International Scholars Journals

African Journal of Plant Breeding ISSN 2375-074X Vol. 4 (5), pp. 001-007, December, 2017. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Effect of *Eucalyptus* pollen isolation methods on pollen viability, debris content, quantity isolated and pollen density per stigma

V. Girijashankar[1,2]

[1]Associate Scientist, Plant Breeding and Genetics, ITC R and D Centre, SP Biotech Park, Turkapally, Shameerpet - 500078, RangaReddy (DT.), Andhra Pradesh, India.

[2]Center for Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological University (JNTU), Kukatpally, Hyderabad-500085, Andhra Pradesh, India. E-mail: vgirija_shank@yahoo.com. Tel: 91-9959599890. Fax: 91-40- 23058729.

Accepted 21 September, 2017

Success of interspecific hybridization in *Eucalyptus* depends on the availability of contamination free, clean and viable pollen from elite paternal trees. Unlike the natural process of open pollination, control pollination (CP) in *Eucalyptus* orchards, is a slow, tedious and labour-intensive procedure that involves high costs, but results in low seed yields. In order to overcome these short-comings, *Eucalyptus* breeders use various methods of pollen isolation (PI) procedures and pollination techniques. Here, we report an efficient method of PI, named as wet -lyophylisation method (WL) and compared the same with the conventionally followed dry sieving (DS) method. Clean pollen isolated by WL method resulted in a reduced debris content, in which the percent pollen viability and quantity of pollen obtained/gram of stamen were not significantly different between WL and DS methods at P < 0.05. During wide-interspecific hybridization, the use of clean pollen isolated by WL method resulted in an enhanced pollen density per receptive stigma. When compared to the conventional dry sieving method, the new (WL) method of PI was efficient in reducing the debris content and enhanced the number of pollen deposited on the receptive stigmas. In addition, the amount of pollen obtained per gram of stamen and percent pollen viability are not significantly different between the two methods of pollen collection under study.

Key words: *Eucalyptus*, hybridization, control pollination, wet-lyophylisation method, pollen viability.

INTRODUCTION

The total area of *Eucalyptus* plantation has been estimated to be 20 million hectares worldwide (GIT Forestry, 2009). It is one of the most widely planted forestry tree (Eldridge et al., 1993) for paper pulp, fuel wood, timber, amenity plantings and land rehabilitation. *Eucalyptus* is being used in hybrid breeding programmes for improving wood yield and quality besides drought and salinity tolerance. This genus is adaptable to tropical, arid and temperate regions of the world (Butcher et al., 2009). In order to meet the demands of the increasing population, it is of priority to develop desirable clones and hybrids in *Eucalyptus*.

The wood properties of *Eucalyptus* are known to vary between species and individuals of the same species (Eldridge et al., 1993). This variability can be tapped in breeding programmes through control pollination and hybridization of desirable species of *Eucalyptus*. Pollen is a product of genetic recombination and it forms a reliable source of nuclear genetic diversity at the haploid stage. Collection of clean and viable pollen is one of the important prerequisites in tree breeding programmes. However, *Eucalyptus* pollen is known for its resistant to osmotic stress imposed by immersion in water and is extremely heat tolerant (Heslop and Heslop, 1985).

An efficient pollen isolation (PI) method should produce large quantities of viable pollen that are free from debris. Different procedures have been followed earlier by different research groups that fall short of one or the other above mentioned requirements. Van Wyk (1977) and Hodgson (1975) reported the use of fresh *Eucalyptus* flowers in carrying CP, but the main hurdle in this procedure is the asynchronous flowering of *Eucalyptus*. Further, different species and subspecies of *Eucalyptus* show variations in the climatic requirements to grow and

