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Breeding Polyploid Varieties of Acacia: Reproductive and Early Growth Characteristics of the Allotetraploid Hybrid (*Acacia mangium* × *A. auriculiformis*) in Comparison with Diploid Progenitors

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Abstract: Diploid clones of the hybrid acacia (*Acacia mangium* × *A. auriculiformis*) are widely planted in Vietnam because of their high productivity, adaptability, and commercial wood yields. Polyploid breeding offers possibilities for further enhancing hybrid vigor and generating new high value genotypes. In a field trial, we compared three diploid hybrid clones with their respective colchicine-induced tetraploid lines. Flowering and seed production of each cytotype were observed and open pollinated seed collected for determination of outcrossing rate and ploidy, inbreeding depression and marker inheritance in the progeny. Comparisons are also made with published characteristics of autotetraploids derived from *A. mangium*. Compared with their corresponding diploid cytotypes, the allotetraploids flowered slightly later but more intensely; produced the same number of seeds per pod but larger seeds; and showed a greatly reduced level of outcrossing (an average of 14% compared with 87%). Inbreeding depression for height growth was less for progeny from the allotetraploid lines (17%) than for those from the original diploids (33%). 96% of seeds from the allotetraploid clones were also tetraploid, but we observed triploids at low frequency at both the seed and field progeny stages. The segregation of the molecular markers in outcrossed allotetraploid progenies demonstrated both disomic and tetrasomic inheritance, indicating that the hybrid behaves as a segmental allotetraploid. Results suggest that an open pollinated breeding strategy is a practical option for improving polyploid acacia hybrids.

Keywords: polyploid breeding; acacia hybrid; inbreeding depression; outcrossing; segmental allotetraploid; disomic inheritance; tetrasomic inheritance

1. Introduction

Acacia mangium Willd., *A. auriculiformis* A. Cunn. ex Benth., and their natural interspecific hybrid (referred to subsequently as *A.* hybrid) are important for plantation forestry in SE Asia because of their high growth rate, wood properties suitable for both pulp and furniture production and wide environmental adaptability [1,2]. Over 2 million ha have been planted in Indonesia, Malaysia, and Vietnam [1]. Acacia hybrid can be propagated as clones, while the parental species are grown from open pollinated seed [1]. In Vietnam, approximately 0.5 M ha of *A.* hybrid had been established by 2014, a similar area to that of *A. mangium* at that time [3]. Breeding and clonal development programs for diploid *Acacia mangium*, *A. auriculiformis*, and *A.* hybrid were established in Vietnam in the 1990s and research into the potential for developing polyploid varieties commenced in 2003 [4], with

the long-term aim of producing triploid (3*x*) genotypes that would be highly productive, effectively sterile [5] (and therefore non-weedy), and with the potential for expression of novel traits [6] such as increased fiber dimensions and modified pulp properties [7]. The initial focus was on the production of autotetraploid (4*x*) *A. mangium* for mating with diploid (2*x*) selections [4], but given the commercial success of the diploid hybrid we also decided to explore the potential for producing and breeding from 4*x* cytotypes of selected F_1 *A.* hybrid clones [8].

Colchicine-induced 4*x* lines (or allotetraploid lines) from three diploid *A.* hybrid clones were produced in Australia [4,9] and transferred to Vietnam for propagation. The clones were all confirmed to be true F_1 hybrids based on their allelic profiles for species-diagnostic SSR markers [10]. These allotetraploid lines were field planted together with their diploid precursors at Bau Bang in southern Vietnam, for performance assessment and production of open pollinated seeds.

The design of an efficient, advanced generation breeding strategy is contingent on the understanding of many biological and genetic attributes. Since controlled pollination is technically difficult and expensive in Acacia, it is particularly important to determine whether an open pollinated breeding strategy is feasible. In this paper, we report observations of flowering, open pollinated seed production, and germination; mating system parameters of the two *A.* hybrid cytotypes together with ploidy of their progeny; and early survival, growth, and inbreeding depression of selfed compared with outcrossed progenies. We were also able to compare the attributes of this allopolyploid *A.* hybrid with earlier reports of autotetraploid *A. mangium* [11–13].

Since this F_1 hybrid does produce some seed, there is clearly a degree of chromosomal homology between the parental species; however, we have no detailed knowledge of the cytogenetics, making it difficult to make predictions regarding inheritance of economically important traits. Our study contributes to such understanding, by comparing observed segregation of SSR genotypes in F_2 outcrossed progeny from the tetraploid lines with expectations under tetrasomic and disomic inheritance models.

2. Materials and Methods

2.1. Germplasm and Field Trial Design

The clonal field trial planted in July 2011 at Bau Bang (Table 1) included three 2*x* clones and twelve 4*x* lines (3–5 lines derived from each 2*x* genotype). The three 2*x* clones (BV10, BV16 and BV33) were originally selected from open-pollinated hybrid progeny from a stand of *A. mangium* (Mossman, Queensland natural provenance) growing adjacent to a stand of *A. auriculiformis* (Darwin, Northern Territory, Australia provenance) in northern Vietnam. These were subsequently developed as production clones [2], and their hybrid status was confirmed using SNP markers [10].

