Stigma Receptivity in *Eucalyptus camaldulensis* DEHNH.

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Summary

Stigma receptivity of *Eucalyptus camaldulensis* DEHNH. grown near Perth, Western Australia, was assessed by seed production. Pollination three days after emasculation, when styles had just turned red and stigmas were enlarged, yellow and sticky gave maximum seed set (45 to 55 seeds per capsule and 95% to 100% capsule set). Reasonably high levels of seed (> 25 seed per capsule and > 65% capsule set) could also be produced when flowers were pollinated at the time of emasculation. The period of stigma receptivity varied between cultivars. Capsules matured 14 to 16 weeks after pollination.

Key words: stigma receptivity, pollination, *Eucalyptus camaldulensis*, seed set.

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Introduction

E. camaldulensis DEHNH. is grown throughout the world for land rehabilitation, paper pulp, timber and firewood (ELDRIDGE *et al.,* 1993). In Australia it has been under utilised as a commercial species and breeding programmes are only now being established to produce genotypes with pulp quality and growth form. To produce intra or interspecific hybrids, it is essential to have an understanding of the reproductive biology of the species of interest. VISUTHITEPKUL and MONCUR (1993) studied the floral biology of a natural stand of *E. camaldulensis* from Petford, Queensland, but gave no information on the timing of stigma receptivity. We report here on the stigma receptivity of two *E. camaldulensis* clones growing in a Mediterranean climate in Western Australia.

Materials and Methods

Controlled pollinations were carried out using a *E. camaldulensis* field trial at Kwinana, 30 Km south of Perth, Western Australia, in December 1993 and December 1994. The trees were made available by the 'Tree Tech project' a cooperative programme between the University of Western Australia, Murdoch University and ALCOA of Australia Ltd. and had been selected for salt tolerance and cloned *in vitro* (BELL *et al.,* 1994; VAN DER MOEZEL and BELL, 1990).

Three trees of each of clones 85 and 87 were used as female parents in experiments to determine the timing of stigma receptivity. Clone 85 originated from Broken Hill, New South Wales and clone 87 from Erudina, South Australia. Both of the clones flowered in December so flowers developed and fruit matured under similar weather conditions. During the period of flowering in 1993 the mean daily temperature maximum was 28.9°C and the minimum 14.8°C. The figures for 1994 were a mean maximum of 29.3°C and a minimum of 15.8°C. Flowers for the trials were emasculated over a 3 day period. For the experiment that examined the time interval between emasculation and pollination, flowers of both clones 85 and 87 were emasculated and pollinated on the same days.

Flowers were emasculated when the operculum had turned from green to yellow and was beginning to lift from the hypanthium (*Figure 1a, b*). Flowers on the selected branch that were at an earlier or later stage were cut off. The stamens of the selected flowers were removed using a thumb nail. They were then washed with deionised water to ensure all pollen was removed from the stigmas. Leaves surrounding the flowers were trimmed back to about three quarters of their original length. The section of branch containing the emasculated flowers was isolated in a double layer of crispy wrap bags (bags made from transparent film with very small perforations). A wire coil was placed inside each bag to protect the stigmas from rubbing against the bag. The bags were then secured onto the branches with electrical ties (*Figure 1d*). Each bag contained about 15 to 25 flowers. All flowers in an isolation bag were pollinated on a particular day following emasculation ranging from day 0 (the day of emasculation) to day 10.

Pollen from clone 84 (originally from Mt. Fouracre, Western Australia) that had been stored for eleven months was used for the experiments. To collect the pollen, branches bearing flowers on which the operculum was turning from green to yellow were placed in jars of water in the laboratory. When the operculum of a flower lifted anthers were removed, placed in gelatin capsules over silica gel and stored at 4°C ± 2°C. Before use the pollen and anthers were transferred to glass vials with rubber bungs. In the field the glass vials of pollen were kept over ice in an insulated container. When the vials were shaken the pollen stuck to the rubber bung, which was then used to apply the pollen to stigmas at the designated times (*Figure 1c*).

