Chapter 15

In Vitro Propagation of *Acacia mangium* and *A. mangium* × *A. auriculiformis*

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Abstract

Acacia mangium and *A. mangium* × *A. auriculiformis* hybrids have gained an increasing interest in reafforestation programs under the humid tropical conditions, mainly for pulpwood production. This is due to their impressive growth on acid and degraded soils, as well as their capability to restore soil fertility thanks to their natural nitrogen-fixing ability. It is crucial to develop efficient methods for improving the genetic quality and the mass production of the planting stocks of these species. In this regard, in vitro micropropagation is well suited to overcome the limitations of more conventional techniques for mass propagating vegetatively selected juvenile, mature, or even transgenic genotypes. Micropropagation of *A. mangium* either from seeds or from explants collected from outdoors is initiated on Murashige and Skoog (MS) basal medium supplemented with 4.4 μM BA. Microshoot cultures produced by axillary budding are further developed and maintained by regular subcultures every 60 days onto fresh MS culture medium added with 2.2 μM BA + 0.1 μM NAA. This procedure enhances the organogenic capacity for shoot multiplication by axillary budding, with average multiplication rates of 3–5 every 2 months, as well as for adventitious rooting. The rooting is initiated on Schenk and Hildebrandt culture medium containing 4 μM IAA. The maintenance of shoot cultures in total darkness for 3 weeks increases the rooting rates reaching more than 70%. The hybrid *A. mangium* × *A. auriculiformis* genotypes are subcultured at 2-month intervals with an average multiplication rate of 3 and rooting rates of 95–100% on a half-strength MS basal medium containing 1.1 μM NAA. The rooted microshoots are transferred to ex vitro controlled conditions for acclimatization and further growth, prior to transfer to the field, or use as stock plants for cost-effective and true-to-type mass production by rooted cuttings.

Key words: *Acacia* hybrids, Adventitious rooting, Axillary budding, Cloning, Legume tree, Micropropagation

1. Introduction

Acacia genus belongs to the Leguminoceae family that encompasses some 1,300 tree and shrub species, mainly native to Australia and to the tropical and warm-temperate regions of both hemispheres including Africa, southern Asia, and the Americas. As

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legumes, *Acacia* species have the natural ability to fix atmospheric nitrogen and hence can thrive in low fertility soils (1, 2). They can be used for combating desertification or for restoring degraded lands (*A. tortilis*, *A. nilotica*), and also for producing gum (*A. senegal*), tannin (*A. mearnsii*), fodder, and also wood for different end-uses (3–6). Both *A. mangium* and *A. auriculiformis* originate from Papua New Guinea (PNG), eastern provinces of Indonesia and northeast Queensland in Australia (7). These two arborescent species can hybridize naturally to give rise to the interspecific hybrid *A. mangium* × *A. auriculiformis*. This hybrid is more site-adaptable, produces higher yield of denser wood and cuttings with higher capacity for adventitious rooting than *A. mangium*. For all these reasons and also due to the severe drawbacks associated with seed propagation of interspecific hybrids, the mass clonal propagation of *A. mangium* × *A. auriculiformis* superior genotypes is of strategic importance. Since their introduction as exotics in many tropical countries, especially in South-East Asia, *A. mangium*, *A. auriculiformis* and the hybrids *A. mangium* × *A. auriculiformis* have gained increasing popularity for reafforestation programs (7–9). This striking expansion of *Acacia spp.* plantations within a few years, mainly for pulp-wood production, is due to their impressive growth performance under humid tropical conditions, especially on acid and degraded soils, as well as to their capability to restore soil fertility thanks to their natural nitrogen-fixing ability (10–14). There is therefore a crucial need to develop efficient methods for mass producing superior-quality or transgenic planting stocks of these species (6, 15). In this respect, the in vitro techniques, specifically micropropagation (16–18), are worth considering for overcoming the limitations of more conventional nursery methods used for vegetatively mass propagating superior genotypes, especially mature ones (19–21).

The aim of this chapter is to provide protocols for sustainable micropropagation of the highly economically important *A. mangium* and the interspecific hybrid, *A. mangium* × *A. auriculiformis*. Stepwise information on the successive phases of primary culture initiation, establishment of organogenic cultures, rooting, and ex vitro acclimatization suitable for juvenile and mature selected genotypes is provided. Such information can also be useful for genetic engineering (6, 22–24).