The trial was laid out in six replicates, each containing three main plots split into two sub-plots of 16 2*x* and 16 4*x* ramets, giving a total of 576 trees. Each 4*x* sub-plot included ramets from all lines derived from its respective diploid progenitor. The spacing between rows was 3 m and the initial spacing between trees within rows was 2 m. The trial was selectively thinned at age 2 years, with about half of the trees removed to promote canopy development and flowering; the equal ratio of 2*x* and 4*x* ramets was maintained. The trial was surrounded by *A. auriculiformis* and *A.* hybrid plantations of flowering age.

Table 1. Total number of ramets of diploid (2*x*) and allotetraploid (4*x*) acacia hybrid of each genotype observed and used as parents in the main study in Vietnam.

Genotype/Line	Ploidy	Number of Ramets Assessed in Flowering Survey	Number of Ramets that Produced Seed	Number of Ramets Where Seed Was Used in This Study
BV33	2*x*	54	6	2
BV33L724	4*x*	16	11	1
BV33L695	4*x*	19	9	1
BV33L684	4*x*	4	2	1
BV33L667	4*x*	8	2	1
BV33L621	4*x*	6	2	1
	Total 4*x*	53	26	
BV16	2*x*	41	7	2
BV16L437	4*x*	20	9	2
BV16L440	4*x*	24	7	2
BV16L438	4*x*	6	1	1
	Total 4*x*	50	24	
BV10	2*x*	57	2	2
BV10L583	4*x*	21	2	1
BV10L590	4*x*	16	2	1
BV10L562	4*x*	19	2	2
BV10L575	4*x*	12	0	0
	Total 4*x*	68	6	
Total		323	62	20

2.2. Phenology Study

In order to assess the potential for open-pollinated crossing among the different clones and cytotypes, the flowering of all remaining trees in the trial was scored every two weeks from September 2015 to February 2016 (Table 1). The number of ramets per genotype that flowered was counted to estimate the percentage of the flowering trees per genotype and per cytotype. This data was aggregated to give the cumulative number of ramets that flowered every month for each genotype/cytotype combination. The flowering intensity of each tree was scored visually using the following categories: 0—no flowering, 1—up to 1/3 of the crown bearing opened flowers, 2—from 1/3 to 2/3 of the crown bearing opened flowers, and 3—more than 2/3 of the crown bearing opened flowers. To obtain the monthly flowering intensity of each genotype/cytotype combination, the scores of all ramets, for a particular month, were summed and divided by the total number of ramets and then divided by 3 (the highest intensity) and expressed as the average percentage of flowering intensity [14].

2.3. Seed Collection, Seed Parameters and Germination

In March 2017, twenty mature pods were collected from two ramets of each 2*x* clone and from one or two ramets of all 4*x* lines except for BV10L575, which did not produce seed (Table 1). Each pod was stored in a separate plastic bag and dried at room temperature until they dehisced and shed their seed.

The total number of normal (fully developed and filled) and abnormal (underdeveloped or empty, unfilled, or with wrinkled appearance) seed, as categorized by Nghiem et al. [13], was recorded for each pod. Normal seeds from each pod were also weighed to estimate mean seed weight. These were then nicked, and those from each pod germinated in separate petri dishes. Nicking the seed coat to permit rapid hydration is accepted as best practice for ensuring uniform germination of research quantities of seed of tropical acacias [13]. The number of germinated seeds was recorded to estimate the germination percentage. Germinated seeds were transplanted to pots containing a mixture of soil, sand and compost. Seedlings from each mother tree were grown together in individual pots

placed within a single tray, and trays were randomized in the nursery in Hanoi, Vietnam, under light shade giving approximately 25% light reduction.

2.4. Ploidy Determination by Flow Cytometry

To assess the range of cytotypes that occurred in the open pollinated seed crop, we analyzed samples of the first seed produced in 2014. Mature pods were collected from one ramet of each of the three diploid F_1 hybrid clones and from three of the 4*x* lines derived from each clone and shipped to Australia. Seed was extracted from eight individual pods per sample and seed weights and colour recorded before nicking with a sharp razor blade for germination in 9 cm Petri dishes lined with moist filter paper with seed identity retained. After four days' germination at 23 °C, the seeds were scored as either imbibed (non-viable), germinated (viable), or empty. Experience has shown that if its radicle has not emerged after four days, a seed is unlikely to develop into a viable seedling. Ploidy of each seed was determined with a CyFlow Ploidy Analyzer using modified woody plant buffer [15] and DAPI. After removing its coat, each seed was co-chopped with 1 cm^2 of leaf from the internal reference standard *Pisum sativum* var. Torstag. A phyllode of diploid and allotetraploid hybrid and a previously determined triploid *A. auriculiformis* clone were used to mark the 2*x*, 3*x*, and 4*x* channels for every run. The ploidy of the seed sample was inferred from the ratio of the sample peak channel to the internal reference standard pea channel.

