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Acacia mearnsii (Fabaceae) reproductive biology: pollen tube viability and growth

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ABSTRACT - *The low seed production in* Acacia mearnsii *De Wild. populations indicates that narrow genetic variability interferes with the reproduction. The viability of polyads of* A. mearnsii *trees from a seed production area was evaluated through in vitro germination and the colorimeter method. A high mean germination (73%) and low mean pollen tube growth (23%) were observed in the germplasm. Only tree 30 presented 60% germinated pollen tubes. The other means were less than 50%. On the other hand, the viability observed by the colorimeter method was high (100% viable polyads in five trees), which indicated that the colorimeter technique overestimated the true viability. This study showed that the germination ability of polyads was influenced by the genotype, contributing to a limited seed formation.*

Key words: black wattle, pollen, fecundation.

INTRODUCTION

Acacia mearnsii is a native species to southern and southeastern Australia (New South Wales and Victoria) and Tasmania (Searle et al. 2000). Natural populations present low correlations between the geographic distribution and genetic variability, which was considered moderate compared to other Australian species of the *Acacia* genus (Searle et al. 2000).

In spite of the great importance in the industrial and economic sectors of Rio Grande do Sul, the commercial stands are derived from plant material introduced in the 1930s (Resende et al. 1991). The lack of information on reproductive biology that would help select the individuals may further contribute to reducing the reproductive success of the species, indicated by a seed production that falls short of its potential.

The inflorescences of black wattle are grouped in bunches containing about fifty yellowish cream-colored, strongly scented flowers that can be hermaphrodite, with functional male and female organs, or only male functional parts (Grant et al. 1994). Sixteen pollen grains are grouped in a polyad structure in groups of four, with bilobed anthers that open vertically in the anthesis period (Grant et al. 1994, Kenrick and Knox 1979).

Acacia mearnsii flowering lasts approximately three months in Rio Grande do Sul, beginning in August and ending in November and peaking in mid-September. Many flower visitors are attracted by the flower fragrance, but studies indicate that a coleoptera (*Macrodactylus suturalis*) is the main pollinating agent of the black wattle populations under study. Seed production however underruns the species' potential, indicating barriers to fecundation efficiency.

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One of the alternatives used in plant genetic breeding programs is the maintenance of pollen collections for controlled crosses. This approach has the advantages of storing the collection in restricted locations and recording the male parents. Data on the functional quality of the pollen grains of the selected individuals, obtained by viability and analysis methods are required to define the strategy. There is no information of this type about *Acacia mearnsii* and a reduction in fruit formation and viable seeds has been detected, even in seed production areas. Pollen viability can be detected by various techniques used according to the species and the relationship between the test and fecundation (Dafni and Firmage 2000), including *in vitro* germination and the colorimeter methods.

The procedures used include *in vitro* germination to assess pollen tube development under specific environment and temperature conditions, showing the state of the male gamete reserves and the plasmatic membrane conditions (Heslop-Harrison 1992). The culture medium proposed by Brewbaker and Kwack (1963) contains essential elements in its formulation for pollen tube germination, such as saccharine and borom, magnesium, potassium, and calcium ions. These elements maintain the extracellular ion flow and the intracellular gradient of protein and calcium gradient that are necessary for pollen tube development (Holdaway-Clarke and Hepler 2003). Because of the non-static character of this system the *in vitro* germination does not reflect the *in vivo* growth perfectly and anomalies can be observed in some species in male gamete germination (Dafni and Firmage 2000, Holdaway-Clarke and Hepler 2003, Taylor and Hepler 1997).

Other analysis methods are the colorimeter techniques that indirectly assess polyad viability. These are sustained by procedures that assess intact pollen grains and indicate cytoplasm presence, plasmatic membrane integrity and active enzymes (Dafni and Firmage 2000, Heslop-Harrison et al. 1984). These methods are however only an approximation of the pollen viability because of their tendency to color dead or nonviable pollen grains (Dafni and Firmage 2000, Rodriguez-Riano and Dafni 2000). The nitroblue tetrazolium substrate in Baker reagent indicates the presence of activity in the dehydrogenase alcohol enzyme by staining pollen grains. Advantages of this procedure are speed and simplicity. The disadvantage is the staining of unviable or dead pollen grains (Dafni and Firmage 2000, Rodriguez-Riano and Dafni 2000).

The objective of the present study was to evaluate variations in *Acacia mearnsii* trees from a seed production area regarding the viable male gamete production through the *in vitro* germination technique and colorimeter method.

