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Baskorowati, L. 2006. Controlled pollination methods for *Melaleuca alternifolia* (Maiden & Betche) Cheel.
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Controlled pollination methods for *Melaleuca alternifolia* (Maiden & Betche) Cheel

Liliana Baskorowati

Australian Government
Australian Centre for International Agricultural Research

Canberra
2006

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Foreword

The production of tea-tree oil from *Melaleuca* species in Australia began some 80 years ago, and the industry has grown steadily, if erratically, since then. The main species of *Melaleuca* grown for foliar essential oil is now *M. alternifolia*.

A tea-tree oil R&D plan for 2006–2011 developed by the Rural Industries Research and Development Corporation (RIRDC) notes that a strength of the industry is the availability of higher-yielding seed to reduce costs of production, and a breeding program to provide continuous improvement. The plan envisages oil yield gains of up to 150% from the breeding program by 2010–2011.

Controlled crosses are part of the breeding strategies for *M. alternifolia*, to provide new, elite genotypes for the breeding program. There is interest too in crossing *M. alternifolia* and its close relatives, to yield productive hybrids suited to a wider range of growing sites.

This report is a practical guide to production of controlled-cross seed of *M. alternifolia*. Some or all of the techniques it describes may be adaptable to related species cultivated for essential oils, and to other locations.

ACIAR acknowledges RIRDC's long-standing commitment to tea-tree breeding research.

Peter Core
Director
Australian Centre for International Agricultural Research

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Controlled pollination methods for *Melaleuca alternifolia* (Maiden & Betche) Cheel

Liliana Baskorowati[1]

Introduction

Melaleuca is a large genus of the Myrtaceae family and comprises over 230 species with about 219 species endemic to Australia (Craven and Lepschi 1999). Within this genus, several species are valuable for commercial production of foliar essential oil: *M. alternifolia* (Maiden & Betche) Cheel, *M. cajuputi* ssp. *cajuputi* Powell and *M. quinquenervia* (Cav) Blake are examples (Brophy and Doran 1996; Doran 1999), with *M. alternifolia* currently of most interest to Australian producers.

Three main chemical varieties (chemotypes) of *M. alternifolia*, rich in either 1,8-cineole, terpinolene or terpinen-4-ol, are recognised. The terpinene-4-ol rich chemotype of low 1,8-cineole content (<5%) has undergone most commercial development (Davis 2003; Southwell 2003).

Limited production (2–20 tonnes/year) of Australian tea-tree oil commenced in 1926 in natural stands of *M. alternifolia* on the north coast of New South Wales (Davis 2003).

Increasing demand for this oil from the late 1980s fostered the development of plantations which now total 4000 ha to meet an annual demand for oil approaching 500 tonnes. Most plantations are in northern New South Wales and northern Queensland.

Improving the quantity and quality of oil produced has been the objective of a plant selection and breeding program for *M. alternifolia* in Australia since 1993 (Figures 1 and 2).

Figure 1. Seedling seed orchard of *Melaleuca alternifolia* near Lismore, New South Wales, Australia

Controlled crosses are part of the breeding strategies for this species, to concentrate the best alleles from a range of selected trees and provide new elite genotypes for the program. In addition, inter-species (hybrid) crosses between *M. alternifolia* and its close relatives, *M. linariifolia* and *M. dissitiflora,* are of interest for

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expanding the range of sites where the terpinen-4-ol oil type can be produced economically.

This report describes the practical steps necessary to produce controlled-cross seed of *M. alternifolia*.

Figure 2. Abundant flowers on *Melaleuca alternifolia* in a seedling seed orchard at West Wyalong, New South Wales, Australia

Flower structures and development

Inflorescences are spikes comprising 8–24 (length 5 mm; width 2 mm) small, individual flowers (Figure 3). Each flower is primarily complete with male and female reproductive organs, consisting of 4 sepals, 4 petals, 5 staminal columns from which numerous anthers are attached by short filaments and a small stigma on the end of the style (Figure 4).

All flower parts just before opening are enclosed by white petals. When the petals open, the staminal columns uncurl, exposing the stamens which have a white to creamy feathery appearance.