To investigate whether viable triploids might survive through into field planting, additional observations were made on seedling progeny from the allotetraploid lines, raised in Hanoi, which were established in the field trial at Ba Vi (see below). At age three years, foliage samples were collected from fifteen progenies from each of two 4*x* lines derived from each of the 2*x* clones and assessed using the above cytotype determination methodology.

2.5. Microsatellite Analysis of F_2 Progeny

Twenty progenies per ramet (each seedling derived from a different pod) were genotyped with SSR markers to estimate outcrossing rates. Phyllodes from a total of 400 seedlings derived from the three 2*x* clones (BV10, BV16, BV33) and eleven induced 4*x* lines were collected at the nursery, dried with silica gel (or overnight in an oven at 50 °C), and stored at room temperature before DNA isolation. DNA was extracted using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), where 20 mg of dried phyllode tissue was crushed to a powder in liquid nitrogen and then the standard Qiagen protocol was used for DNA extraction (www.qiagen.com/handbooks, Accessed on 12 October 2014). DNA concentration and purity were assessed using gel electrophoresis, and comparison with Lambda HindIII molecular weight standard.

Microsatellite analysis was conducted as described in Le et al. [16] using 15 of the 16 markers developed to discriminate hybrids from pure *A. mangium* and *A. auriculiformis* (including 5 species diagnostic markers and 10 highly polymorphic markers). The marker AH3_17 was dropped due to poor amplification. PCRs were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) with a final volume of 12.5 µL, consisting of 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each forward and reverse primer, 0.5U Taq DNA polymerase (Invitrogen, MA, USA) and 20 ng of genomic DNA. The 15 pairs of SSR primers were multiplexed in four mixes with the annealing temperature (Tm) ranging from 50 °C to 60 °C depending on marker [16]. The SSR primers were tagged with fluorescent dyes NED, 6-FAM, HEX, or ROX on their forward primers and PCR was conducted for all 400 samples. PCR products were separated using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) by the Australian Genome Research Facility (http://www.agrf.org.au, Melbourne, VIC 3000, Australia). Raw data were analyzed using GeneMapper 3.7 (Applied Biosystems, Foster City, CA, USA) and Geneious R10 (http://www.geneious.com, Accessed on 8 June 2015) software to score genotypes.

2.6. Inheritance of SSR Markers in 4x Acacia Hybrid Progenies

Allotetraploid F_1 hybrids between species with non-homologous chromosomes are expected to show disomic inheritance where the two homologous chromosome sets (e.g., AA and BB) only recombine with their strict homolog [17]. In this case, only one gamete (AB) is produced, and when this gamete is fertilized with an outcross gamete (e.g., CD), there is only one progeny genotype that can be produced (ABCD). Note that in this case, selfing can only produce individuals with the AABB genotype (i.e., no homozygous individuals are produced), which cannot be distinguished from the diploid genotype AB using SSR markers. Deviations from disomic inheritance expectations, such as tetrasomic inheritance, can provide evidence of partial homology between parental genomes resulting in chromosome instability in later generations and following recombination [18].

In the tetrasomic model, four homologues pair at metaphase. In this case, the genotype AABB produces three different diploid gamete genotypes with the allelic composition AA, AB, and BB at a ratio of 1:4:1, respectively. When crossing with a different genotype (e.g., CCDD) nine progeny genotypes (AACC, AACD, AADD, ABCC, ABCD, ABDD, BBCC, BBCD, and BBDD) corresponding to nine progeny phenotypes (A_C_, A_CD, A_D_, ABC_, ABCD, ABD_, B_C_, B_CD, and B_D_) will be produced with a final ratio of 1:4:1:4:16:4:1:4:1, respectively. If the parents share one allele in common, five phenotypes will be produced (A_ _ _, A_ _ C, A_B_, AB_C and B_C_) with the ratio of 1:5:5:24:1. When the parents have the same genotype or in cases of selfing, five genotypes (AAAA, AAAB, AABB, ABBB, and BBBB) will be produced and if gene copy number cannot be ascertained (e.g., as with SSRs) these will be detected as three phenotypes (A_ _ _, A_ _B and B_ _ _) with the ratio of 1:34:1, respectively. To evaluate the segregation in $4x$ F_2, we compared the observed SSR genotypes of 42 outcrossed (see below) progenies with the expected frequencies predicted based on tetrasomic and disomic inheritance models.

2.7. Outcrossing Rate Estimation

The outcrossing rate for seed from each maternal genotype was estimated using SSR data and simple exclusion principles (seedlings with non-maternal alleles at two or more loci were declared as outcross) as well as using maximum likelihood method developed for diploids, MLTR [19], and for allotetraploids, MLTET [20].

