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Full Length Research Paper

Pollen isolation method affects interspecific hybridization in eucalyptus

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The ultimate test for utility of a pollen sample is the seed yield following control pollination (CP). In the present study, two different methods of eucalyptus pollen isolation (PI) namely: wet-lyophylisation (WL) and conventionally dry-sieving (DS) were followed. The pollen thus isolated was used to carry interspecific hybridization in order to evaluate and compared the efficiency the above two methods of PI in eucalyptus. Apart from testing the efficiency of the above two methods of PI, CP was carried to generate new combinations of alleles towards developing hybrid eucalyptus for commercial use. Wide-interspecific hybridization was performed between important eucalyptus species following a partial diallel mating design. Pollen from three paternal species namely E. globulus (5 trees), E. pellita (1 tree) and E. urophylla (1 tree) were used to CP three elite commercial clones of E. camaldulensis. Except in few occasions, the percent capsule set and number of seeds obtained per 10 capsule (≥30 seeds) with the PI through WL method was comparatively high and significantly different from conventional DS method (≤30 seeds) at P<0.01. The results of this experiment prove that the method of PI affects the outcome of wide-interspecific hybridisation in eucalyptus.

Key words: Wet-lyophylisation, pollen isolation, control pollination, interspecific hybridization, eucalyptus.

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INTRODUCTION

Eucalyptus is the second most widely planted (20 million hectares) forestry tree species in the world (GIT Forestry, 2009). Among the available 700 species and hybrids of eucalyptus, *Eucalyptus camaldulensis* Dehn forms one of the major sources for paper pulp, fuel wood and timber (Eldridge et al., 1993). This species is used in eucalyptus breeding programmes because of its wide adaptability to grow under drought and rain-fed conditions in tropical and semi-arid regions of the world (Butcher et al., 2009).

The wood properties of eucalyptus are known to vary between species, which could be exploited through introgression of useful alleles from other species into the desired ones towards enhancing wood yield and quality. Interspecific hybridization of eucalyptus can address this requirement through CP of elite trees having desirable traits of interest. Hodgson (1975) and Van Wyk (1977) reported the use of fresh eucalyptus flowers in carrying CP. Conventionally; pollen is isolated by dry sieving (DS) method which involves brushing of clipped or unclipped stamens of mature flowers on to nylon sieve. This method is largely followed because of its simplicity. Recently, it has been reported that the use of sieved pollen in breeding program of *Acacia mangium* can result in outcross contamination up to 19.1%. Further, this process substantially decreases the working labour productivity when compared to the use of the entire inflorescences from the male parent (8.7%) as the pollen applicator (Griffin et al., 2010).

Earlier, Griffin et al. (1982) proved the efficiency of VP method of PI in CP studies of *E. regnans* and reported improvement in mean seed yield per capsule. However, this method was associated with problems such as clogging during filteration as well as sticking of pollen to filter papers. To overcome these shortcomings associated with Griffins VP method and conventional DS methods, we developed and reported Wet-lyophilisation (WL) method of PI. The ultimate test of pollen utility is the capsule set and seed yield following control pollination.