Figure 3. The inflorescence of *Melaleuca alternifolia* (scale 1:1 mm)

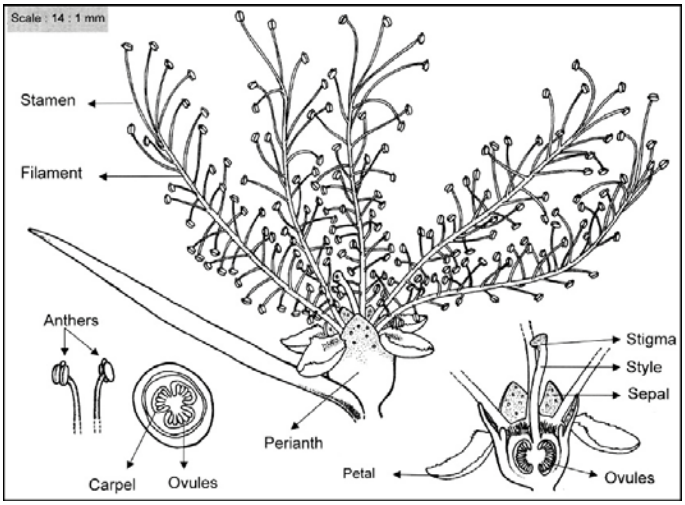

Figure 4. Flower of *Melaleuca alternifolia* (illustration by Teguh Triono 2005)

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Pollen of individual flowers is shed before the stigma is receptive (protandry), and thus self-pollination within the same flower is minimised.

Large numbers of flowers are produced per tree during a 2–3 week period (Figure 5) usually from mid October to late November. There is therefore plenty of opportunity for receptive stigmas to receive pollen from nearby flowers of the same tree, providing potential for self-pollination. However, despite this potential, the outcrossing rate in *M. alternifolia* has been reported to exceed 90% (Butcher et al. 1992).

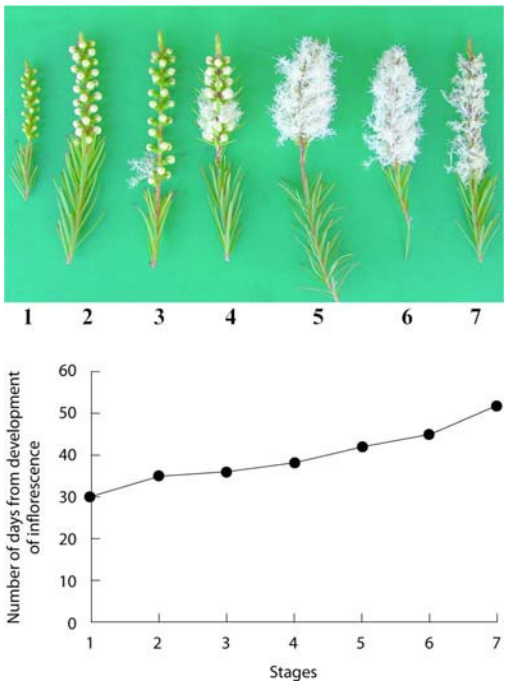

Figure 5. Stages of flower development of *Melaleuca alternifolia*. The x-axis gives the stage of flower development and the y-axis the number of days from initiation of inflorescence

To produce controlled crosses, pollen (Figure 6) from a selected male parent is placed on the receptive stigma of a female parent ('pollination') (Figure 7). Pollen germinates on the stigma and the pollen tube grows down the style to the ovary and fertilises the ovules ('fertilisation') (Figure 8). *Melaleuca alternifolia* pollen is triporate in structure.

Knowledge of flower structure and floral development, especially identifying the important stages of development to control pollination (Figures 5–10) is essential for manipulating crosses (both pollen collection and emasculation of flowers).

The flowering times of plants chosen for parents might vary. This is especially so when hybridisation is being attempted between different species, or when crosses are being undertaken between individuals of the same species but growing under different environmental conditions.