2.8. Field Trial of F_2 Progeny

A field trial was established at Ba Vi, Hanoi province, northern Vietnam, in 2016 to compare the growth of the identified sets of self and outcross progenies produced from open pollination among the diploid and allotetraploid parent trees. 290 of the genotyped F_2 seedlings survived to the pre-planting stage. These included 214 $4x$ F_2 (195 selfed and 19 outcrossed from a total of 10 lines) and 76 $2x$ F_2 (33 selfed and 43 outcrossed). The trial was laid out in a randomized complete block design with 3 replicates. Each plot, representing a genotype-by-ploidy/line combination, contained up to ten seedlings. Where fewer than ten genotyped seedlings were available, the plots were completed using un-genotyped seedlings from the respective mother tree. All seedlings in the trial were assessed for survival and tree height 12 months after planting, but only data from genotyped seedlings were analyzed.

2.9. Statistical Analysis

One-way ANOVA was used to compare different genotypes and ploidy levels for seed parameters and germination. These analyses were performed using ANOVA procedures in the R programming language (https://www.r-project.org/, Accessed on 5 August 2014). To provide adequate sample sizes, data from all lines/clones within each cytotype were pooled in order to test for main cytotype effects.

We used contingency chi-square tests based on numbers of outcrossed and selfed offspring to compare the outcrossing rates of the different ploidies, and of the different clones within each ploidy level. Contingency chi-square tests were also used to compare

the field survival of outcrossed and selfed progenies at each ploidy level. T-tests were used to compare the height of outcrossed and selfed progenies at each ploidy level. Inbreeding depression for average height at 12 months after planting was estimated as:

$$ID\ (\%) = ((\bar{}X\ out - \bar{}X\ self))/(\bar{}X\ out) \times 100$$

where, $\bar{}X$ out and $\bar{}X$ self are the mean height of seedlings originating from outcrossing and selfing of each cytotype, respectively.

3. Results

3.1. Flowering Assessments of Diploid and Allotetraploid Acacia Hybrids

Flowering occurred from late September and finished in early March. The proportion of ramets that flowered varied between genotypes and ploidy level. In the diploid (2*x*), only two out of 57 BV10 ramets (4%) flowered during the 2015–2016 season at age 4–5, whereas 41% of BV16 and 39% of BV33 ramets flowered (Tables 1 and 2). The limited flowering of BV10 was also found in allotetraploid (4*x*) trees, where it had a lower proportion of flowering ramets (up to 26%) than 4*x* BV16 (81%) and 4*x* BV33 (63%). On average, the proportion of flowering individuals in allotetraploid lines (56%) was higher than that in their diploid cytotypes (26%).

Table 2. The percentage of ramets flowering in the Bau Bang trial each month for the three diploid clones and their combined derived tetraploid lines.

Genotype	Ploidy	Number of Ramets	September	October	November	December	January	February	March
BV33	2*x*	54	4	18	39	21	6	0	0
	4*x*	53	0	0	4	25	63	15	1
BV16	2*x*	41	0	12	41	29	7	0	0
	4*x*	50	0	0	15	44	81	22	3
BV10	2*x*	57	0	0	2	4	2	0	0
	4*x*	68	0	0	0	9	26	9	1
Mean	2*x*	152	1	13	27	17	5	0	0
	4*x*	171	0	0	6	26	56	15	2

There were variations in flowering intensity between genotypes and ploidy levels (Table 3). For 2*x*, flowering intensity was highest in November (with 26% in BV33 and 28% in BV16) then declined in December to 14% and 20%, respectively. For 4*x*, the flowering intensity was highest in January (44% in 4*x* BV33 and 49% in 4*x* BV16). An overlap of flowering between ploidy levels occurred from early November to late December with a peak of flowering overlap from middle to late December (Tables 2 and 3), showing the potential for open pollination among clones and also between 2*x* and 4*x* cytotypes in the trial.

Table 3. Monthly flowering intensity [a] of diploid (2*x*) and tetraploid (4*x*) *A.* hybrid genotypes in the Bau Bang field trial.

Genotype	Ploidy	September	October	November	December	January	February	March
BV33	2*x*	2	11	26	14	4	0	0
	4*x*	0	0	3	21	44	12	1
BV16	2*x*	0	8	28	20	5	0	0
	4*x*	0	0	7	22	49	11	2
BV10	2*x*	0	0	1	2	1	0	0
	4*x*	0	0	0	6	17	6	1
Mean	2*x*	1	9	18	11	3	0	0
	4*x*	0	0	3	16	37	10	1

([a] value expressed as percentage of mean monthly observation for all ramets: <33.3%: little flowering, 33.3–66.6%: moderate flowering and >66.6%: heavy flowering).

3.2. Seed Parameters and Germination

There were significant differences in the number of seeds per pod within cytotypes with a significant interaction between genotype and ploidy. The number of abnormal seeds per pod varied at all levels, being higher in 4*x* lines than 2*x*, but the majority of seeds were normal. Across all 4*x* lines, there was an average of one abnormal per pod compared with 5.6 normal seeds (Table 4).

Table 4. Seed parameters and germination from open-pollinated progenies of diploid (2*x*) and tetraploid (4*x*) *A.* hybrid genotypes (2017 collection).