MATERIAL AND METHODS

Branches with inflorescences were collected in October 2003 from 20 *Acacia mearnsii* trees (codes 03, 04, 05, 06, 08, 28, 29, 30, 31, 32, 33, 40, 41, 42, 44, 45, 46, 47, 48, and 49). They were cut in the early morning and the branch base was covered with paraffin. This black wattle population stands on the seed production area (APS) of the Locatelli Farm, which belongs to the SETA S.A. company, municipality of Butiá, Rio Grande do Sul. The analyses were carried out in the Forestery Biotechnology Laboratory (Federal University of Santa Maria, Santa Maria, Rio Grande do Sul). Under laboratory conditions, the inflorescences were excised from the branches, placed on Petri dishes, opened individually and placed in a desiccator containing silica gel to separate the polyads. The inflorescences were selected at the peak of flower opening while senescent or newly opening inflorescences were discarded.

In vitro germination technique

The Brewbacker and Kwack (1963) culture medium was used containing agar (0.5%), saccharine (10%), calcium nitrate (0.03%), potassium nitrate (0.01%), magnesium sulfate (0.02%) and boric acid (0.01%). After placing the culture medium on Petri dishes, the polyads were distributed homogeneously on the surface using cotton swabs. The plates were then placed in a germination chamber (B.O.D.) at 25 °C in the dark.

A randomized block design was used consisting of 21 treatments of fresh polyads from the 20 trees described above and a control with a polyad mixture representing all trees, distributed onto four blocks. To meet the assumptions of the mathematical model, the values were transformed by the square root method for analysis of variance. The hypothesis of treatment differences was tested by the F statistic and considered significant at the level of a 5% when the calculated F value was greater or equal to the tabled F value. The means were compared by the Tukey test at the level of 5% probability.

After 48 hours, germination was assessed by microscope counts of the pollen tubes grown on the four quadrants of each dish. At least 40 polyads per quadrant were evaluated. A pollen tube was considered germinated

when its length was twice the diameter of the pollen grain. The presence of 12 germinated pollen tubes was considered 100% growth, due to the difficulty of counting pollen grains in underlying positions.

Viability by the colorimeter method

The Baker reagent was the colorimeter method used to analyze viability (Rodriguez-Riano and Dafni 2000). The reagent also contained distilled water and phosphate buffer 0.1 M with pH from 7.3 to 7.0 in its formulation at the 2:1 ratio; Nitroblue Tetrazolium (NBT); 0.9 mM Nicotinamide Adenin Dinucleotide (NAD) and 50% ethanol. The polyads were spread on glass slides with 25µL Baker reagent, which were placed in a germination chamber (B.O.D.) at 25 °C for 20 minutes. They were then dried at room temperature. The samples were fixed in glycerol and assessed under an optical microscope. The polyads stained with blue tones were considered viable, while the unstained indicated non-viability. The viability was analyzed by the Chi-square independence test at the level of 5% significance.

RESULTS AND DISCUSSION

In vitro germination and pollen tube growth

The *Acacia mearnsii* polyad structure contains 16 pollen grains, a similar number to that of the ovules (12 and 14) that would theoretically enable a polyad to fertilize an ovary completely (Grant et al. 1994, Kenrick and Knox 1979, Moncur et al. 1989). However, a unique possibility of fecundation would be a disadvantage for the species in the case of incompatible polyads adhering to receptive stigmas, because barriers against self-pollination in *Acacia mearnsii* contribute to the reduced seed production (Kenrick and Knox 1989), which is further influenced by the high proportion of male flowers (Grant et al. 1994).

The present study showed that the mean of *in vitro* germinated polyads was high (73%), but the pollen tube growth per polyad was reduced (23%). The trees differed significantly ($P \leq 0.05$) regarding *in vitro* germination and pollen tube growth, demonstrating the genotype influence on polyad vigor (Figure 1).

A comparison of the mean germinated polyads (Table 1) showed that the trees 30 and 32 (99%) and tree 31 (96%) did not differ significantly (P d" 0.05). The means observed in trees 32, 30, 31, 8, 28, 48, 4 and 5 were higher than in the control (83%). A lower mean of *in vitro* germinated polyads was observed in tree 47 (5%). Tree 30 differed significantly from the others by the Tukey test at 5% probability (Table 1) regarding pollen tube growth. Means superior to the control were observed in the trees 30, 40, 8, 32, 8, 31, 4, and 29 (24%). The pollen tube mean observed in tree 47 was lower than in the others.