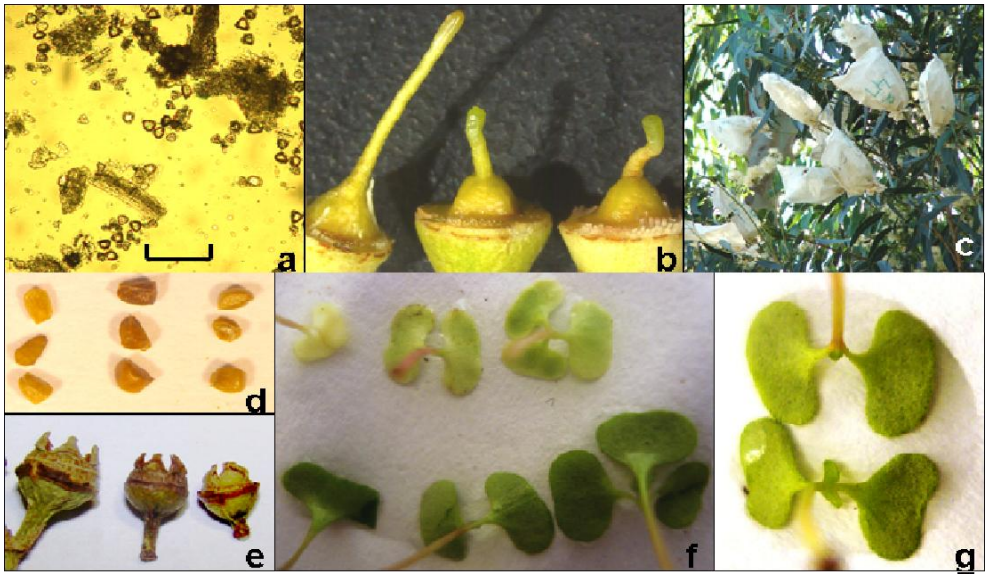


Figure 1. Wide-interspecific hybridization of eucalyptus. a) Eucalyptus pollen collected through conventional DS method and viewed at random under 10X objective of compound microscope. The occurrence of dead cell mats and sparsely distributed pollen can be seen. Scale = 120 µm. b) Emasculated flowers of E. camaldulensis clone 27, 71 and 3 (left to right) used as maternal parents in interspecific hybridization studies. Variation in style length, shapes and flower size can be observed. c) Control pollinated flowers, bagged on E. camaldulensis clone 71. d) Difference in hybrid seed size starting with smaller seed of clone 3 (left) followed by larger seed belonging to clone 27 (center) and medium sized seed of clone 71 (right).) e) Variation in capsule size among the three E. camaldulensis clones namely 27, 71 and 3 (left to right) which resulted in variation in seed set and percent capsule set. f) E. camaldulensis x E. globulus hybrid seedlings showing variations in number of cotyledons and shapes - intermediary to their parents. g) Difference in the cotyledon shape of hybrid seedlings. Lower: hybrid seedling showing E. globulus character - bilobed, chordate, heart shape. Upper: hybrid seedlings cotyledon showing characteristic E. camaldulensis shape – kidney or bean shape.

In the present study, in order to compare the efficiency between WL and DS methods of PI, wide-interspecific hybridization was carried between 4 important species of eucalyptus. The widely adaptable drought tolerant tropical species *E. camaldulensis,* was used as maternal parent while *Eucalyptus globulus* (a temperate species), *Eucalyptus pellita* and *Eucalyptus urophylla* were used as the pollen donors. The efficiency of PI methods was evaluated in terms of percent capsule set and seed set /10 capsules.

MATERIALS AND METHODS

Pollen isolation methods

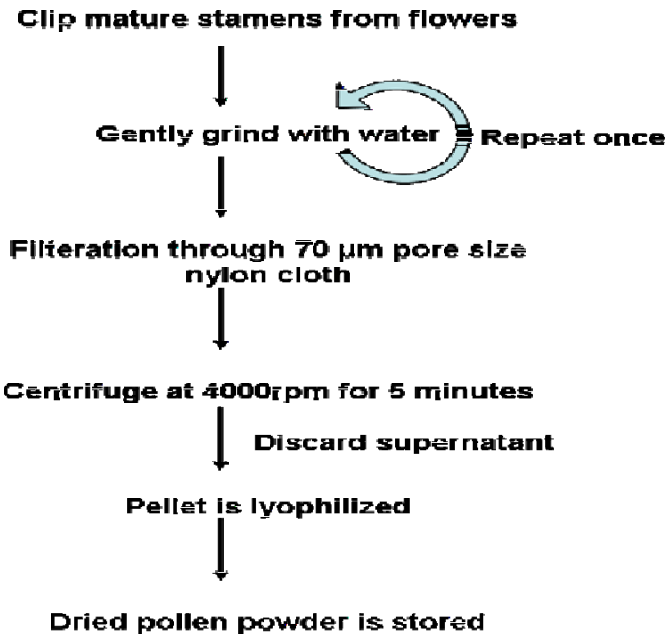
The WL method involves tender grinding of stamens twice in water followed by filtration, centrifugation and lyophilization as described in Girijashankar (2010). This method is also depicted as a flow chart in Figure 2. DS method of PI on the other hand, involves clipping of stamens from mature, unopened flowers followed by shade drying and tender rubbing of the same stamens against a 70 µm pore size nylon sieve which results in filtration of fine pollen powder. This pollen filtrate may often result in dead cell mats and debris (Figure 1a).