and flower (Savva et al., 1988; Butcher et al., 2009). Even if the time of flowering coincides, the availability of germplasm and parents proximity hinders the ready availability of paternal flowers for CP. Collection of flowers at anthesis stage and storing them under freezing conditions until the flowers on maternal parent become receptive (Boden, 1958) is a cumbersome procedure. Sieving, also known as dry sieving method of PI, is a widely followed conventional method that involves brushing of clipped or unclipped stamens of matured flowers on a 70 µm pour size sieve. This method is largely followed because of its simplicity, though it is often accompanied with debris and is dependent on the handling practice that is followed during sieving. Mostly, the fresh unopened matured flower stamens are gently rubbed against the sieve for PI. If proper care is taken, this process yields less amount of good quality pollen. On the contrary, the matured flowers are shade dried for few days at room temperature following which the stamens are clipped and rubbed against the sieve. However, this conventionally followed method yields debris along with pollen. Gentle and careful handling at the time of sieving can prevent debris formation, but the content of pollen collected reduces and vice versa. This becomes more problematic when the matured flowers of required paternal species are in short supply.

Recently, it has been reported that the use of pollen isolated by sieving in breeding program of *Acacia mangium* results in outcross contamination rates up to 19.1% and substantially decreases the working labour productivity when compared with the use of the entire inflorescence from the male parent (8.7%) as the pollen applicator (Griffin et al., 2010). The PI method described by Griffin et al. (1982), named as vacuum pump method (VP) is followed; however, the pollen got clogged on 3 µm filter paper during vacuum separation of water. In addition, sticky pollen tightly adhered to the filter papers and as a result reduced the efficiency of Griffin's vacuum pump method. The requirement for pollen (approximately 3 g) with reduced debris content from each paternal tree towards *Eucalyptus* interspecific hybridization programme forced us to develop the present WL method of PI. The two methods (WL and DS) of PI were compared for their efficiency in terms of debris percentage, quantity of pollen isolated per gram of stamens, pollen viability (Figure 1) and number of pollen deposited per receptive stigma.

MATERIALS AND METHODS

Plant material and interspecific hybridization

Eucalyptus species, belonging to three different sections of the major subgenus *Symphyomyrtus*, are used in the present study. Pollen is collected following dry sieving (DS) and WL methods from matured unopened flowers of *Eucalyptus camaldulensis* (section: *Exsertaria*)*, Eucalyptus globulus* (section: *Maidenaria*)*, Eucalyptus pellita* (section: *Transversaria*) and *Eucalyptus urophylla* (section: *Transversaria*). For comparison of efficiency between WL and DS methods of PI, observations on debris percentage, pollen obtained/gram of stamens and pollen viability are estimated using trees from the above four species, that is, *E. camaldulensis* (6 trees), *E. urophylla* (4 trees)*, E. pellita* (5 trees) and *E. globulus* (5 trees) (Table 1). The *Eucalyptus* species used in this experiment are spread across two south Indian states namely Andhra Pradesh and Tamilnadu. Interspecific hybridization experiments are carried at Andhra Pradesh State Forest Research Center, Mulugu, Medak, and Andhra Pradesh.

Pollen isolation methods

Wet lyophylisation

The operculum is separated from the matured unopened flower and the stamens were clipped off; however, the stamens were mixed with deionised (Millipore) water and ground gently in a pestle and mortar so that the pollen comes into the surrounding water. Later, the partially ground mixture was carefully filtered through a 70 µm pore size nylon cloth. Water is again added to these clipped stamens and the above processes is repeated. Both the filtrates were pooled and centrifuged at 4,000 rpm for 5 min. Kubota 6500 centrifuge with rotor Ag-6512C that can hold 50 ml Tarson tubes are used for centrifugation. The yellow coloured supernatant water should be discarded and the pollen pellet is lyophilized overnight till the last trace of water is removed with the help of a bench top freeze-dryer (VirTis benchtop K). This pollen powder can be labeled and stored at -20^{o}C in a desiccator.

Dry sieving and vacuum pump methods of PI

Stamens of matured, unopened flowers are clipped off from the flower whorls and shade dried for 2 - 4 days. Later, the stamens are gently rubbed or brushed against the 70 µm nylon sieve and the resultant filtrate is used as pollen powder for experimental purpose. However, vacuum pump method of PI has been followed initially which is in accordance with the procedure described by Griffin et al. (1982).