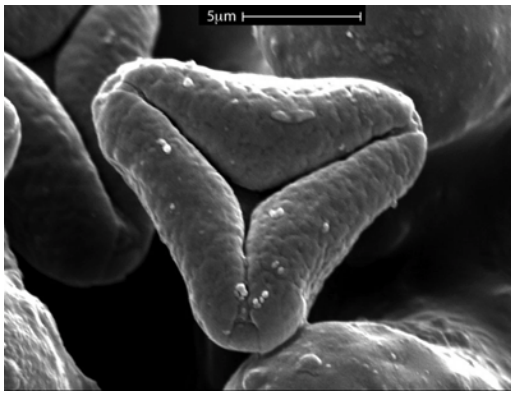

Figure 6. Pollen of *Melaleuca alternifolia*

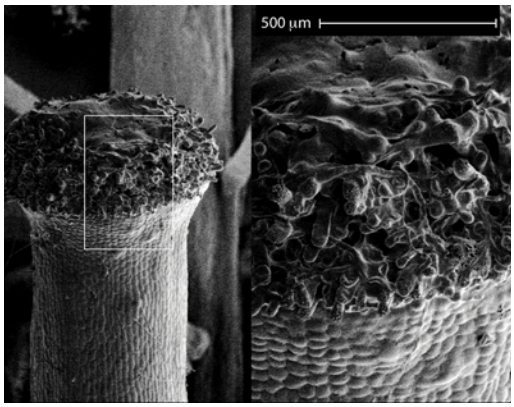

Figure 7. Receptive stigma (female organ) of *Melaleuca alternifolia* with abundant secretion

Pollen will often need to be collected in advance and stored so that it is available when required. When attempting pollen transfer between species (hybridisation) it is essential to understand the barriers that might inhibit success; there may be a degree of incompatibility between species.

Unopened flower buds must be isolated to ensure there is no contamination from other pollens. Melaleucas are insect-pollinated and, unless flowers are

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covered, some pollen transfer may occur following insect visits (Figures 11 and 12).

Pollen collection and storage

The pollen must come from the correct source and be uncontaminated by stray pollen.

Step 1 All open flowers are removed and the unopened flowers covered by pollination bags. The flowers inside the bag open and the staminal columns uncurl exposing the anthers, giving a white to creamy feathery appearance. The white anthers slowly change to a light brown colour over 3–4 days.

Step 2 The colour change from white to brown coincides with the splitting open of the anthers, exposing the pollen grains (Figure 9). At this stage, the flowers should be collected.

Step 3 Flowers should be placed on a piece of paper or flat dish, and placed in desiccators to dry out (Figure 13), or bottled for freeze-drying (Figure 14).