Plant materials

Eucalyptus sp. belonging to three different sections of the major subgenus, *Symphyomyrtus,* was used in the present study. Pollen was collected separately following both methods (DS and WL) from mature, unopened flowers of *E. globulus* (section: *Maidenaria*), *E. pellita* (section: *Transversaria*) and *E. urophylla* (section: *Transversaria*). The pollen thus collected is used to cross pollinate three elite commercial clones of *E. camaldulensis* (section: *Exsertaria*). The trees and species used were spread across two South Indian states namely Andhra Pradesh and Tamilnadu. Interspecific hybridization was carried at Andhra Pradesh State Forestry Research Center, Mulugu, Medak (district), Andhra Pradesh, India.

Control pollination and interspecific hybridization

For comparing the efficiency of WL and DS methods of PI, interspecific hybridization was carried out with pollen collected separately from 5 trees of *E. globulus* and one tree each from *E. pellita* (EP-HP-26) and *E. urophylla* (single tree pollen: EU-S6). CP was carried out with mature flowers of *E. camaldulensis* (belonging to ITC, Badhrachalam) elite clones namely 3, 27 and 71 (15 years old) (Figure 1b). For each clone, the best two out of the crossing trees were selected for hybridization experiments. This is because within the same clone, each individual tree in the orchard showed



Flow diagram of Wet Lyophylisation method of pollen isolation

Figure 2. Flow diagram of Wet-Lyophylisation method of pollen isolation.

variation in the crossing rates which was revealed during our earlier experiments. Thus, three maternal clones were crossed with seven pollen donors, eventually giving rise to a total of 21 different combinations of wide-interspecific hybridizations (Table 1).

For CP, the matured unopened flowers are carefully emasculated. Later, they are washed with a sharp jet of water using a squeeze bottle and immediately bagged in a cotton cloth bag (30 X 10 cm) (Figure 1c). On the fourth day, after emasculation, the cotton bags were opened and the receptive stigmas which turned pinkish with sugary secretion were dipped in the respective pollen powders and bagged immediately. The capsules were harvested ≥100 days after CP. The number of capsules and seeds were manually collected and counted (Figures 1d and e).

Statistical analysis

Paired T test was performed in order to ascertain the efficiency of WL and DS methods of PI. In this study of wide-interspecific hybridizations, a total of 42 combinations of CP were evaluated (21 each for WL and DS methods, respectively). This lead to the development of 21 different families of hybrid seed as mentioned in Table 1. Each value in Table 1 is an average of 5 bags, that is, 5 replications per each combination of CP. Further, a minimum of 20 receptive flowers were control pollinated and maintained per bagged inflorescence. Thus, a minimum of 100 receptive flowers (5 bags X 20 flowers/bag) were control pollinated with pollen from each paternal tree. The seed set was expressed as number of seeds obtained per 10 capsules because not all the receptive flowers that were control pollinated could set seed (few capsules contain only chaff). As reported by Meddings et al. (2003), success of interspecific hybridization (*E. camaldulensis* x *E. globulus*) was confirmed by observing cotyledon shapes of hybrid seedlings which were intermediate to that of the respective parent species (Figures 1f and g).

RESULTS

Interspecific hybridization

WL method of PI resulted in enhanced capsule set (significantly different at $P<0.01$) over the conventional DS method. With few exceptions, the number of seeds obtained per 10 capsules was also found to be significantly different between the two methods of PI at $P<0.01$ (Table 1). The average number of seeds obtained per 10 capsules was less in the DS method when compared to the WL method of PI. The number of seeds obtained per capsule following DS method is in the range of 20-30 seeds per 10 capsules. On the other hand, using the same crossing combinations, WL method resulted in 30 to 40 seeds per 10 capsules. Among the 21 combinations of crosses, it was observed that few capsules did not set seed, while few capsules dried or withered off, irrespective of the pollen source or method of collection. As such, the withered capsules were not taken into account for analysis. The percent capsule set and number of seeds/10 capsules was high for clone 27 which has comparatively larger flowers and developed bigger capsules.

Contrary to this, lowest values for the above traits was observed in case of clone 3 which produced smaller

Table 1. Comparison of pollen isolation methods and their effect on interspecific hybridization.