Five styles from each treatment were harvested for histological processing when the first signs of style abscission were observed. About 14 weeks after pollination (March) the capsules were harvested. Each capsule was placed in a vial and stored over silica gel to dry. The percent capsule set was calculated and the number of seeds in each capsule was counted.

In 1993 the flowers pollinated on day 0 in both clones 85 and 87 were lost because of parrot damage or broken branches. For this reason pollination on day 0 was repeated the following year. Clone 84 pollen was unavailable so fresh clone 42 (Capelis, WA) pollen was used for the crosses. There was no significant difference ($P < 0.05$) in the number of seeds produced per capsule between 85 x 42 and 85 x 84 and between 87 x 42 and 87 x 84 when pollinated at peak receptivity. Five styles of the day 0 crosses were harvested 6, 24, 48, 72 and 96 hours following pollination to examine pollen germination. For comparison, 5 styles were also harvested from flowers pollinated 3 days after emasculation 0, 3, 6, 24 and 48 hours after they had been pollinated.

Histological processing

Styles were fixed in Carnoy's fixative (6:3:1 absolute alcohol: chloroform: glacial acetic acid) and stored at 4°C ± 2°C for at least 24 hours. The tissue was then hydrated through an ethanol series (70% ethanol, 30% ethanol, 2 changes of distilled water for at least 10 minutes), softened in 0.8 N NaOH at 60°C for 30 to 60 minutes and then stained in 0.1% water soluble aniline blue in 0.1 N K_3PO_4. Aniline blue solution was prepared by dissolving the stain then placing it in the dark

Figure 1. – Controlled pollination of *E. camaldulensis.* a. Buds at operculum shed, the stage of emasculation, b. Emasculated buds, c. Pollination using pollen on a rubber bung, d. Pollinated buds isolated in crispy wrap bags. Bars represent 1 cm.

for 24 hours before filtering through number 1 Whatman filter paper. Styles were stained for at least 10 minutes then the tissue was squashed in 80% glycerol and viewed under a Zeiss photomicroscope III utilising violet excitation with an exciter filter, 390 nm to 420 nm and a barrier filter, 450 nm.

The styles of *Eucalyptus* species are covered by a thick cuticular layer (BOLAND and SEDGLEY, 1986) that obscures the observations of pollen tubes. To enable clear observations a longitudinal slit was made through the styles. The style was placed with the slit uppermost on the slide before the tissue was squashed to ensure the cuticular layer was beneath the pollen tubes when microscopic observations were made.

Statistical analysis

The number of seeds per capsule was log transformed and analyses of variance and Tukey's B tests were performed to determine statistical differences between crosses and pollination times using Statistical Package for Social Sciences X (Anon., 1988).

Results

Appearance of E. camaldulensis flowers from emasculation to style abscission

The nature and timing of the visual changes that took place from emasculation to style abscission were almost identical in

the two clones. When the flowers were emasculated the style, top of the ovary and stigma were green and dry, the receptacle was yellow / orange and anther dehiscence had commenced. By the following day nectar was present in the receptacle. Two to four days (most usually three days) after emasculation, the style and the top of the ovary had turned red and the stigma had become enlarged, sticky and yellow. The first signs of style abscission were observed on day 10 to 11 (after emasculation) in clone 85 and day 9 to 10 in clone 87.

The timing of stigma receptivity in E. camaldulensis - capsule and seed set

Capsules matured in March 14 to 16 weeks after pollination. The timing of stigma receptivity as assessed by capsule set and the number of seeds per capsule varied between clones 85 and 87 (*Table 1*). In clone 85 the capsule set was high (above 65%) and there were more than 25 seeds per capsule when flowers were pollinated from zero to five days after emasculation. The mean number of seeds produced per capsule peaked when flowers were pollinated on day three (*Table 1*). When flowers were pollinated more than five days after emasculation seed production was very low (*Table 1*).

Table 1. – Capsule set and the mean number of seeds produced per capsule in *E. camaldulensis* controlled pollinations from 0 to 10 days after emasculation. Clones 85 and 87 were used as the female parents and clone 84 as the male parent except the day zero crosses for which the male parent was clone 42.