Debris percentage and percent pollen viability

To ascertain the debris percentage, pollen powder collected from DS method is weighed. Later, it is mixed with water and filtration is done through 70 µm nylon cloth sieve and the debris is eliminated by following the WL method. Consequently, the difference in weight [(DS – (DS+WL)/DS) X 100] is taken as debris percentage. Percent pollen viability is estimated by Acetocarmine staining and fluorochromatic procedure. In fluorochromatic method, fluorescein diacetate (FDA) dissolved in high sucrose is used (Heslop and Heslop, 1985) to assess the intactness of the plasma membrane in viable pollen. Moreover, FDA stain taken up by pollen grains is not trapped in membranes that are not intact. As a result, nonspecific esterases cleave the diacetate portion of the molecule, rendering the fluorescein photoactive under ultraviolet (UV) light. Under UV light, the viable pollen grains having intact membrane can be seen as fluorescing, while the nonviable pollen grain will be faintly/non-fluorescing. Percent pollen viability (FDA test) in this study is an average of 10 random microscopic field observations (>100 pollen per view). Microscopic observations are carried out using Leica DM1000 at 10X, 40X and 100X. However, the software used for visualizing the images is Leica Application Suit edition 1.0.

Pollen density/stigma

In order to assess the number of pollen adhering to the initial 2 mm

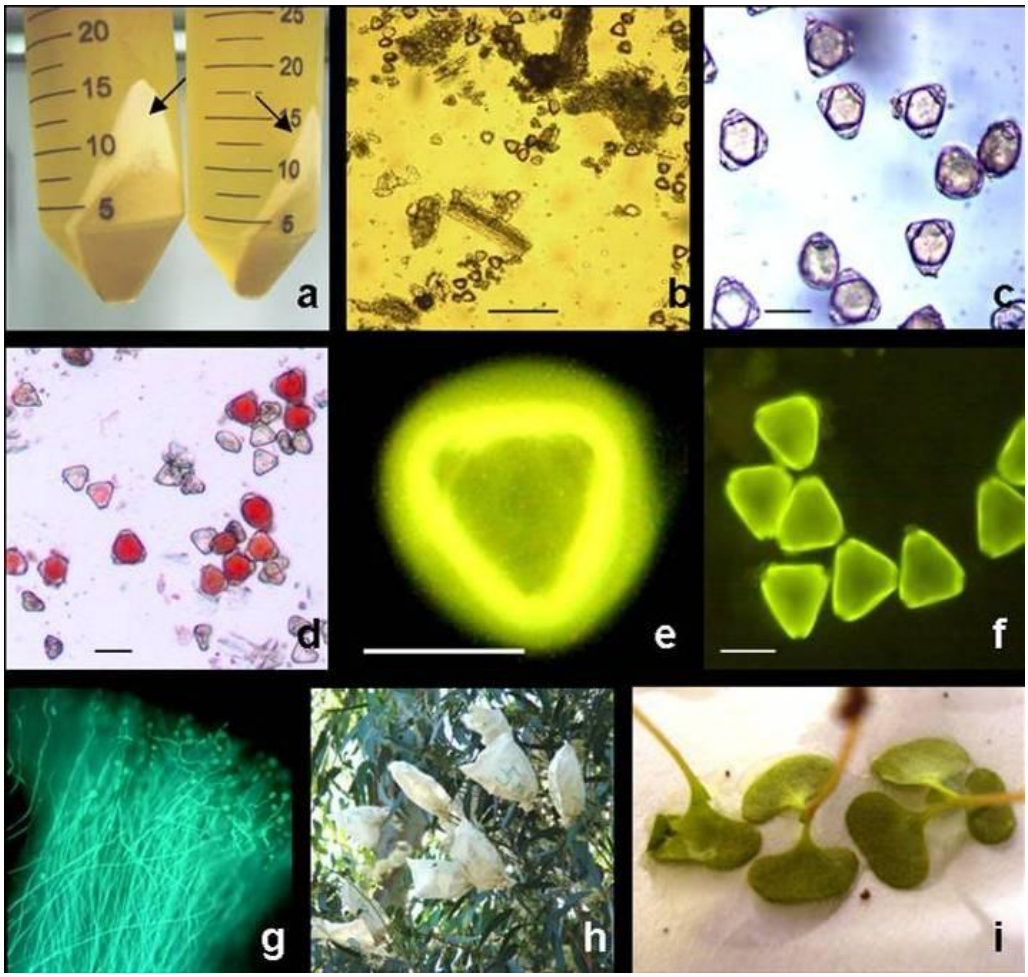

Figure 1. Method of pollen isolation, evaluation of pollen viability and interspecific hybridization. (a) Pollen pellet at the bottom of centrifuge tubes with yellow coloured water as supernatant. Above the pollen pellet, a layer of white precipitate is formed as indicated by arrows. The thin white layer is formed by cell debris of 5 µm size generated because of excess or repeated grinding. At the bottom of 50 ml centrifuge tubes is pure pollen pellet along with yellow coloured water as supernatant. (b) *E. camaldulensis* pollen, collected through the conventional DS method viewed at 10X magnification, showing the occurrence of dead cell mats and sparsely distributed pollen. Scale is 120 µm in length. (c) Clean and debris free triangular *E. globulus* pollen viewed at 25X. Scale represents 25 µm. (d) Acetocarmine staining and pollen viability assessment in *E. globulus*. Pollen