Although the mean percentage of germinated polyads was expressive (Table 1), the pollen tube growth quantification showed that the analyzed plant material

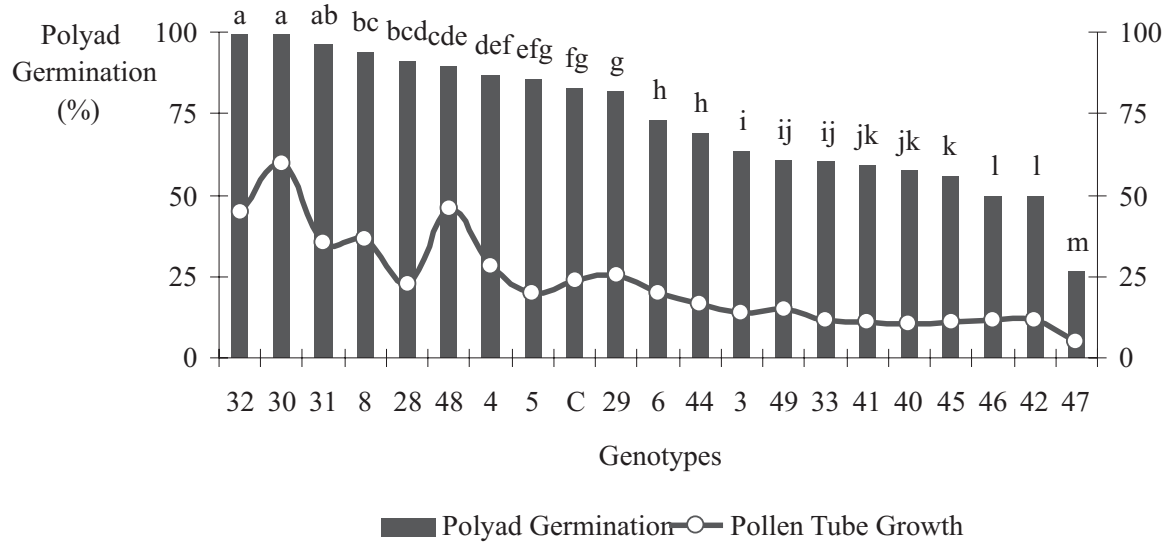

Figure 1. Comparison among the means verified for *in vitro* germination and pollen tube growth of *A. mearnsii* polyads from trees in the seed production area. Means followed by the same letter did not differ by the Tukey test (5%). C = control

Table 1. Results of the analysis of variance (ANOVA) and comparison among the germination and pollen tube growth means observed in *A. mearnsii* polyads from trees in the seed production area

	Polyad Germination	Pollen-Tube Growth
F treatment	63.61**	55.84**
CV	3.7	8.4
Mean (%)	72.9	22.8
Tukey (5%)	0.104msd	0.128msd

Polyad Germination Means			Pollen-Tube Growth Means		
Tree 32	99.4	A	Tree 30	59.6	A
Tree 30	99.4	A	Tree 48	45.7	B
Tree 31	95.9	AB	Tree 32	45.0	B
Tree 8	94.0	BC	Tree 8	36.2	C
Tree 28	91.3	BCD	Tree 31	35.4	CD
Tree 48	89.3	CDE	Tree 4	28.2	D
Tree 4	86.7	DEF	Tree 29	25.4	DE
Tree 5	85.9	EFG	Control	23.9	E
Control	82.7	FG	Tree 28	22.5	F
Tree 29	81.7	G	Tree 5	20.1	F
Tree 6	73.0	H	Tree 6	19.9	F
Tree 44	68.8	H	Tree 44	16.7	G
Tree 3	63.4	I	Tree 49	14.8	GH
Tree 49	61.0	IJ	Tree 3	13.9	HI
Tree 33	60.4	IJ	Tree 33	11.7	IJ
Tree 41	59.0	JK	Tree 42	11.6	IJ
Tree 40	57.3	JK	Tree 46	11.4	J
Tree 45	55.6	K	Tree 45	11.1	J
Tree 46	50.0	L	Tree 41	10.9	J
Tree 42	49.6	L	Tree 40	10.6	J
Tree 47	26.4	M	Tree 47	4.7	K

CV. = Coefficient of experimental variation; ** = $P \leq 0.05$; msd = minimal significant difference detected by the Tukey test (5%).

presented low capacity for *in vitro* pollen tube development, because, with exception of tree 30, the percentage of pollen tube growth was less than 50% of the rest of the study germplasm.