Pollination - days since emasculation	Number of flowers pollinated [a]	% capsule set	Mean no. seeds per capsule (se)
Clone 85			
0	6	66.7	26.0 (3.67)
1	10	100.0	42.4 (5.28)
2	14	71.4	35.0 (4.83)
3	21	95.2	54.6 (1.58)
4	17	82.4	33.8 (2.06)
5	24	70.8	26.5 (3.36)
6	0	0	
7	0	0	
8	12	8.3	1
9	0	0	
10	0	0	
Clone 87			
0	26	69.2	42.11 (2.05)
1	17	88.2	45.9 (3.52)
2	16	100.0	35.9 (4.56)
3	28	100.0	45.1 (2.52)
4	22	22.7	4.6 (1.50)
5	9	66.7	15.0 (4.06)
6	19	31.6	3.7 (1.91)
7	15	13.3	5.0 (3.00)
8	0	0	
9	0	0	

[a]) Flowers that were pollinated but capsules lost due to parrot damage or broken branches were excluded from data.

In clone 87 the number of seeds produced per capsule was highest when flowers were pollinated zero, one, two or three days after emasculation with capsule set peaking on day two and three (*Table 1*). If flowers were pollinated after day three seed production was very low, but some seeds were set up to day seven (*Table 1*).

The timing of stigma receptivity in E. camaldulensis - pollen tube growth

In both *E. camaldulensis* clones pollen tubes grew the entire length of the style (4 mm) when flowers were pollinated on day zero, one, two or three (*Table 2*). In most of these styles the number of pollen tubes present was so prolific they could not be quantified.

Table 2. – Pollen tube growth in the styles of clones 85 and 87 when pollinated from 0 to 7 days after emasculation. Five styles were examined at each harvest which was made when styles showed the first signs of style abscission.

Time of pollination (days since emasculation)	Pollen tube growth
Clone 85	
0	Numerous [a] pollen tubes to the base of all styles
1	Numerous pollen tubes to the base of all styles
2	Numerous pollen tubes to the base of all styles
3	Numerous pollen tubes to the base of all styles
4	4 styles - numerous pollen tubes to the base of style 1 style - numerous pollen tubes 0.5 - 0.7 mm long
5	4 styles - numerous pollen tubes to the base of style 1 style - numerous pollen tubes 0.3 - 0.5 mm long
6	4 styles - numerous pollen tubes 0.1 – 0.2 mm long 1 style - 16 pollen tubes 1 mm long
7	Numerous pollen grains on stigma, few pollen tubes observed
Clone 87	
0	4 styles - 25 - 50 pollen tubes to the base of the style 1 style - numerous pollen tubes to the base of style
1	Numerous pollen tubes to the base of all styles
2	Numerous pollen tubes to the base of all styles
3	Numerous pollen tubes to the base of all styles
4	4 styles - numerous pollen tubes 2.5 - 3.0 mm long 1 style - 11 pollen tubes reached the base of the style
5	3 styles - numerous pollen tubes about 1 mm long 2 styles - numerous pollen tubes 2.5 - 3.0 mm long
6	Numerous pollen tubes 0.25 mm long 1 style with a few pollen tubes 1.0 mm long
7	Numerous pollen tubes 0.1 - 0.2 mm long

[a]) When pollen tube numbers were too prolific to count they were recorded as numerous.

In clone 85 numerous pollen tubes grew the entire length of the style in 80% of flowers pollinated four or five days after emasculation (*Table 2*). In the remaining styles pollen tubes stopped after 0.3 mm to 0.7 mm of growth. If flowers were pollinated after day five pollen tube growth was arrested before tubes reached the base of the style.

In clone 87 the number of pollen tubes reaching the base of the style fell dramatically if flowers were pollinated more than three days following emasculation. If pollination was delayed to day five or later, pollen tube growth was arrested before tubes reached the base of the styles.

When flowers were pollinated on day zero immediately after emasculation the pollen remained on the stigma for about three days without germinating (*Table 3*). Pollen germination

coincided with styles turning pink / red and stigmas beginning to enlarge, turn yellow and release a sticky exudate. It should be noted that the flowers used in this experiment developed at a slightly slower rate (by about one day) than was usually observed.