viewed at 10X. Pollen grains stained in red colour are considered viable, while unstained pollen grains are nonviable. Scale represents a distance of 25 µm. (e) Fluorescent *E. pellita* pollen stained with fluoresine diacetate (FDA) and illuminated under UV light. The intact live plasma membrane that is present near the periphery emits yellow fluorescent colour due to esterase activity. Scale represents 20 µm in length. (f) *E. globulus* pollen isolated through WL method and stained with FDA for assessment of percent pollen viability. Pollen grains are easily scored as viable (fluorescing) by observation at 25X magnification. Scale indicates a distance of 20 µm. (g) Dense concentration of germinated pollen tubes of *E. globulus* pollen isolated by WL method (seen as florescent treads) passing through the stigma and stylar canal, as observed under UV light. (h) Control pollinated flowers bagged on *E. camaldulensis* clone 71 tree. (i) *E. camaldulensis* X *E. globulus* hybrid seedlings showing variations in number of cotyledons. The variation in the cotyledon shape was observed as varying from the entire and orbicular dumb bell (a character of *E. camaldulensis*) to reniform with a notch (*E. globulus*).

distance of the receptive stigmatic surface, the styles are dipped in the pollen powder isolated by the two methods under investigation. Following the deposition of pollen, 10 random flowers for each species cross combination are collected in Petri plates. The pollen sticking to other parts of the styles was gently removed using camlin brush. However, the stigmatic surface of the flower is dipped in a drop of water placed on the microscopic slide. As a result, all the pollens were manually counted under 10X magnification and a girded eyepiece. Three species namely *E. camaldulensis*, *E. urophylla* and *E. globulus* are used as paternal parents to cross pollinate the receptive stigmas of *E. camaldulensis*. The observation on the number of pollen deposited per stigmatic surface has been reported as range with upper and lower limits because the size of pollen is small and the pollen density per receptive stigma varied drastically (few hundreds). Moreover, *E. camaldulensis* is used as the female parent (Table 2).

Table 1. Comparison of WL and DS methods of PI using *E. urophylla, E. pellita*, *E. camaldulensis* and *E. globulus*.