Genotype	Ploidy	Number of Seed Per Pod	Normal Seed/Pod [a]	Abnormal Seed/Pod [b]	Seed Weight (mg) [c]	Germination (%)
BV10	2*x*	3.6	2.9	0.7	14.2	80.0
	4*x*	6.7	5.6	1.1	16.5	82.0
BV16	2*x*	7.0	6.5	0.5	16.3	93.7
	4*x*	7.0	6.0	1.0	16.8	85.4
BV33	2*x*	7.5	7.0	0.5	15.4	94.0
	4*x*	6.1	5.2	0.9	19.4	85.0
Overall	***2x***	***6.0***	***5.5***	***0.6***	***15.2***	***89.2***
	4x	***6.6***	***5.6***	***1.0***	***18.5***	***84.1***
S.e.d means		0.08	0.08	0.04	0.13	
			Significance of differences			
Genotype (G)		***	***	***	***	**
Ploidy (P)		Ns	ns	***	***	***
G X P		***	***	***	***	*

([a] = number of normal seeds per pod, [b] = number of abnormal seeds per pod, [c] = calculated for normal seeds only. Asterisks indicate statistical significance * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Genotype, ploidy, and the interaction between genotype and ploidy all had a significant effect on seed weight. 4*x* of each genotype produced larger seeds compared to each of their 2*x* progenitors. 4*x* BV33 produced the largest seed (19.4 mg), while the smallest seeds were produced by 2*x* BV10 (14.2 mg). Genotype, ploidy, and the interaction between genotype and ploidy had small but significant effects on seed germination. The percentage of germination was high for all genotype/ploidy combinations (≥80%), however, BV10 had the lowest germination and its 2*x* and 4*x* lines had very similar germination whereas for the other genotypes (BV33 and BV16), there were greater differences between 2*x* and 4*x*.

3.3. Ploidy Determinations

Almost all open pollinated seeds taken from diploids were diploid (160 out of 161 seeds assessed; Table 5). A total of 94.5% of the 458 seeds from the nine 4*x* lines were 4*x*, but eight (1.8%) were classified as triploid and one as diploid. Six of the allotetraploid lines produced at least one triploid. Seven non-euploid seeds were also identified, five of which came from just one 4*x* line (BV10L583). In the follow-up survey of field grown progenies issued from 4*x* parents and open pollination, four of 91 sampled plants (4.4%) were classified as triploid (Table 6). These derived from 3 of the 6 lines representing 2 of the 3 diploid precursors.

3.4. Segregation Abnormality in the Allotetraploid Acacia Hybrid

The observed segregation of 15 loci in 42 outcrossed 4*x* F_2 is listed in Table 7. Among the SSR markers examined, eight were consistent with disomic inheritance. This is because at these eight loci, all the outcross F_2 progenies presented the same unique genotype. For seven loci, the observed segregations among F_2 seedlings were in agreement with the expectations for tetrasomic inheritance.

Table 5. Ploidy determination for open pollinated seed harvested from 2*x* and 4*x* lines in the Bau Bang trial during 2014.

		Seeds by Cytotype				
Clone/Line	**Maternal Ploidy**	**2*x***	**3*x***	**4*x***	**Other**	**Total Assayed**
BV10	2*x*	37	0	0	1	38
BV16	2*x*	60	0	0	0	60
BV33	2*x*	63	0	0	0	63
Total 2*x*		160	0	0	1	161
%		99.4			0.6	100
BV10L583	4*x*	0	1	36	5	42
BV10L590	4*x*	0	1	43	0	44
BV10L562	4*x*	0	1	60	0	61
BV16L437	4*x*	0	3	49	1	53
BV16L437	4*x*	0	0	61	0	61
BV16L440	4*x*	0	1	52	0	53
BV33L667	4*x*	0	1	41	0	42
BV33L695	4*x*	0	0	54	1	55
BV33L724	4*x*	1	0	46	0	47
Total 4*x*		1	8	442	7	458
%			1.8	94.5	1.7	100

Table 6. Ploidy determination from the field trial of OP progeny from 4*x* parents sampled at age 3 years.

		Progeny by Cytotype			
ID	**Maternal Ploidy**	**2*x***	**3*x***	**4*x***	**Total**
BV10L562	4*x*	0	1	14	15
BV10L583	4*x*	0	1	14	15
BV16L437	4*x*	0	0	12	12
BV16L440	4*x*	0	0	17	17
BV33L667	4*x*	0	2	15	17
BV33L695	4*x*	0	0	15	15
Total 4*x*		0	4	87	91
%		0	4.4	95.6	

Table 7. Summary of inheritance pattern for 15 SSR loci in outcross progenies (n = 42) of allotetraploid (4*x*) acacia hybrid.