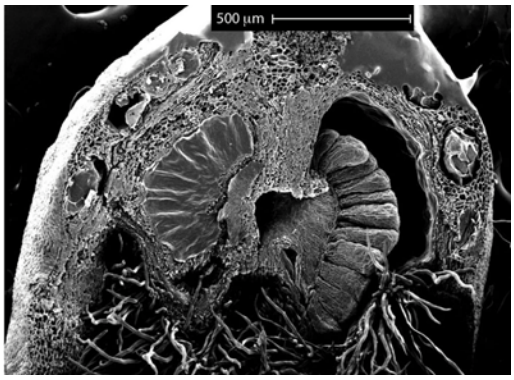

Figure 8. Ovary and ovules of *Melaleuca alternifolia*

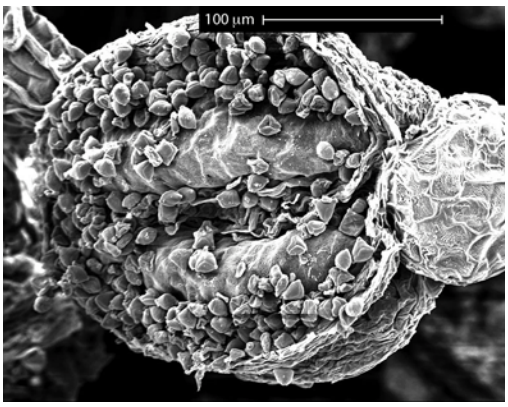

Figure 9. Open *Melaleuca alternifolia* anther, shedding pollen

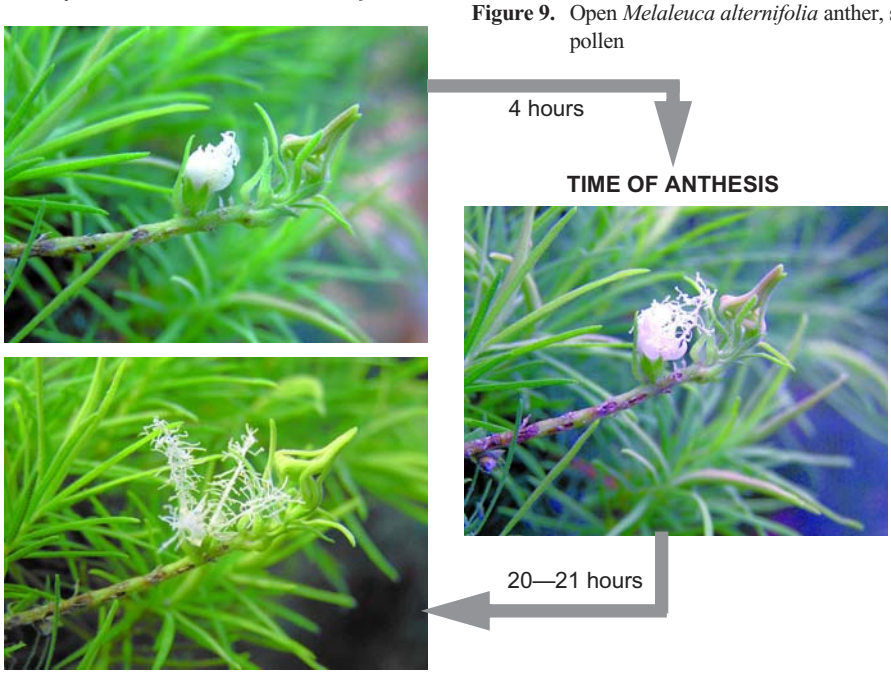

Figure 10. Time of anthesis of *Melaleuca alternifolia*

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Figure 11. Insects visiting *Melaleuca alternifolia* flowers: a. honey bee (*Apis mellifera*); b. butterfly 'blue' family Lycaenidae; c. butterfly family Nymphalidae; d & e. wasp family Sphecidae; f. wasp family Vespidae; g. brown beetle family Lycidae; h. fly family Calliphoridae

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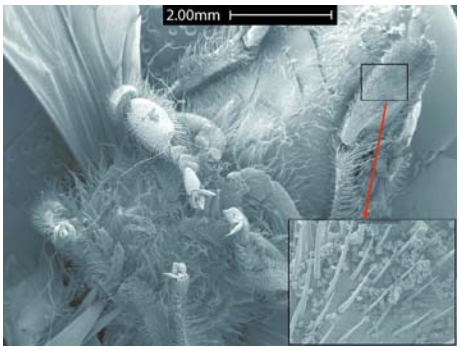

Figure 12. Insect (honey bee) carrying pollen on its body

Drying pollen over silica gel in a desiccator

- Prepare the flower buds for sieving by drying over silica gel in a desiccator for 3 days at room temperature.
- The base of the desiccator should be filled with fresh silica gel to assist the drying process.
- The plant material is then passed through a 45 micron sieve to remove the pollen.
- Pollen is placed in a glass bottle or vial, sealed, labelled and stored at sub-freezing temperatures (e.g. –18°C). Sub-freezing storage is best combined with a desiccant to maintain low humidity.
- Between flower sampling, all equipment must be cleaned by washing or spraying with alcohol.

Figure 13. Flowers drying in a desiccator

Drying pollen using a freeze-dryer

If available, a freeze-dryer (Figure 14) is a useful method of drying a large number of pollen samples quickly. The method of use is as follows, though operators should also familiarise themselves with the machine and read the operating procedures:

- mature flowers should be collected and loosely packed into glass vials (Figure 15)
- the vials are then placed in the specific chamber with rubber stoppers positioned for later sealing (Figure 16)
- freeze-dry the pollen for about 24 hours
- seal vials and store as described above.

Figure 14. A freeze-dryer

Figure 15. Collecting flowers for pollen extraction

Pollen for cross-pollination should be as fresh as possible. It can be stored for a few days under low humidity in desiccators, but its viability may drop considerably after 10 days of storage.

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Melaleuca pollen has been successfully stored for short periods at temperatures of 3–5°C. For long-term storage, however, temperatures of –18°C to –20°C are recommended. Pollen of *M. alternifolia*, for example, will retain acceptable viability for 11 months if stored at –18°C in sealed vials (Figures 16 and 17).