Species and tree no.	Pollen + debris (mg) / gram of stamens		Debris (%)		True pollen quantity (mg) / gram of stamens		Pollen viability (%)	
	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet
E. urophylla								
EU-1 M	310	193	40	5	186	183	62	64
EU-S3	250	120	50	10	125	108	68	72
EU-S4	155	123	40	5	93	117	75	69
EU-S6	250	120	50	5	125	114	65	64
E. pellita								
EP-M	224	110	50	5	112	105	70	68
EP-BP-6	200	100	50	5	100	95	75	73
EP-BP-21	175	76	50	5	87.5	72	63	64.5
EP-HP-21	230	120	50	5	115	114	71	69
EP-HP-26	290	167	50	5	145	159	62	66
E. camaldulensis								
3 - 1	115	95	20	5	92	90.25	70	76
3 - 2	125	108	20	5	100	102.6	59	72
27 - 1	105	91	20	5	84	86.45	68	73
27 - 2	113	98	20	5	90.4	93.1	68	74
71 - 1	78	75	15	5	66.3	71.25	71	73.5
71 - 2	55	53	20	5	44	50.35	58	61
E. globulus								
1	138	120	15	5	117	114	66	64
2	128	104	15	5	109	99	62	68
3	115	99	20	5	92	95	64	55
4	121	113	15	5	103	107	76	78
5	124	98	20	5	99	95	62	66
Paired T test			T calculated		0.743		0.1236	
			df		19		19	
			P < 0.05		NS		NS	

The results in Table 1 clearly show that WL method of PI is more beneficial than dry sieving method because the WL method produces less debris and at the same time the quantity of pollen isolated and percent pollen viability are not significantly different (P < 0.05).

Statistical analysis

In order to ascertain the efficiency between WL and DS methods of PI, the paired T test is performed with the amount of pollen obtained/gram of stamen and percent pollen viability. For percent pollen viability, the mean is calculated for 10 microscopic field observations at random.

RESULTS

Debris percentage and methods of PI

Application of pressure or repeated grinding of stamens during pollen collection process can lead to 5% debris formation and the debris size can be to a maximum of 5 µm in WL method (Table 1). The debris percentage was found to be high in DS method (ranging between 20 and 50%) when compared to WL (5%) (Table 1 and Figures 1b and c). Thus, when compared to the conventionally followed sieving method, wet-lyophylisation method of pollen collection is efficient in reducing the debris percentage. In DS method especially delicate rubbing of stamens on to the sieve with less pressure resulted in reduction of debris percentage. This is evident in the case of *E. camaldulensis*, where stamens are rubbed gently onto the sieve for 60 s and the process of rubbing is not repeated (Table 1). However, this delicate rubbing procedure resulted in a reduction of quantity pollen, isolated per gram of stamen.

The mean diameter of few debris (dead cell mats) extended between 30 to 200 µm in the DS method; whereas it is not Clear as to how the dead cell mats >100 µm get filtered through 70 µm pore size sieve. On the contrary, the 5 µm debris, generated in the WL method, was

Table 2. Pollen density on the initial 2 mm length of the stigma after cross pollination.

Cross between	Wet lyophylisation	Dry sieving
E. c X *E. c*	250 - 500	150 - 350
E. c X *E. globulus*	450 - 800	260 - 500
E. c X *E. urophylla*	250 - 500	190 - 300

The number of pollen deposited on stigmatic surface are given with observed upper and lower limits. The size of pollen is small and the pollen density varied drastically by *E. c - E. camaldulensis*.

formed as a thin separate superficial layer during centrifugation and it got deposited on the top of the yellow pollen pellet due to differences in densities and can be separated easily using fine paint brush.

Pollen viability and pollen obtained per gram of stamen

Percent pollen viability values across the four species used in the study did not differ significantly between the two methods of PI ($P < 0.05$). When the pollen is processed within 4 days after flower collection, the pollen viability was generally in the range of 60 to 75% (Figures 1d to f and Table 1). During storage, exposing pollen frequently to room temperatures led to a decrease in *E. globulus* pollen viability from 60 to 10% in a period of six months. Matured, unopened flowers were shade-dried for 2 to 4 days before PI was followed in our studies and the average pollen viability was around 60% in most of the cases. The pollen viability varied between trees of the same species (Table 1). Earlier, in an independent experiment, *E. grandis* pollen, kept immersed in water for 48 h and was later collected by WL method, resulted in viable PI. CP and hybridization with this water immersed *E. grandis* pollen during winter, 2007 season, led to the successful development of interspecific hybrids where *E. camaldulensis* clone 71 was the maternal parent (data not shown).