Table 3. – Pollen tube growth in the styles of clones 85 and 87 pollinated with clone 42 at the time of emasculation. Five styles were harvested and examined from 0 to 4 days following pollination.

Time of harvest (since pollination)	Appearance of style / stigma at time of harvest	No. of stigmas with pollen grains present	No. of styles with pollen tubes present
Clone 85			
0	green	0	0
6 hours	green	0	0
1 day	green	2 (3 - 8 pollen grains)	0
2 days	styles pink, stigmas green	1	0
3 days	styles pink, stigmas beginning to enlarge, produce sticky exudate and turn yellow	4	0
4 days	styles pink / red, stigmas enlarged, yellow and producing sticky exudate	5	5 (tube length 2 mm)
Clone 87			
0	green	0	0
6 hours	green	1 (3 pollen grains)	0
1 day	green	0	0
2 days	green	0	0
3 days	styles pink, stigmas beginning to enlarge, produce sticky exudate and turn yellow	5	0
4 days	styles pink / red, stigmas enlarged, yellow and producing sticky exudate	5	5 (tube length 1 mm)

The timing of pollen germination and pollen tube growth in the style was also examined when flowers were pollinated three days after emasculation (when the stigma was yellow, enlarged and sticky). In both crosses examined, 85 x 84 and 87 x 84 numerous pollen grains had germinated on the stigmas three hours after pollination. After six hours pollen tubes had grown 0.2 mm to 0.7 mm long. By 24 hours they had grown half way down the style (about 2 mm) and by 48 hours they had grown beyond the base of the style.

Controls

No capsules were produced in the controls that were emasculated and bagged but not pollinated for either clone 85 or 87.

Discussion

Stigma receptivity based on seed production peaks in *E. camaldulensis* three days after emasculation which was carried out at the time of operculum shed. Three days after emasculation the style turned red and the stigma became enlarged, yellow and sticky. Similar associations between changes in stigma appearance and peak receptivity have been observed in other *Eucalyptus* species (CAUVIN, 1984; GRIFFIN and HAND, 1979; HODGSON, 1976; SAVVA *et al.,* 1988; SEDGLEY and SMITH, 1989; TIBBITS, 1986). The rate of development from operculum shed to style abscission varies considerably between species. *E. camaldulensis* has the fastest development recorded to date, with stigma receptivity peaking at day three and style abscission occurring from day eight to nine. *E. urnigera* has one of the slowest rates of development with stigma receptivity being observed from day thirteen to twenty eight after operculum shed (SAVVA *et al.,* 1988). These differences may be partly explained by differences in habitat and flowering time. *E. urnigera* is found in high altitudes and flowers during winter (SAVVA *et al.,* 1988), while *E. camaldulensis* flowers in much warmer environments from spring to summer.

Although peak receptivity was the same for the two *E. camaldulensis* clones examined the pattern of receptivity varied. The period of receptivity that produced a reasonably high amount of seeds (greater than 65% capsule set and 25 seeds / capsule) was two days longer in clone 85 than in clone 87 when both clones experienced the same climatic conditions.

The stigmas of flowers pollinated after day five in clone 85 or after day three in clone 87 were capable of sustaining the germination of numerous pollen grains but most or all the subsequent pollen tube growth was arrested before reaching the base of the style (*Table 2*). The decline of receptivity was not associated with visual changes in flowers. A change in the composition or amount of exudate secreted from the stigmas or physiological changes in the stylar tissue or both may have been responsible for blocking pollen tube growth.

Seed was produced from *E. camaldulensis* flowers pollinated immediately after emasculation. This has also been observed in *E. grandis* (HODGSON, 1976) and *E. gunnii* (CAUVIN, 1984). However in these two species the amount of seed produced from such early pollination was very low. *E. camaldulensis* on the other hand produced a high number of seeds, with clone 87 producing a comparable number of seeds per capsule when pollinated at the time of emasculation and at peak stigma receptivity.