2. Materials

2.1. Sterilization of Outdoor-Grown Selected Plant Material

1. Select mother plants growing naturally outdoors, or hedged stock plants in the nursery, or seeds.

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2. Shears, secateurs (or averruncator or a ladder or a climber for not easily accessible shoots), ice box, plastic bags, moistened newspaper, permanent markers or pencils, notebooks.
3. Tap water.
4. Ethanol 70% and 95% (v:v).
5. Wetting agent such as dishwashing liquid soap or Tween 20.
6. Commercial bleach solution (NaClO) diluted 1:10 (v:v) with tap water, mercuric chloride ($HgCl_2$ 1 and 2 mg/L).
7. Autoclaved pure or ultrapure water (distilled, reverse-osmosis water) in 250 mL screw capped bottles.
8. Autoclaved absorbing paper (filter paper, newspapers).
9. Magnetic stirrer, magnetic bar.
10. Usual tissue culture facilities, equipment and instruments—laminar flow hood, bunsen or ethanol burner, scalpel, forceps, glass culture tubes (21 × 150 mm covered with polypropylene caps).
11. Tea strainer.
12. Binocular microscope and cold light source for shoot apex excision.
13. Cellulosic or autoclave-resistant and chemically neutral 20 × 30 mm plugs (Sorbarod).
14. Specific dissecting tools (mounted razor blade splinter).

2.2. In Vitro Culture

1. Glass culture tubes held in appropriate racks with polypropylene or polycarbonate caps.
2. Glass culture jars and flasks (total capacity of 300–800 mL) with appropriate polypropylene or polycarbonate caps.
3. General medium preparation equipment (pH meter, precision and analytical balances, macro- and equipment micro-salts required for Murashige and Skoog (MS (25)), and Schenk and Hildebrandt (SH (26)) media (see Table 1).
4. Culture rooms with automatic temperature control (26 ± 2°C) and a 16 h photoperiod (50–60 μmol/m^2/s, "ORSAM L36W/77" fluorescent lamps).

2.3. Acclimatization to Ex Vitro Conditions

1. Bowls and plastic containers, that can be filled with water and aqueous solutions of fungicide for soaking the collected plant material and for transfer from in vitro to ex vitro conditions.
2. Forceps.
3. Rooting substrate (river sand, peat, composted bark) that can be used in mixture.
4. Shade-, lath-, or greenhouse facilities equipped with polyethylene-covered benches or beds, automatic mist or fog system.

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Table 1
Composition (in mg/L) of the basal and rooting media used for micropropagating *Acacia magium* and the hybrid *A. mangium* × *A. auriculiformis*

	Acacia mangium		*A. mangium* × *A. auriculiformis*
	Basal medium	**Rooting medium**	**Basal medium**
KNO_3	1,900	830	950
NH_4NO_3	1,650	100	825
KH_2PO_4	170		85
$CaCl_2 \cdot 2H_2O$	440	70	220
$MgSO4 \cdot 7H_2O$	370	130	185
$FeSO4 \cdot 7H_2O$	27.8	13.7	27.8
Na_2EDTA	37.3	18.6	37.3
H_3BO_3	6.2	3.1	6.2
$ZnSO_4 \cdot 7H_2O$	8.6	4.3	8.6
$MnSO_4 \cdot H_2O$	16.9	8.4	16.9
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.12	0.25
KI	0.83	0.41	0.83
$CuSO_4 \cdot 5H_2O$	0.025	0.012	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.012	0.025
Myo-inositol	50	50	50
Glycine	2	2	2
Thiamine HCl	1	1	1
Pyridoxine HCl	1	1	1
Nicotinic acid	1	1	1
Casein hydrolysate	200	200	200
BA	0.5 (2.2 μM)		
NAA	0.02 (0.1 μM)	0.7 (4 μM)	0.2 (1.1 μM)
Sucrose	20,000	20,000	20,000
Phytagel	2,500	2,500	2,500

5. Fungicides (thiram, benomyl).
6. Recyclable or disposable containers of various kinds, e.g., plastic bags, root trainers, plastic pots, potting cells, Jiffy pots.

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Fig. 1. Shoot apex micrografting of mature *A. mangium*. (**a**) shoot apex (*arrow*) newly top grafted in aseptic conditions onto an in vitro-grown seedling rootstock; (**b**–**d**) scion elongation; (**e**) acclimatized micrograft in outdoor conditions. Scale bars correspond to 1 mm for **a**–**c**, and to 2 mm for **d**.

3. Protocols

Acacia spp. can be micropropagated from in vitro germinated seeds (27, 28), transgenic cells (6, 24), and outdoor explants (18). In the latter case, in vitro introduction can be done directly on the culture medium (29), or by shoot apex micrografting (see ref. (30); Fig. 1).

3.1. Culture Medium Preparation

1. For *A. mangium* culture initiation, prepare MS medium (for details see Table 1) (see Note 1) by using stock solutions (see Note 2). Increasing BA concentration to 4.4 μM during culture initiation stimulates axillary bud development from nodal explants, collected from mature trees. Interspecific hybrid *A. mangium* × *A. auriculiformis* cultures require modified MS

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medium containing half strength of macronutrients and 1.1 μM NAA (see Table 1 (14, 31)).

2. Add 20 g/L sucrose and stir the medium continuously. Adjust the pH to 5.5–5.7 using 1 M KOH or NaOH.
3. Add 0.25% Phytagel (Sigma, St Louis, w/v) before heating the medium to about 80°C. Dispense 10 mL into glass culture tubes (21 × 150 mm), covered with polypropylene caps (see Note 3).
4. Sterilize culture media by autoclaving at 120°C and 95 kPa for 20 min (see Note 4).
5. Culture explants on solidified culture media under the laminar flow hood to prevent any contamination.
6. For *A. mangium*, the rooting medium SH/3 contains 1/3 strength SH macronutrients, half-strength MS micronutrients, and 4 μM indole-3-acetic acid (IAA) (see Table 1). *A. mangium* × *A. auriculiformis* shoot explants root on their basal medium.