There is no significant difference in the quantity of pollen isolated/gram of stamen in both methods of PI ($P < 0.05$) (Table 1). The amount of pollen produced by both WL and DS methods fall in the range of 90 to 120 mg per gram of stamens. Also, the amount of pollen collected by these two methods generally differed by ± 5%. However, there can be few exceptions, where the difference in amount of pollen collected between these two methods oscillates around 15%. Generally, the amount of pollen that can be collected by these two methods vary between 5 and 18% of the total stamens weight. The experimental results show that pollen viability and pollen obtained per gram of stamens did not differ significantly between the two methods of PI.

Pollen density per stigma

When compared to DS (150 to 350 pollen/stigma), in *E. camaldulensis* X *E. camaldulensis* cross, the number of pollen deposited on the receptive stigma was high with pollen isolated by the WL method (250 to 500 pollen/stigma). Also, a similar trend was observed in other cross combinations such as *E. camaldulensis* X *E. globulus* (Figures 1g to i) and *E. camaldulensis* X *E. urophylla* (Table 2) . Pollen deposited per stigma varied drastically because of the debris content, minute size of pollen and type of stigma.

White mycelial mats of fungal contamination occurred even when low moisture levels were retained in the pollen pellet after centrifugation. So, it is important to continue lyophilizing of pollen pellet until the last trace of moisture is completely removed. The moisture status during lyophylization can be easily monitored by observing the pollen pellet from the sides of the transparent centrifuge tubes. Tapping the centrifuge tube leads to splitting of the pellet into small fragments or powder, if it is dried completely. A general comparison is made among the three methods of PI in Table 3.

DISCUSSION

Interspecific hybridization is one of the various tools available for genetic improvement of plants, as it creates new combinations of genes which would otherwise not exist in nature. Controlled CP help in combining superior wood quality with pulp yield, especially in trees species to meet industrial requirements. Successful interspecific hybridizations are accomplished in *Eucalyptus* with difficulty because of incompatibility barriers as well as asynchronous flowering (Savva et al., 1988).

Proteins and lipids, on the surface of pollen grain, are involved in the pollen-stigma interaction (Stephenson et al., 1997; Hulskamp et al., 1995). However, treating pollen with organic solvents (toluene, carbon tetrachloride, etc.) can reduce the cross incompatibility barriers among woody trees like willow (Stott, 1984), but at the cost of reduced pollen viability (Kopp et al., 2002). In WL method, deionized water (Millipore) which is used as solvent for extracting pollen, eventually developed yellow colour. Unlike the above mentioned reports, in our studies, there is no significant variation in pollen viability between WL and DS methods when water is used. Centrifugal force, lyophylisation and use of water as solvent have no significant effect on pollen viability among the four *Eucalyptus* species used in this study. Earlier studies have proven that use of water to extract pollen from different *Eucalyptus* species would not affect pollen germination under *in vitro* conditions (Griffin et al., 1982).

Further, WL method of PI resulted in reduction of debris percentage when compared to DS method and increased the number of pollen being deposited on the receptive stigmatic surface. Number of seeds formed per capsule as well as percent capsule set, were improved by using the pollen from WL method (data not shown). Further, the quantity of pollen isolated did not differ significantly

Table 3. General comparison of dry sieving, vacuum pump and wet lyophylisation methods.

Observations	Dry sieving	Vacuum pump	Wet lyophylisation
Pollen yield	Medium	Good	Good
Debris	High	Low	Low
Easy to perform	Yes	No	No
Time required	More time required for drying flowers	More time due to clogging of filters	Less
Amount of pollen per stigma	Medium	Medium	High
Cost of PI	Less	High	High
% capsule set	Medium	-	High
Seed / 25 capsules	25 - 45	-	40 - 80

These observations were made during interspecific hybridization studies when *E. camaldulensis* is used as the female parent and *E. globulus* as the male parent.

between both methods of PI. Unlike Griffins VP method, which results in clogging of filter paper, WL method can be regularly followed to obtain more amounts (5 g) of pollen in a single extraction attempt from desirable trees of *Eucalyptus* species.