Figure 16. Flowers in vials in freeze-dryer

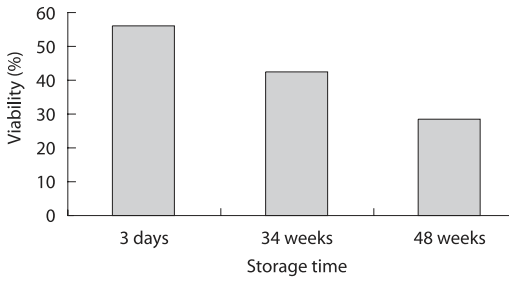

Figure 17. Decline in viability of pollen of *Melaleuca alternifolia* over time when stored in sealed vials at –18°C

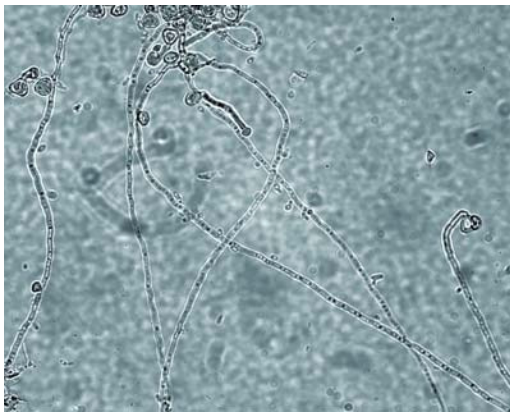

Figure 18. Germinated pollen (×20 magnification) of *Melaleuca alternifolia*

Testing pollen viability

A pollen viability test should be carried out as soon as possible after collection of flower buds and extraction, before the pollen is stored. Testing should also be undertaken before the pollen is used for pollinating flowers, especially if it has been held in storage for some months. Adequate fertilisation can sometimes be obtained using pollen with a germination rate as low as 10%, but best results are obtained with higher viabilities. There are numerous methods of testing pollen viability. The method below is simple and has given consistent results.

Use a small screw-capped glass vial that has been sterilised. Prepare a medium consisting of 30% (300 g) sucrose and 150 ppm (150 mg) of boric acid in 1 litre of distilled water. Place 3–4 drops of the medium (sufficient to cover the base) into the vial. Add a small amount of pollen mix using a toothpick. Secure cap, shake and label the vials, and place them in a germination cabinet at 20–25°C for 24 hours.

After germination, which usually takes 1–2 days, use a 1 mL pipette to extract from the vial a drop of liquid containing medium and pollen. Place a drop on a microscope slide and view at about ×20 magnification. Germinated (Figure 18) and ungerminated pollen can be counted and a germination percentage calculated.

Selection of trees and branches for pollination

The selection of trees and branches on which flowers will be pollinated is an important step. Consider the following factors:

- Trees must be healthy. There is no point in spending a lot of time and effort on pollination if the tree aborts its flower buds because of stress; or worse, dies.
- Trees with a medium to heavy crop of flower buds should be chosen, as this will give greater opportunities for selection of quality flowers. In cases where trees must also serve as pollen parents, an initial survey and ranking of individuals based on the number of branches suitable for emasculation (removal of male flower parts) and as a pollen source aids selection.
- Select unshaded, strong branches where growth is vigorous. Branches low in the crown or close to the ground run the risk of damage by passing traffic.

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Branches need to be strong, as they will be supporting a pollination bag for about 14 days. Strong winds may break the branch because of the added weight of the bag. In some cases, the bag may have to be tied to a nearby branch for support. Too much branch movement may result in the stigma coming in contact with twigs and leaves or the pollination bag and being damaged.

- Be sure to replicate each cross on each mother tree as security against breakage. The replicates (bags) of each cross should be spread around the crown for added security.

Emasculation and isolation

A branch that has about 10 inflorescences, each subtended by a healthy vegetative bud, is selected. Inflorescences without a healthy vegetative bud at their apex may fail to grow, inducing capsule abortion. Any advanced, open flowers are removed. The aim is to emasculate about 50 flowers in each bag. Any immature capsules from last year's crop should also be removed at this point. To ensure there is no contamination from outside pollen, a pollination bag should be fitted to cover the inflorescences. Cotton wool or foam should be used at the base of the bag as insulation for the branch and to ensure exclusion of insects.