At the time of emasculation the stigmas of *E. camaldulensis* flowers were green and dry and did not sustain the germination of pollen grains. Ungerminated pollen was washed from the stigmas during histological processing. The pollen remained on the stigma for about three days until it began to enlarge and produce an exudate. The pollen then began to germinate. HODGSON (1976) also demonstrated that pollen grains remain ungerminated on the stigmas of *E. grandis* for several days. The ability of *E. camaldulensis* flowers to produce high amounts of seed when pollinated at emasculation may be partly due to the rapid development of stigma receptivity in this species. The only other species studied that produce some seed when pollinated at emasculation, *E. grandis* and *E. gunnii,* also have a fairly fast rate of flower maturation, with stigma receptivity peaking about day five. A delay in stigma receptivity may reduce the viability of pollen remaining on the stigma or increase the likelihood of the pollen being knocked off the stigma. These factors and variation in the rate of development of stigma receptivity in individual flowers was probably responsible for the reduction in the capsule set observed in *E. camaldulensis* flowers pollinated at emasculation.

Stigma morphology may also be important in allowing pollen to remain on the stigma for a certain time before germination (GRIFFIN and HAND, 1979). Generally the stigmas of *Symphyomyrtus* (of which *E. camaldulensis* is a member) are made up of many papillae (BOLAND and SEDGLEY, 1986). Such stigmas would have a larger surface for pollen to contact than the

stigmas of other subgenera that have few papillae such as *Monocalyptus*. The timing of control pollination would be more crucial in such species.

There would be commercial benefit in being able to pollinate flowers at the time of emasculation. It would cut down on the cost of labour and travel. From this study it is apparent that a considerable amount of seed can be produced from *E. camaldulensis* flowers pollinated at the time of emasculation. The benefits of such action compared to the increased seed produced when flowers are pollinated at peak receptivity would have to be analysed. Further studies on different genotypes in various environments are also required. HODGSON (1976) and GRIFFIN and HAND (1979) have shown a cool change in the weather can delay peak receptivity by up to two days. Such a delay in *E. camaldulensis* may significantly reduce the amount of seed produced when flowers are pollinated at emasculation.

This study indicates the optimal time to pollinate *E. camaldulensis* is just as the style turns red and the stigma becomes enlarged, yellow and sticky (three days following emasculation). However receptivity falls dramatically after this time without displaying any visual changes. Therefore pollination should be carried out before, or at peak receptivity rather than following it. Results indicate that there is potential to emasculate and pollinate *E. camaldulensis* flowers on the same day with only relatively small losses in seed production. This factor combined with relatively high seed production and the rapid maturation of capsules (14 to 16 weeks under conditions at Kwinana, Western Australia) makes *E. camaldulensis* highly amenable to controlled pollination techniques.

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Study of Early Selection in Tree Breeding

1. Advantage of Early Selection through Increase of Selection Intensity and Reduction of Field Test Size

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Summary

There are three main advantages for early selection in tree breeding: 1.) increased selection intensity or reduced field-testing size; 2.) a shortened generation interval; and 3.) genetic information from early testing can be used to enhance selection efficiency at mature age. The first advantage is realized when early testing results can be used for culling families with the poorest performance prior to field testing. The expected genetic gain formula is derived for early plus mature two-stage successive selection. This formula is used to study the first advantage of early selection, which results in an increase in total selection intensity or reduction of field-testing size. The gain increase from early selection for a larger base population and gain decrease from early culling of the poorest families is a function of heritabilities, selection intensities on early and mature traits and their phenotypic and genetic correlation. Both early-mature genetic correlation and heritability of the early trait affect the magnitude of genetic gain increase for the mature trait from early selection. The formula is also used to answer the following three questions: (1) is it possible that early selection can be used to reduce the size of field testing without any loss in ultimate gain for the mature trait? (2) are there any conditions where more gain can be obtained when both early and mature selection are practiced than when selection is only practiced at the mature stage? (3) what is the condition where any selection at the early stage will result in less gain than if all selection is postponed to the mature stage? Depending on genetic parameters, all above three conditions are possible. The relationships of genetic parameters for satisfying one of the three conditions were derived from the formula and the theory is applied to a lodgepole pine retrospective early selection study.

Key words: Early selection, indirect selection, two-stage successive selection, genetic gain, lodgepole pine.

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