Low capsule set is known to limit hybrid seed production in *Eucalyptus*. Compared to DS method of PI, in the present study, it is observed that the pollen collected through WL method resulted in enhanced deposition of more number of pollen along with less amount of debris on the receptive *Eucalyptus* stigmas. It is hypothesized that increase in pollen density per stigma could result in improving the number of competitive pollen grains to germinate which in turn leads to successful fertilization. In *Eucalyptus*, enhanced pollen tubes entry into the ovary can result increased viable seed formation (Trindade et al., 2001). It is not possible for all the pollen which land on the stigmatic surface to successfully travel the stylar canal, fertilize the ovules and develop into seeds. Earlier works with *Eucalyptus* have proved that genotypes allowing higher proportion of pollen tubes to grow till the base of the style have higher fertilization rates and increase reproductive success (Suitor et al., 2007, 2009). Resource competition has been shown to exist between ovules within the individual capsule. Further, the presence of larger capsules can increase the chance of capsule set in *Eucalyptus* (Griffin et al., 1987; Suitor et al., 2007, 2009).

In the present study, it is observed that the pollen isolated by dry method (DS) contain more debris resulting in low pollen density on stigma. Interference of dead cell mats can interfere and prevent the pollen tube to penetrate the receptive stigmatic surfaces. Use of pollen basal germination medium not only diluted the pollen number on the stigma but also attracted cockroaches, ants, flies and other insects which eat the sweet stigmas eventually leaving the inflorescence with bare branches inside the cotton cloth bags.

DS method is simple, inexpensive and effective especially in collecting non-sticky pollen (Potts and Gore, 1995) or when the flowers are adequately available. Griffins (1982) VP method is used for collecting sticky pollen and has drawbacks like slow pace of operation, followed by adherence of pollen to filter papers which eventually shed little pollen on receptive stigma. Unlike the above two methods, WL method described here can be used for isolating pollen from all types of *Eucalyptus* (irrespective of their sticky or non-sticky nature) and produces good quantity of clean and pure pollen along with less debris, eventually leading to successful hybridization under the given conditions. It is known that *Eucalyptus* interspecific hybridization through CP is a labour-intensive and costly exercise which produces lower seed yields (Horsley et al., 2010; Meddings et al., 2003). Under such low seed- set conditions, any effort towards the enhancement of at least one seed per capsule may be a significant contribution to exploit the combination of different alleles available (for a given trait) in a tree breeding programme. General application of this procedure is likely to have beneficial impact on the efficiency of large-scale genetic improvement programs.

Conclusion

Wet-lyophylization method of PI methods is found to be efficient over DS method in terms of reducing the debris content in pollen powder and enhanced pollen density/stigma without significantly altering the pollen viability and quantity being collected. For successful interspecific hybridization in the future, especially *E. camaldulensis* breeding in tropical arid climates, it is advantageous to follow (a) WL method of pollen collection and (b) dip the receptive stigma in pollen powder rather than using pollen germination medium and applying as a paste on the receptive stigmas. Based on the present study, it is concluded that WL method of PI is beneficial over the conventional dry sieving method followed in *Eucalyptus* breeding programme. However, WL method of PI needs to be further evaluated using

other species combinations and across agro-climatic regions in order to prove its efficiency.

ACKNOWLEDGEMENTS

The author is thankful to Samresh Dwivedi for his guidance and technical advices during *Eucalyptus* hybridization works. The constant support rendered by Hareesh TS, Sunita Lakshmi, Raj Kumar and Kamal Kannan towards pollen isolation and controlled pollination studies is highly appreciated.

REFERENCES

Boden RW (1958). Handling and storage of pollen in *Eucalyptus* breeding. Aust. J. For., 22(2): 73-81.