Removal of all male parts can be carried out with a small pair of tweezers. It is very difficult to emasculate the small flowers before they open. As anthers do not shed their pollen for a few days after extension, it is easier to emasculate when the staminal columns have started to uncurl. Advanced buds (Figure 19) can be emasculated by prying open the top of the bud with sharp tweezers (Figure 20) and carefully removing sepals and filaments so as not to damage the style, which is still bent at this stage (Figure 21).

Close examination with a ×10 magnifying glass will show if anthers have been successfully removed. It may be necessary to remove some foliage to avoid a build-up of moisture within the bag.

Once emasculation is completed, the pollination bag should be replaced and tied on (Figure 22). Next, the branch has to be clearly labelled, preferably with a metal tag or 'Dymo' tape. It must be considered that the tag will need to remain firmly attached on the branch for about 14 months.

For various reasons, some tags do fall off. It is thus suggested that two labels per bag be used (Figure 23). This small investment of extra time provides insurance for a process that has cost a lot of effort.

As a high level of outcrossing rate has been identified, it may be possible to pollinate without first emasculating each flower. This would save considerable time and place less stress on individual flowers. Pollination without emasculation has been field tested and is suitable where some (<10%) self-pollination is acceptable.

Applying pollen

Before flower opening, the styles are small and bent over. After flower opening, the style straightens and increases in length. The stigma, on the tip of the style, enlarges and appears shiny, moist and sticky, indicating receptivity. Once the stigma is receptive,

Figure 19. Advanced bud and freshly opened flowers which are used for emasculation

Figure 20. Emasculating flowers of *Melaleuca alternifolia* using tweezers

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pollen can be applied. Pollen is applied using a small brush (Figure 24) or stick, or the lid of the vial.

This procedure should be repeated during the subsequent few days, to ensure all stigmas receive pollen at the receptive stage. It is important to rinse hands and pollen applicator with ethanol between pollinations to ensure no contamination occurs from pollen used in a previous cross. It is recommended that one brush be used for each type of pollen, and labelled as such, to avoid any confusion of pollen source.

Sequence of pollination

Step 1. Select branch. Remove mature capsules, opened flowers and immature flower buds (Figure 25).

Step 2. Label branch with metal and colour tags (Figure 26). If a range of colours is available, use of a colour specific to the pollen parent aids identification for subsequent pollinations.

Step 3. Emasculate flower buds (Figure 27).

Step 4. Place pollination bag over branch (Figure 27). Record event.

Step 5. Inspect after 2–3 days.

Step 6. When the stigma is receptive, apply pollen with brush or vial cap. Replace bag, record event (Figure 28).

Step 7. Repeat pollination after 2–3 days.

Step 8. Once fertilisation is complete, stigmas turn brown and abscise. About 14 days after last pollination, pollination bag should be removed (Figure 29).

Step 9. Collect mature capsules identified by their labels. This occurs at 14–18 months after pollination.

Figure 21. Bent styles after emasculation

Figure 23. Colour tag and metal tag for bag labelling

Figure 22. Bagging the emasculated flowers

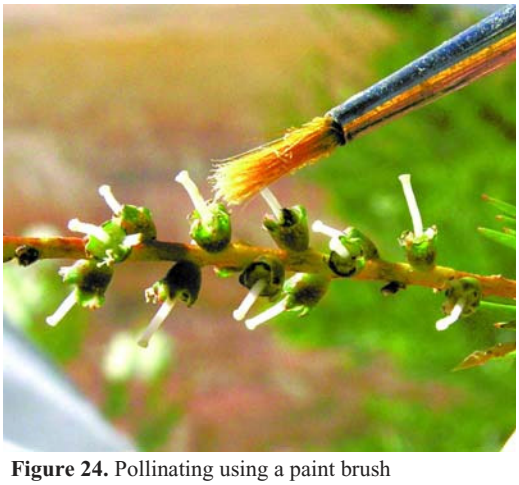

Figure 24. Pollinating using a paint brush

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Figure 25. Removing opened flowers and immature and mature capsules

Figure 26. Labelling the branch

Figure 27. Emasculating the flowers of *Melaleuca alternifolia*; completed branches (inflorescences) are bagged

Figure 28. Applying pollen to receptive stigmas

Figure 29. Removing the pollination bag 14 days after pollination

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Figure 30. Fruit (capsules) of *Melaleuca alternifolia* at different stages of development: a. 2 weeks after anthers shed; b. 4 months old; c. 14 months old and ready to harvest. Capsules may be retained on a tree for some years.