Although the culture medium provided a controlled experimental system, with adequate levels of ions and osmotic components, *in vivo* growth could not be exactly reproduced (Dafni and Firmage 2000, Taylor and Hepler 1997). However, the results could be compared with other studies that assessed pod formation in *Acacia mearnsii* by controlled fecundation.

Moffett (1956) studied the reproductive system of *Acacia mearnsii* progenies planted in South Africa. He detected the presence of areas to prevent self pollination in this species owing to the larger number of pods and seeds formed after cross-fecundation. Kenrick and Knox (1989) observed the presence of a gamete plant system acting together with recessive lethal post-zygotic alleles to control self-pollination in species of the *Acacia* genus, including *Acacia mearnsii*. Post-zygotic control would occur in the presence of a compatible S allele and after endogamous crosses.

The male contribution to seed formation in black wattle may be affected by abiotic factors and by reduced genetic variability in the study population, inducing crosses of related trees. The adaptation of forest species to different locations has led to a reduction in genetic viability through selection processes aiming at genotypes with higher genetic gain (El-Kassaby 2000). In the case of *Acacia mearnsii,* the selection of trees, along with the reproductive isolation of a small sample of the original genepool by a continental barrier could be leading to a continuous allele loss by genetic drift (Seoane et al. 2000), causing inbreeding in the population, expressed in the difficulties with reproduction .

Variation in pollen grain development was described in *Betula pendula* clones (Pasonen et al. 2000), where a correlation was detected between the *in vitro* pollen tube growth and seed formation rates. Evidence suggested that male reproductive success was in fact controlled by the mother plant influenced by the pollen grain genotype and that it differed among trees and among pollen grains from the same individual (Snow and Spira 1996, Traveset 1999).

Polyad viability with Baker reagent

In contrast, the viability detected by the Baker reagent was high in all trees under study (Figure 2), indicating the possibility of staining non-viable or dead polyads by this technique, resulting in overestimation. Dafni and Firmage (2000) observed this disadvantage of the technique in their revision of the practical, ecological and evolutionary aspects of pollen viability.

This hypothesis was confirmed in the present study, because the percentage of *Acacia mearnsii* polyads stained by the technique as an indication of viability was greater than the mean percentage of pollen tube growth (Figure 2). Five trees (3, 5, 30, 32, and 46) presented the maximum pollen tube viability, that is 100% (Table 2), indicated by blue staining. Comparatively, the mean pollen tube growth detected in trees 32 and 31 was, respectively, 45% and 35.4% (Table 1) while pollen tube growth means below 21% were observed in the other trees (3, 5 and 46) (Table 1).

The polyad viability detected by the colorimeter method was also high in the other trees. The trees 30, 28 and 42 presented 98% viable polyads whereas 97% to 85% viable polyads were observed in the rest of the germplasm (Table 2). Comparatively, the mean pollen tube growth in trees 28 and 42 was less than 25% (Figure 2). The trees did not differ significantly by the chi-square test (Table 2) at 5% probability.

Other colorimeter methods used in the *Acacia* genus confirmed the possibility that non-viable polyads can be stained by these techniques, overestimating the results. Polyad viability and storage conditions were tested by Sedgley and Harbard (1993) by the fluorochromatic (FCR) procedure that tests the presence of the active esterase enzyme and the plasmatic membrane integrity, 2,3,5-triphenyltetrazolium chloride (TTC) that stains pollen grains containing active dehydrogenases and 5-bromo-4-chloro-3-indolyl–a-D-galactoside (X-Gal) that detects the presence of the a-galactosidase enzyme. The authors observed high pollen viability (over 80%) when using these methods. However, dead or non-viable polyads were stained by these techniques, especially by the X-Gal and TTC methods.

Staining procedures using the presence of active enzymes to indicate viability can lead to an overestimation of pollen grain potential, resulting in positively biased results (Heslop-Harrison et al. 1984). Viability loss would be a continuous process of gradual reduction in enzyme activity. In spite of the inability to germinate and grow pollen tubes, active enzymes could be stained by these methods (Dafni and Firmage 2000). However, the use of the Baker reagent to analyze *Acacia mearnsii* polyad viability was justified by the time savings, since samples with reduced viability could be detected and discarded in an initial analysis.