Marker	**Mother Genotype**	**Segregation Information**	**Inheritance**
		Species diagnostic markers	
AH3_6	AABB	3A_ _ _:37A_ B_:2 B_ _ _	Tetrasomic
AH54	AABB	1A_CC:2A_CD:1A_D_: 3ABC:22ABCD:5ABD_:2B_C_:4B_CD:2B_D_	Tetrasomic
AH08	AABB	2A_ _ _:5A_C_:6A_B_: 26AB_C:3B_C_ _ _	Tetrasomic
ancp16a&ancp17s	AABB	All A_B_ except for one A_ _ _ *	Disomic, except for 1 abnormal (A_ _ _)
ancp54a&ancp55s	AABB	All A_BC	Disomic
		Other markers	
AH29	AABB	All A_C_	Disomic
AH69	AABB	All A_B_	Disomic
AH76	AABB	All A_B_	Disomic
AH16	AABB	All ABCD	Disomic
PCT4&ancp8a	AABB	2A_ _ _:6A_C_:5A_B_: 25AB_C:3B_C_:1C_ _ _	Tetrasomic, but 1 abnormal (C_ _ _)

Table 7. *Cont.*

Marker	Mother Genotype	Segregation Information	Inheritance
ancp29s&ancp30a	AABB	All A_BC	Disomic
ancp69a&ancp70s	AABB	All A_BC	Disomic
Am041	AABB	1A_ _ _:5A_C_:4A_B_: 30AB_C:1B_C_:1B_ _ _	Tetrasomic, but 1 abnormal (B_ _ _)
Am387	AABB	2A_ _ _:5A_C_:6A_B_: 26AB_C:3B_C_:	Tetrasomic
Am465	AABB	1 A_CC:2 A_CD:1 A_D_:3 ABC_:25 ABCD:3 ABD_:2 B_C_:3 B_CD:2 B_D_	Tetrasomic with 9 genotype groups, 25 F_2 having 4 different alleles

One abnormal genotype was detected at each of three loci (ancp16a&ancp17s, PCT4&ancp8a, and Am041), where only one SSR allele was detected; the expectation was for at least two different alleles. These abnormal genotypes were found in three different seedlings (one for each locus), and the abnormal genotype for PCT4&ancp8a did not have maternal alleles.

3.5. Outcrossing Rates

The outcrossing rates derived by the exclusion method were consistent with those calculated using maximum likelihood (data not shown), but usually slightly lower (maximum 4% difference between the two methods). This is not unexpected because there may be some outcrosses that happen not to carry any non-maternal alleles. Because the varying mode of inheritance of the markers shown above could have affected the outcrossing rates calculated using maximum likelihood, those estimates will not be considered further.

The mean outcrossing rate of 4*x* was significantly ($p < 0.001$) lower (14%) than that of 2*x* (69%, Table 8). Among diploids, the outcrossing rate of 2*x* BV10 (37%) was significantly ($p < 0.001$) lower than that in the other two 2*x* genotypes (83% for 2*x* BV16 and 87% for 2*x* BV33). Variation in outcrossing rates among the 4*x* genotypes (range of 12 to 15%) was not significant. No contamination through outcrossing with pollen from outside the trial was found in the progeny of 4*x*, whereas in 2*x*, 4% of the seedlings (5 out of 120) had alleles not found in the three genotypes within the trial (data not shown). A total of 79% of outcrosses across both cytotypes were produced by the crossing of BV16 and BV33 (in both combination directions).

Table 8. Outcrossing rates (%) for clones and ploidy levels.

	BV10	BV16	BV33	Mean
2*x*	37	83	87	69
4*x*	13	13	15	14

Chi-square value for comparing ploidy levels = 148.5 ($p < 0.001$, 1 d.f.). Chi-square value for comparing diploid clones = 38.72 ($p < 0.001$, 2.d.f.). Chi-square value for comparing tetraploid clones = 0.33 (not significant, 2 d.f.).

3.6. Survival and Height of Selfed Compared to Outcrossed Progenies in the Field Trial

Survival and height of the progeny in the trial at 12 months are given in Table 9. The survival of outcrossed 2*x* (88.5%) was significantly higher than selfs (56.3%) However, there was a no significant difference in the survival of outcrosses (91.4%) and selfs (86.0%) for progeny from 4*x* lines.

Selfing significantly affected the height growth of 2*x* and 4*x* progenies (Table 9). The height of outcrossed F_2 was higher than that of selfed F_2 in both 2*x* (2.7 m and 1.8 m, respectively) and 4*x* (2.9 m and 2.4 m, respectively). Inbreeding depression of 2*x* selfed progenies was significantly (DF = 14.2, $p = 0.02$) greater (33%) than in 4*x* selfed progenies (17%).

Table 9. Field survival and growth of outcross and selfed progenies of 2*x* and 4*x* lines after 12 months and estimated inbreeding depression.

Ploidy	Status	Number of Planted Seedlings	Number Surviving	Survival (%)	Height at 12 Months (m)	ID [1] (%)	CV [2] (%)
2*x*	F_2 Outcrosses	61	54	88.5	2.7		25
	F_2 Self	16	9	56.3	1.8	33	28
S.e.d. mean					0.23		
	Significance (outcrosses vs. *selfs)*			**	**		
4*x*	F_2 Outcrosses	35	32	91.4	2.9		11
	F_2 Self	178	153	86.0	2.4	17	23
S.e.d. mean					0.12		
	Significance (outcrosses vs. *selfs)*			*Ns*	***		

([1] = inbreeding depression, [2] = coefficient variation. Asterisks indicate statistical significance: ** $p < 0.01$, *** $p < 0.001$).

