysis were collected from 160 surviving seedlings that were generated following pollination by interspecific pollination. The PCR analysis based on the SCAR marker

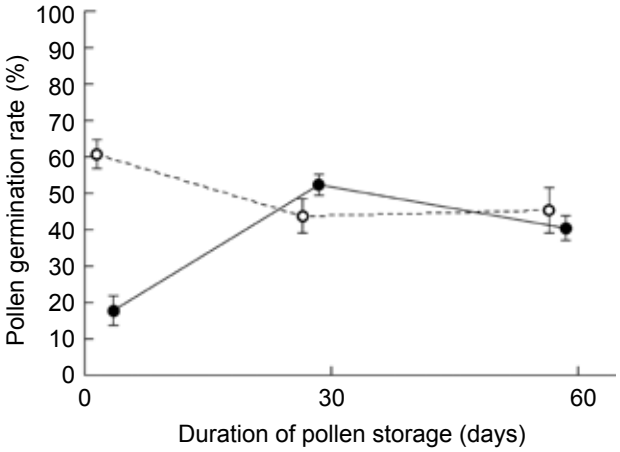

Figure 2 Mean germination rate of pollen following different storage days in *A. mangium* (●) and *A. auriculiformis* (o); error bar indicates ± SE

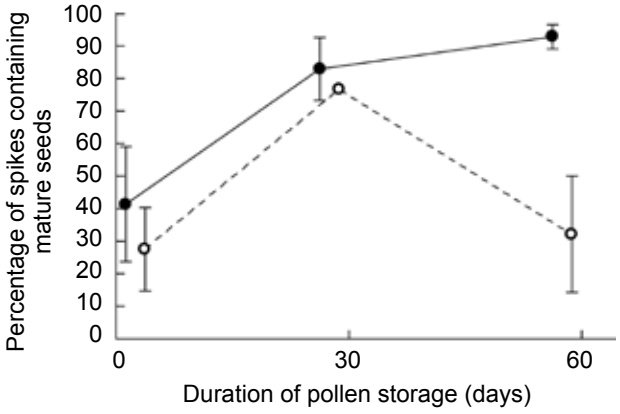

Figure 3 Mean percentage of spikes containing mature seeds per cross combination in each pollination condition versus the length of pollen storage for intraspecific (○) and interspecific (●) pollination; error bar indicates mean ± SE

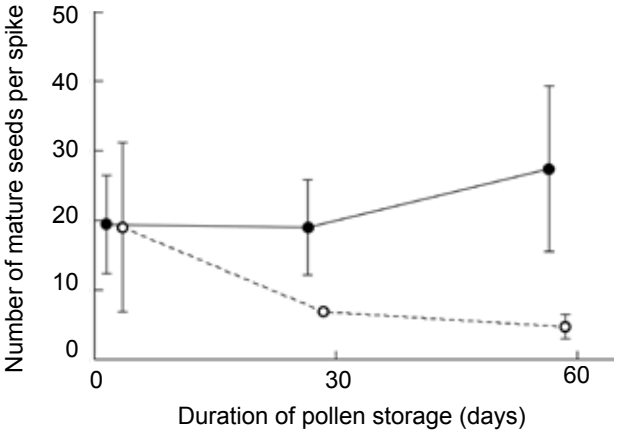

Figure 4 Mean number of mature seeds per spike per cross combination in each pollination condition versus the length of pollen storage for intraspecific (○) and interspecific (●) pollination; error bar indicates ± SE

Table 2 Number of harvested seeds for each pollination

Female tree of *A. auriculiformis*	Mating scheme							
	1	2	3			4		
			Storage of *A. auriculiformis* pollen (days)			Storage of *A. mangium* pollen (days)		
			1	30	60	1	30	60
A 1	0	0	173			15	313	
A 2		0	102		49	255	1277	714
A 3		0	0		9	188	100	273
A 4	0	0	263	157			485	130
Total	0	0	538	157	58	458	2175	1117