Bag removal and seed collection

Pollination bags must be left in place until fertilisation is complete. When fertilisation is completed, the stigma is no longer sticky and shiny and has turned brown. This occurs about 7 days after pollination, at which time the pollination bag should be removed. Delay increases the chances of wind damage and/or stress to the developing flower buds by high temperatures.

In *M. alternifolia* it takes about 14–18 months for the seed to mature and be ready for harvest. Each mature capsule, which is dark brown in colour (stage C, Figure 30), consists of 20–25 seeds on opening.

Record keeping

It is important to keep a record of pollination details. Data to be recorded should include male and female parent, date of emasculation, date of pollen application and number of flowers pollinated at each treatment, and date of bag removal. Using a special Control Pollination Records sheet (Appendix 1) facilitates this process.

Materials

A list of the materials required for pollinating melaleucas follows. The materials are shown in Figure 31.

- magnifying headset
- magnifying glass
- labels (metal + wet-strength paper of various colours)
- ethanol
- metal ties + wire
- pollination record sheets
- small paint brushes
- pencil

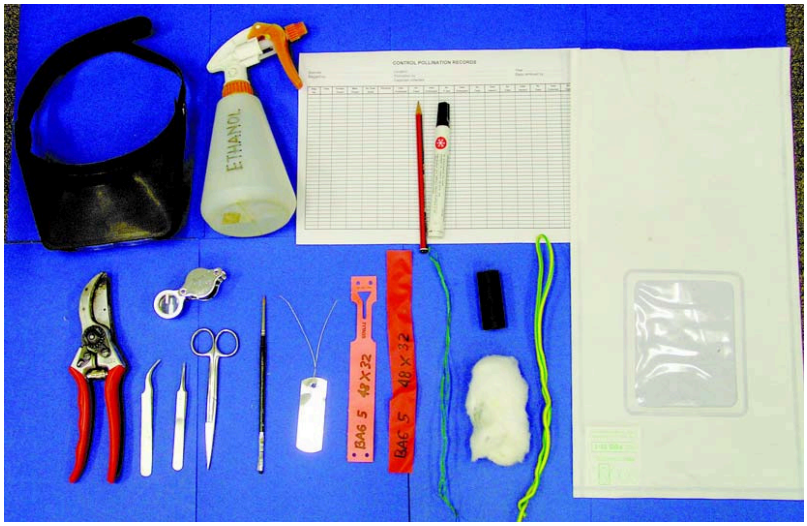

Figure 31. Materials used in controlled pollination

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- scissors
- waterproof marker
- tweezers
- pollination bags
- rope
- secateurs
- cotton wool or foam
- ladders (if required).

Acknowledgments

This technical report describes methods used in my PhD research. I am very grateful to ACIAR, ANU and CSIRO for institutional support. I am indebted to John Doran (CSIRO), Mike Moncur, Paul Warburton (CSIRO), Garry Baker (NSW, DPI) and Mauro Davanzo (ANU) for introduction to the technique and field assistance, and to Roger Heady and Cheng Huang (RSBS, ANU) for scanning electron microscopy. I am also grateful to my supervisory panel—Peter Kanowski, John Doran, Mike Moncur and Saul Cunningham—for comments and discussion during the preparation of this report.

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Appendix 1

CONTROL POLLINATION RECORDS

Species ……..……..……..
Bagged by ……..……..……..

Location ……..……..……..
Pollination by ……..……..
Capsules collected ……..……..

Year ……..……..
Bags removed by ……..……..

Bag No	Date	Female Parent	Male Parent	No flowr /buds	Remarks	Date Pollinated	No Flwrs	Date Pollinated	No Fl wrs	Date Debagged	No Caps	Date Inspected	No Caps	Date harvested