Butcher PA, McDonald MW, Bell JC (2009). Congruence between environmental parameters, morphology and genetic structure in Australia's most widely distributed eucalypt, *Eucalyptus camaldulensis*. Tree Genet. Genomes 5(1): 189-210.

Eldridge K, Davidson J, Harwood C, Wyk G (1993). *Eucalyptus* domestication and breeding. Clarendon, Oxford. pp. 60–71.

GIT Forestry (2009). Available at http://git-forestry.com/download_git_eucalyptus_map.htm.

Griffin AR, Ching KK, Johnson KW, Hand FC, Burgess IP (1982). Processing eucalyptus pollen for use in controlled pollination. Silvae Genetica, 31(5-6): 198-203.

Griffin AR, Morgan GF, Fripp YJ (1987). Preferential outcrossing in *Eucalyptus regnans* F. Muell. Aust. J. Bot., 35: 465-475.

Griffin AR, Tran Duc Vuong, Harbard JL, Wong CY, Brooker C, Vaillancourt RE (2010). Improving controlled pollination methodology for breeding *Acacia mangium* Willd. New Forests. DOI 10.1007/s11056-010-9188-x.

Heslop HJ, Heslop HY (1985). Germination of stress tolerant eucalyptus pollen. J. Cell Sci., 73: 135-157.

Hodgson LM (1975). Some aspects of reproductive behaviour in *E. grandis*. D. Sc. Thesis, Dept General Botany, University of Pretoria, South Africa.

Horsley TN, Johnson SD, Myburg AA (2010). Comparison of different control-pollination techniques for small-flowered eucalyptus. New For., 39(1): 75-88.

Hulskamp M, Kopczak SD, Horejsi TF, Kihl BK, Pruitt RE (1995). Identification of genes required for pollen-stigma recognition in *Arabidopsis thaliana*. Plant J., 8(5): 703–714.

Kopp FR, Charles AM, Patricia RDN, Lawrence BS, Lawrence PA (2002). Collection and storage of pollen from Salix (Salicaceae). Amer. J. Bot., 89(2); 248–252.

Meddings AR, McComb AJ, Michael CC, Sandra RT, Richard AM (2003). *Eucalyptus camaldulensis* × *E. globulus* hybrids. Aust. J. Bot., 51(3): 319–331.

Potts B, Gore P (1995). Reproductive biology and controlled pollination of Eucalyptus- a review. In: Techniques of controlled pollination, Ch 6, School of Plant Science, University of Tasmania, Hobart, Tasmania. pp. 50-68.

Savva M, Potts BM, Reid JB (1988). The breeding system and gene flow in *Eucalyptus urnigera.* In: Pollination '88, University of Melbourne, Parkville. (eds RB Knox, MB Singh L Troini). pp. 176–182

Stephenson AG, Doughty J, Dixon S, Elleman C, Hiscok S, Dickinson HG (1997). The male determinant of self-incompatibility in *Brassica oleracea* is located in the pollen coating. Plant J., 2(6): 1351–1359.

Stott KG (1984). Improving the biomass potential of willow by selection and breeding. *In* K. Perttu [ed.], Ecol. Manage. For. Biomass Prod. Syst., 15: 233–260.

Suitor S, Potts BM, Brown PH, Gracie AJ, Gore PL (2007). Factors limiting capsule set, seed set and reproductive success in *Eucalyptus globulus* seed orchards. In: IUFRO conference on Eucalyptus and diversity: balancing productivity and sustainability. Durban, South Africa, 22-26, October, 2007.

Suitor S, Potts BM, Brown PH, Gracie AJ, Gore PL (2009). The relationship of the female reproductive success of Eucalyptus globulus to the endogenous properties of the flower. Sex. Plant Reprod., 22(1): 37-44

Trindade H, Boavida C, Borralho N, Feij JA (2001). Successful fertilization and seed set from pollination on immature non-dehisced flowers of *Eucalyptus globulus*. Ann. Bot., 87: 469-475.

Van Wyk G (1977). Pollen handling, controlled pollination and grafting of *Eucalyptus grandis*. South Afr. J. For., 101: 47-53.