Mating scheme: 1 = open pollination, 2 = tube method with no pollination, 3 = tube method with intraspecific pollination, 4 = tube method with interspecific pollination

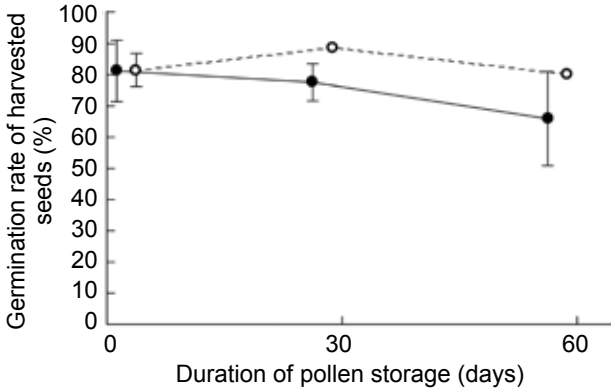

Figure 5 Mean germination rate of harvested seeds per cross combination in each pollination condition versus the storage days of pollens on intraspecific (○) and interspecific (●) pollination; error bar indicates ± SE

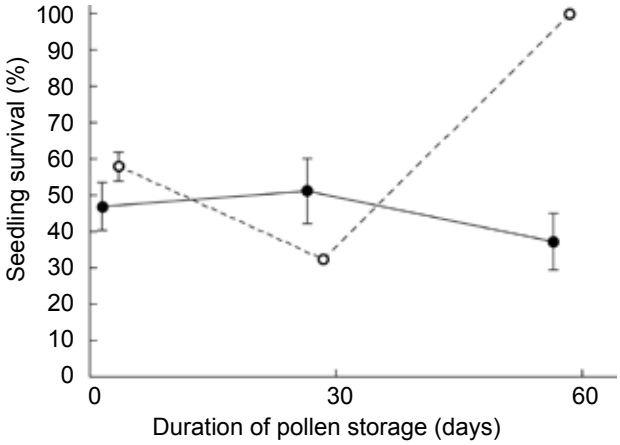

Figure 6 Mean survival of seedlings after six months from germination per cross combination in each pollination condition versus the length of pollen storage for intraspecific (○) and interspecific (●) pollination; error bar indicates ± SE

revealed that 158 (98.7%) of these seedlings were hybrids (Table 3). The two seedlings detected as non-hybrid *A. auriculiformis*, which were obtained using pollen stored for one month, were harvested from the identical female parent tree.

Finally, for both intraspecific and interspecific pollination, the number of seedlings or hybrid seedlings acquired per spike six months after germination was less than five when mating was performed using pollen stored for one day (Figure 7). However, with longer storage, the seedlings showed opposite trends, i.e. for interspecific pollination, the number of hybrid seedlings increased with increasing length of pollen storage while for intraspecific pollination, the number decreased. The value for pollen stored for one month was higher in interspecific than intraspecific pollination ($t = 2.0$, $p = 0.051$).

DISCUSSION

In our study, we demonstrated that hybrid seeds of *Acacia* could be harvested by artificial pollination using a tube method, even if the

Table 3 Proportion of seedlings (%) recognised as hybrids by DNA analysis for each cross combination

Female tree of *A. auriculiformis*	Duration of pollen storage (days)		
	1	30	60
A 1	100 (3)	100 (17)	
A 2	100 (12)	95.8 (48)	100 (20)
A 3	100 (4)	100 (16)	100 (18)
A 4		100 (19)	100 (3)
Total	100 (19)	98.0 (100)	100 (41)