4. Discussion

This study has examined the reproductive biology and growth characteristics of allotetraploid *A.* hybrid, allowing comparison with previously published results for autotetraploids of one of the parents, *A. mangium*, and assisting consideration of the practicality of polyploid breeding on an operational scale.

4.1. Flowering, Seed Production and the Mating System of Allotetraploid Acacia Hybrid in Comparison with Autotetraploid A. mangium

Nghiem et al. [12,13] described the flowering phenology, seed production, and germination characteristics of 2*x* and 4*x A. mangium*, which is one of the parent species of *A.* hybrid. Peak flowering in allotetraploid acacia hybrid was approximately one month after peak flowering in the diploid acacia hybrid clones from which they derived (Tables 2 and 3) in contrast with Nghiem et al. [12], who did not find any significant difference in flowering time between *A. mangium* cytotypes in a nearby trial at Bau Bang. Cytotype differences in flowering time have been reported in many other taxa [21–24]. We know that in southern Vietnam, although there is some overlap in flowering times, *A. mangium* generally flowers 1–2 months before *A. auriculiformis* [25], so it is not unreasonable to speculate that the expression of the alleles contributed by each parent may vary in the 2*x* and 4*x* cytotypes of *A.* hybrid.

In our study, there were no significant differences in total number of seeds and normal number of seeds per pod between tetraploid and diploid *A.* hybrid cytotypes (Table 4). In contrast, the *A. mangium* autotetraploid produced half as many seeds per pod compared to diploid cytotypes [13,26]. In many crop taxa, tetraploid varieties produced fewer seeds than diploids [27–30] with variation in number of flowers per plant, lower pollen viability, or higher rates of embryo abortion in tetraploids invoked as possible causes [30]. In our study, flowering intensity of tetraploid *A.* hybrid was higher than that in its diploid progenitors (Table 3). We did not study pollen viability or rate of embryo abortion, but it is possible that the autotetraploid *A. mangium* differed from the allotetraploid in these attributes. We would expect a higher frequency of viable gametes in the allo-polyploid (fewer multivalence problems during meiosis) compared to the auto-polyploids [31]. However, the segregation abnormality that was encountered could indicate chromosome instability that could lead to low pollen viability and embryo abortion (discussed below).

There were major differences in the outcrossing rate between the two cytotypes of *A.* hybrid. The diploid clones averaged 69% outcrossing and the allotetraploid lines 14%. The mean diploid estimate is consistent with the 86% reported for *A.* hybrid by Ng et al. [32], however there was large variation in outcrossing rate between clones. BV10 had 37% outcrosses compared with 84% to 87% for BV16 and BV33, respectively (Table 8). This may be explained by variation in opportunity for outcrossing within the trial since the peak flowering time of BV10 was one month later than the other two clones (Table 2). To

analyze the allotetraploid data, we pooled lines within genotypes with mean values varying between 12 to 15% outcrossing. This is a higher rate than observed for the autotetraploid *A. mangium* where only 2% of the seeds were outcrosses [11]. The literature contains many examples of higher selfing (lower outcrossing) in tetraploids compared with equivalent diploids [33–35]. The floral biology of Acacia is such that self-pollination must occur at a high frequency, and Griffin et al. [11] concluded that the higher yield of viable selfed seed in the autotetraploid could be ascribed to reduced expression of post-zygotic genetic load relative to that following selfing of diploids. Tetraploid pollen is usually larger than diploid [36], and this was found to be the case in the autotetraploid *A. mangium* study of Nghiem et al. [12]. Polyads from 4*x* trees were 42 µm in diameter compared with 33 µm in diploid *A. mangium*. However, it was concluded that these differences did not affect the ability of cytotypes to cross-pollinate.

Among the 458 open pollinated seeds from the allotetraploid lines that were assayed by flow cytometry, we found 15 (3.5%) which differed from expectation that viable progeny would also be 4*x* (Table 5). Of these, eight (1.8%) were classified as 3*x* on the basis of intermediacy between known 2*x* and 4*x* lines. In a similar study of 758 open-pollinated seeds derived from 49 seedlots, collected from a polyploid hybridizing orchard at Bau Bang, Vietnam [11], Harbard and Nghiem [37] found 3 triploid genotypes (frequency of 0.4%). Based on microsatellite genotyping, those were all derived from 2*x* mothers and were most likely produced by the fusion of an unreduced and a haploid gamete rather than inter-cytotype outcrossing. It has, however, proved possible to produce viable triploid seedlings by controlled inter-cytotype pollination [13], and the small study of ploidy of field planted progeny from 4*x* hybrid mothers (Table 6) confirms that viable triploid hybrids can also be produced by open pollination.