Paternal species and tree number	Maternal parent	Wet lyophilisation		Dry sieving	
	E. CAMALDULENSIS	% capsule set	Hybrid seeds /10 capsules	% capsule set	Hybrid seeds /10 capsules
E. globulus 1	Clone No. 3	42 ± 9	32	35 ± 8	23
2		52 ± 7.1	32	26 ± 8	26
3		38 ± 4.3	30	24 ± 6.5	25
4		42 ± 5.4	24	33 ± 7.5	20
5		45 ± 6	30	22 ± 4.5	20
1	Clone No. 27	95 ± 1.2	36	70 ± 8.5	33
2		94 ± 1.7	39	65 ± 7.6	31
3		92 ± 2.3	39	59 ± 7.5	36
4		84 ± 2.8	31	61± 7	33
5		100 ± 0.0	40	76± 6.4	31
1	Clone No. 71	63 ± 4.1	33	60± 4.5	20
2		70 ± 9	38	62± 8.6	21
3		54 ± 6.3	31	50± 3.4	19
4		52± 4.2	30	46 ± 5	31
5		55 ± 9	34	46± 3.1	30
E. pellita EP-HP-26	Clone No 3	75 ± 8.5	23	15± 8	22
EP-HP-26	Clone No 27	93 ± 2.5	31	37± 5	31
EP-HP-26	Clone No 71	76 ± 8.1	35	35± 4.3	24
E. urophylla EU-S6	Clone No 3	75 ± 9.1	26	43± 8.0	22
EU-S6	Clone No 27	96 ± 3.3	34	65± 4.2	27
EU-S6	Clone No 71	95 ± 2.4	31	70± 5.4	23
	T cal	6.68	5.85		
	DF	20	20		
	P<0.01	S	S		

Paired T test: T cal-values between WL and DS methods of pollen isolation affecting the % capsule set and number of hybrid seeds obtained/10 capsules. Hybrid seeds were manually counted from 10 randomly selected capsules.

flowers and capsules (Figures 1b and e and Table 1). The size of the flower and capsule from the three female clones used in the present study was in the order of 27>71>3 (Figures 1b and e). The percent capsule set and the size of hybrid seeds also (in general) followed a similar pattern (Figure 1d). In this experiment, it was observed that the size of the capsule as well as the hybrid seeds was dependent on the maternal parent that is *E. camaldulensis*.

Generally, *E. camaldulensis* blooms twice a year, with the first flowering occurs between September and January (winter flowering) while the second bloom occurs during February to May (summer flowering). After carrying interspecific hybridization for two consecutive years, it has been observed that the percent capsule set and the number of seed/10 capsules was high on winter flowers, that is, September to January. In contrast, summer flowers result in higher flower drop, capsule withering and empty capsule formation that eventually lead to failure of our earlier hybridization attempts.

A maximum of 11 hybrid seeds per individual capsule were developed in *E. camaldulensis* x *E. globulus* cross combination. Open pollination in these three *E. camaldulensis* clones resulted in 10 to 24 seeds per capsule, while the capsule set was in the range of 75 - 100%. These observations have been recorded during winter flowering. However, CP and wide-interspecific hybridization which involves manual handling resulted in a reduced capsule set (35 to 70% in most cases) as well as decrease seed set per capsule (2 to 4 seeds per capsule). Furthermore, the success of interspecific hybridization between *E. camaldulensis* x *E. globulus* in the present study was confirmed by the cotyledon shapes which were observed to be inter-mediate between the parent species (Figure 1f). In few occasions, the hybrids seedlings completely developed either the *E. globulus* cotyledon shape (bilobed, heart and chordate shape)

or the maternal parent *E. camaldulensis* shape (bean, kidney) (Figure 1g).

DISCUSSION

Hybridization efforts toward eucalyptus germplasm enhancement are hindered due to asynchronous flowering and incompatibility barriers (Savva et al., 1988). In order to overcome these barriers, different PI methods have been developed to collect pollen from desirable trees. Earlier, we have reported that WL method is efficient when compared to conventional DS method of PI. WL method resulted in a reduction of debris content in pollen powder and en-hanced pollen deposition per stigma along with minimal affect on pollen viability and quantity being isolated (Girijashankar, 2010).

As in the present study, partial diallels have been used earlier in eucalyptus breeding programmes towards improving cellulose and wood quality traits (Bison et al., 2007). It is known that eucalyptus interspecific hybridisation via CP is a labour-intensive, costly exercise and produces lower seed yields (Horsley et al., 2010; Meddings et al., 2003). Similar observations have been made between natural open pollination and CP in our interspecific hybridization experiments.