Number of samples is written in parenthesis

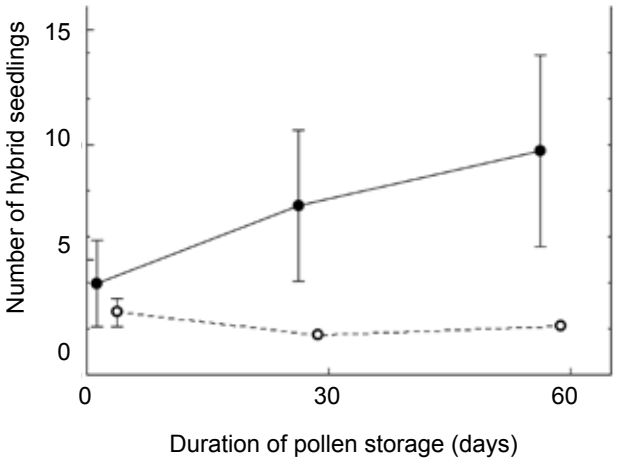

Figure 7 Mean number of hybrid seedlings acquired per spike per cross combination at age six months in each pollination condition versus the length of pollen storage for intraspecific (○) and interspecific (●) pollination; error bar indicates ± SE

pollen used for pollination was stored at -18 °C for two months. However, several points must be considered when using this method. First, the germination rate of *A. mangium* pollen that was stored for one day was lower than that of *A. mangium* and *A. auriculiformis* pollen stored for one or two months (Figure 2). A low pollen germination rate immediately after collection was also reported by Sedgley et al. (1992a), suggesting that the presence of immature pollen grains after dehiscence might be a general trait of *A. mangium*. Second, as artificial pollination using *A. mangium* pollen stored for one day also resulted in a low percentage of spikes containing seeds (Figure 3), it appeared that pollen germination rate was linked to the development of mature seeds in the spikes. This proves the importance of using pollen with a high germination rate during artificial pollination for maintaining a high percentage of mature seeds.

The mesh house used in this study to cultivate female trees effectively prevented natural pollination as no spikes containing seeds were obtained from the untreated trees (self-pollination). In addition, no spikes containing mature seeds were found in the no-pollination study, suggesting that in the mesh house self-pollination rarely occurred on spikes of *A. auriculiformis*. However, 2.6% of harvested mature seeds obtained from interspecific pollination were recognised as *A. auriculiformis* (Table 3). This finding was supported by Sedgley et al. (1992a) who also reported that 4.1% seedlings were not hybrids after interspecific artificial pollination on *A. auriculiformis* trees using the paintbrush method. Taken together, these results suggest that self-pollination occasionally occurs in *A. auriculiformis* trees, the frequency of which may differ with the pollination method or existence of pollen from other *Acacia* species. Therefore, if only hybrid seeds are harvested by interspecific pollination, manipulation of emasculating spikes must be performed (Sedgley et al. 1992a, Otsuka et al. 2010).

Although the percentage of spikes containing mature seeds in the case of using pollen stored for one day or one month was consistent between mating for intraspecific and interspecific pollination, the percentage resulting from pollen stored for two months in the latter showed low level compared with that for interspecific pollination (Figure 3). Similarly, the number of harvested seeds per spike was also considerably lower for intraspecific pollination using pollen stored for longer than one day (Figure 4), suggesting that the tube method might be more appropriate for *A. mangium* pollen than for *A. auriculiformis*. It was reported that no mature

seeds were harvested on 9 of 13 *A. auriculiformis* trees produced using paintbrush pollination method (Sedgley et al. 1992b), whereas in the present study, mature seeds were harvested from all *A. auriculiformis* trees. It was clear that this method depended on the skill of the technician because pollen must be collected with the brush and transferred to the stigma without fail. On the other hand, the tube method required no skill and assured a higher level of success. Therefore, the tube method may be more appropriate for harvesting hybrid seeds from *Acacia* than paintbrush pollination method.

This study also showed that for interspecific pollination, the percentage of seed germination and the survival of seedlings decreased with increased length of pollen storage (Figures 5 and 6). This suggested that although the number of hybrid seedlings acquired per spike increased with increasing pollen storage (Figure 7), the optimal balance might occur at a later stage of storage. Hence, we are currently examining the number of hybrid seedlings acquired per spike six months after germination following artificial pollination performed using *A. mangium* and *A. auriculiformis* pollens stored for longer than two months.

In conclusion, the tube method represents a simple approach for the establishment of *Acacia* hybrids using stored pollen and thus focuses on the useful implications of this technique for future *Acacia* research.

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