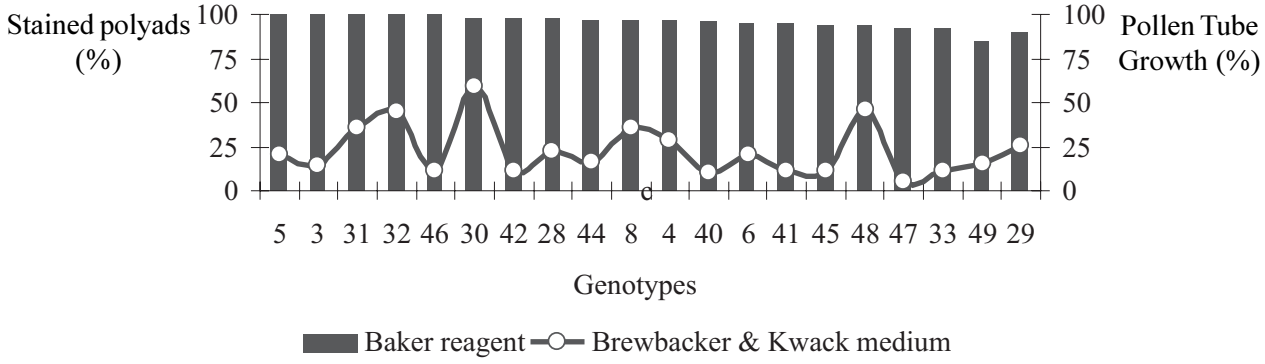

Figure 2. Viability percentage (Baker reagent) and pollen tube growth [Brewbaker and Kwack (1963) culture medium] observed in *A. mearnsii* polyads from trees in the seed production area

Table 2. Results of the Chi-square test (χ^2) and percentage of polyad viability in *A. mearnsii* from trees in the seed production area, observed by the colorimeter method

	Number of analyzed polyads	Stained polyad frequency		Error	% viable polyads
		Observed	Expected		
Tree 29	50	45	47.9	-2.9	90.0
Tree 5	92	92	88.2	+3.8	100.0
Tree 28	44	43	42.2	+0.8	97.7
Tree 3	73	73	70.0	+3.0	100.0
Tree 31	35	35	33.5	+1.5	100.0
Tree 30	47	46	45.0	+1.0	97.9
Tree 32	79	79	75.7	+3.3	100.0
Tree 8	36	35	33.5	+1.5	97.2
Tree 33	71	65	68.0	-3.0	91.5
Tree 4	64	62	61.3	+0.7	96.9
Tree 44	154	150	147.6	+2.4	97.4
Tree 42	90	88	86.3	+1.7	97.8
Tree 6	168	160	161.0	-1.0	95.2
Tree 46	37	37	35.5	+1.5	100.0
Tree 45	62	58	59.4	-1.4	93.5
Tree 41	113	107	108.3	-1.3	94.7
Tree 49	54	46	51.8	-4.7	85.2
Tree 40	94	90	90.1	-0.1	95.7
Tree 48	94	85	87.2	-0.1	93.4
Tree 47	63	58	60.4	-2.4	92.1
χ^2 estimated		35.4 ns			
χ^2 critical		53.4			

"ns – $p \leq 0.05$"

The present study provided an estimate of the male contribution to fruit formation in an *Acacia mearnsii* population on a seed production area. Limitations in *in vitro* pollen tube growth were observed in the analyzed population, suggesting that the formation of viable male gametes is one of the factors that limits seed production in the *Acacia mearnsii* study population. Genotype interference in *Acacia mearnsii* polyad germination was shown in the present study because great variation in *in vitro* pollen tube growth was observed in the trees. The Baker reagent (Rodriguez-Riano and Dafni 2000) overestimated the true *Acacia mearnsii* polyad viability in the study population.

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Biologia reprodutiva de *Acacia mearnsii* De Wild. (Fabaceae): viabilidade e crescimento do tubo polínico

RESUMO - *A baixa produção de sementes em populações de* Acacia mearnsii *De Wild. tem sugerido a existência de variabilidade genética restrita interferindo na reprodução. Através da análise da germinação* in vitro *e da viabilidade por método de colorimetria avaliou-se a contribuição dos gametas masculinos de árvores de uma área de produção de sementes para a fecundação da acácia-negra. Observou-se no germoplasma analisado média de germinação elevada (73%), porém, média de*

emissão de tubos polínicos baixa (23%). Apenas a árvore 30 apresentou 60% de tubos polínicos germinados. As demais médias ficaram abaixo de 50%. Por outro lado, a viabilidade observada pelo método de colorimetria foi alta (100% de políades viáveis em cinco árvores), indicando que a técnica de colorimetria superestima a viabilidade real. Através deste estudo surgiram evidências de que a habilidade de germinação das políades foi influenciada pelo genótipo, contribuindo com a restrita formação de sementes.

Palavras-chave: acácia-negra; pólen; fecundação.

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