4.2. Mixed Inheritance Pattern Was Detected Showing the Complexity of Segregation and Recombination in Allopolyploid Acacia

We analyzed the segregation of 15 SSR loci in 42 outcrossed progenies of allotetraploid acacia. Eight SSR loci showed segregation consistent with disomic inheritance as expected for our allotetraploids, while seven loci had segregation consistent with tetrasomic inheritance (Table 7), which is more typical of autotetraploids. Evidently, the tetraploids derived from the F_1 combination of *A. mangium* and *A. auriculiformis* do not behave like a 'classic' allopolyploid [38] where chromosomes pair faithfully as bivalents, do not recombine between species sets, and display stable disomic inheritance. It is best described as a segmental polyploid with mixed or intermediate inheritance [18,39]. This inheritance model was also detected in a polyploid of *Coffea arabica* × *C. canephora* by Lashermes et al. [31], where nine RFLP markers segregated with tetrasomic inheritance while two markers followed disomic inheritance. In allotetraploid Brassica napus, Grandont et al. [40] found that only 50% of meiotic cells exclusively showed synaptic bivalents while the rest had one or two synaptic tetravalents per meiosis.

Because the chromosomal positions of the markers are unknown, we do not know how many chromosomes show tetrasomic inheritance, and further research is required to determine this. Irrespective of the exact number of chromosomes involved, tetrasomic inheritance is likely to result in aneuploidy [24]. A high number of multivalents in polyploids can result in high rates of homologous recombination, which can eliminate the contribution of one parent in a genomic region [41] producing aneuploid gametes. Aneuploid plants are often less vigorous and fertile than euploids (those with complete chromosome sets) [17,42]. We found three abnormal genotypes, each containing only one allele, which was not consistent with either the disomic or tetrasomic inheritance model. Since no other errors (mislabelling, scoring error) were found with these genotypes, aneuploidy is considered as the best explanation as we also found a number of non-euploid seeds in the initial flow cytometry survey (Tables 5 and 6). These three seedlings grew poorly and did not survive (data not shown). Aneuploids are frequently found in synthetic autotetraploids, with 30–40% of the progenies of autotetraploid maize reported as aneuploid [42] with low fertility.

4.3. Inbreeding Depression in the Two Cytotypes

In this study, we found stronger inbreeding depression in diploid than in allotetraploid F_2 hybrids, both in terms of survival and growth after 12 months in a field test (Table 9). Our study was not designed to elucidate the genetic basis for the observed inbreeding depression, but since this will influence expectations from advanced generation breeding, the issue is worthy of further research. Two interpretations are commonly presented in the literature (52). Under the dominance hypothesis, the effect is primarily due to increased homozygosity of deleterious recessive genes [43], while the overdominance hypothesis emphasizes the reduction in heterosis consequent on reduced heterozygosity of inbred progeny. As homozygosity of deleterious alleles increases, there is a loss of biological fitness and the lower inbreeding depression in polyploids can be explained by the lower chance of fixation of recessive alleles compared to diploids and subsequent expression of this genetic load. The alternative hypothesis emphasizes the importance of overdominance, whereby the doubling of genome size in allotetraploids causes an increase in heterozygosity (due to fixed differences between species), which reduces the influence of inbreeding depression in tetraploid progenies [43]. Under this hypothesis, if all the chromosomes have bivalent inheritance, and heterozygosity is completely fixed, then inbreeding depression should be absent. In the case of allotetraploid *A.* hybrid, the presence of a significant but lower level of inbreeding depression could be explained by mixed or intermediate inheritance. There is evidence suggesting that diploids are likely to experience more inbreeding depression than tetraploids [44], however the genetic basis for this effect may vary between auto and allopolyploids, and possibly between neo and advanced generations of polyploidy [45]. A deeper understanding of the genetic basis for the observed inbreeding depression in our polyploid *A.* hybrid germplasm would help optimize the breeding strategy.

4.4. Implication for Acacia Breeding Strategies

Because the flowers of Acacia are small, difficult to emasculate, and have low flower-to-pod ratios [12,46], controlled pollination is difficult and open pollinated breeding strategies are much cheaper and simpler to manage. It is therefore of practical importance to consider whether the data reported in this paper are consistent with open pollination being a suitable strategy for polyploid breeding of *A.* hybrid [4].

The 4*x* hybrid lines flowered heavily and produced as many normal seeds per pod as their F_1 diploid progenitors (Table 4). Although the majority of the seeds were selfs with some associated inbreeding depression, an average of 16% were outcrosses (Table 8) with an average growth rate equivalent to the outcrosses from diploid progenitors (Table 9), which are therefore candidates for forward selection and importantly for clonal evaluation and deployment [1].

The potential for superior growth and wood properties and reduced fertility and consequent weediness of triploid hybrid acacias is now well established [47–49], and the findings presented here encourage the adoption of this methodology for further breeding. The chromosomal instabilities, including aneuploidy, which we have identified make it unlikely that we can use forward selection based on simple quantitative genetic predictions, and further research is required to optimize the breeding strategy. An appropriate size for the breeding population is also an issue, as production of stable neo-tetraploid hybrid lines is technically feasible but not simple [9,50].

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