In this study, the pollen collected by WL method resulted in enhancing the numbers of seeds obtained per capsule as well as the percent capsule set when compared to the DS method ($P<0.01$). Earlier, Griffin et al. (1982) carried CP studies using *E. regnans* and reported that the pollen collected through the VP method which also involves immersion of pollen in water (as in the case of WL method) did not affect pollen viability. Further, they also reported that PI, through VP method, resulted in an enhancement of pollen deposition per stigma along with a mean increase of 0.5 seed/capsule. Similarly, WL method of PI also improved the eucalyptus breeding efficiency when compared to the DS method of PI by enhancing the number of seeds / 10 capsules and percent capsule set. Thus, Griffins VP method, as well as WL method of PI stands proof for the increased eucalyptus breeding efficiency.

Resource competition has been shown to exist between ovules within an individual capsule and the presence of larger flowers can increase the chance of the capsule set in eucalyptus (Griffin et al., 1987; Suitor et al., 2007). Similar observations were recorded in our experimentation especially with clone 27 which produced larger flowers and capsules that resulted in higher seed set (3.8) and percent capsule formation (90%) when compared to clone 3 with smaller flowers (2 to 3 seeds/ capsule and 40 to 60% capsule set), irrespective of the pollen viability and paternity.

In eucalyptus, increase in the number of pollen tubes reaching the ovary can enhance viable seed formation (Trindade et al., 2001). It is not possible for all the pollen that land on the stigmatic surface to successfully fertilize the ovules or all the ovules that get fertilized can form seeds. In eucalyptus, only few pollen tubes travel successfully till the end of the stylar canal and eventually fertilize the ovules (Suitor et al., 2007). Earlier works in eucalyptus have proven that genotypes allowing higher proportion of pollen tubes to grow till the base of the style have higher fertilization rates and increased reproductive success (Suitor et al., 2007).

Proteins and lipids on the surface of the pollen grain are involved in the pollen–stigma interaction (Stephenson et al., 1997; Hulskamp et al., 1995). Treating pollen with organic solvents (toluene, carbon tetra chloride, etc.) proved to reduce cross compatibility barriers among woody trees like willow (Stott, 1984) but at the cost of reduced pollen viability (Kopp et al., 2002). In the present study of PI i.e. WL method, mixing the pollen with water or in other terms use of water as solvent for extracting pollen resulted in the development of a yellow colour to water that was finally discarded. It is hypothesize that the water used in the WL method of PI removes some of the chemical molecules present on the pollen grains which may play an important role in eucalyptus interspecific hybridization. Stanley and Search (1971) reported that immersion of tree pollen in water can cause elution of proteins from pollen walls. Unlike the above mentioned reports, Girijashankar (2010). These observations on pollen viability between WL and DS methods of PI. These observations on pollen viability are in agreement with earlier findings of Griffin et al. (1982) and Heslop and Heslop (1985) who reported that eucalyptus pollen are resistant to immersion in water.

Reproductive success of *E. camaldulensis* and *E. globulus* in terms of number of seeds/capsule and % capsule set are known to get influenced by physical and physiological parameters which in turn are dependent on genotype, season and moisture availability (Suitor et al., 2007; Varghese et al., 2009; Butcher et al., 2009). How-ever, success of interspecific hybridization between *E. camaldulensis* x *E. globulus* was rarely reported (Meddings et al., 2003). In the present study, considering the cotyledon shape of hybrid seedlings (intermediate between the two parents) as an excellent way of checking hybrid status (Meddings et al., 2003), we report that our CP experiments successfully resulted in a generation of new hybrids. In such a rare crossing combination, we could develop 15 families of hybrid eucalyptus and also show that the method of PI does affect percent capsule set as well as the number of hybrid seeds developed per capsule.

For successful interspecific hybridization, especially with *E. camaldulensis* (if used as a maternal parent) in tropical and semi-arid climates, it is beneficial to follow the WL method of pollen collection and winter flowering season for improving hybrid seed yields. Based on this study, we conclude that wet-lyophylisation method of PI is beneficial when compared to the conventional DS

method followed in eucalyptus breeding programme. WL method of PI needs to be further evaluated using other species and genera in order to confirm its efficiency across different regions.

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