3.2. Initiating the Cultures

3.2.1. From Seed

1. Place seeds in tea strainers before soaking 5–10 s in boiling water, followed by immersion for 5 min in 70% ethanol and then for 3 min in 1 mg/L $HgCl_2$ aqueous solution (see Note 5).
2. Rinse three times in sterilized ultrapure water under laminar flow hood.
3. Inoculate 2–3 seeds per tube (see Note 6) filled with Phytagel solidified culture medium, or place these seeds on liquid imbibed plugs in the test tubes for rootstock production (see Note 1). Transfer the seed-containing tubes to the culture room under a 16 h photoperiod (50–60 μmol/m^2/s, "ORSAM L36W/77" fluorescent lamps) at 26 ± 2°C. Maintain one germinated seed per tube; the germinations in excess can be transferred, upon the emergence of the radical, to other individual tubes.

3.2.2. From Outdoor Developed Plants

1. For field or nursery-grown selected acacia trees of any age, collect elongating shoots from the more accessible parts of the tree, preferably close to the root system. Epicormic shoots, excised or micro-grafted shoot apices (0.2–0.4 mm), can also be used (see Notes 7 and 8, Fig. 1).
2. Upon collection, maintain soft ramets in suitable humid conditions, wrapped for instance in moistened tissue paper and placed in cool boxes to avoid hydric stress until utilization in the shortest delays (see Note 9).
3. Cut shoots into small pieces of 2–3 cm long with at least one node. Wash under running tap water for several minutes.
4. Soak explants in 70% ethanol for 5 min, followed by immersion in 1 mg/L $HgCl_2$ (see Note 5) for 3 min, and 3 rinses in sterile distilled water.

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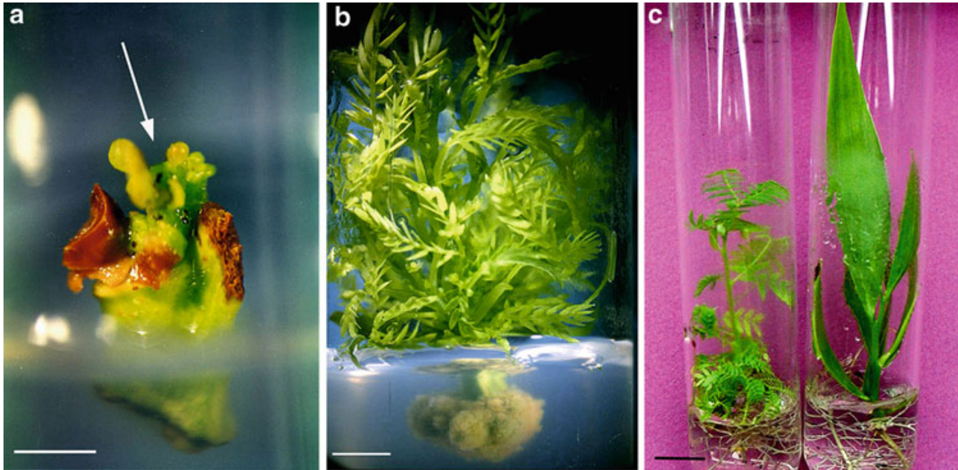

Fig. 2. Micropropagation of mature selected *A. mangium* genotypes. (**a**) Primary culture using a mononodal explant with an elongating axillary bud (*arrow*); (**b**) well-established explant showing a lot of axillary shoots with juvenile morphology; (**c**) in vitro rooted microshoots from the same mature clone exhibiting the compound-leaf juvenile morphology (*left*) and the phyllode mature morphology (*right*). Scale bars correspond to 0.5 mm for **a** and **b**, and 1 cm for **c**.

5. Cut upper and basal parts of the nodes to obtain 1 cm-long single node explants with a longer portion of segment below than above the node. This is done using scalpels on sterilized pieces of absorbing paper, under the laminar flow hood.
6. Culture explants vertically and individually in the tubes by digging 1–3 mm basal portion of the explant into the culture medium (see Figs. 2a and 3a), and transfer them to the culture room under above mentioned conditions for further development (see Note 10).

3.3. Developing and Maintaining the Cultures

1. After 2 months of primary culture, the base of every explant must be refreshed by a basal cut (see Note 11).
2. Trim the newly regenerated shoots in 1 cm-long microcuttings with at least one node or terminal bud, then transfer these microcuttings to tubes (1 or 2 per tube) containing fresh basal culture medium (see Note 12).
3. Place the newly inoculated tubes under the standard culture room conditions (16 h photoperiod and 26 ± 2°C).
4. The subcultures must be carried out regularly after every 2–2.5 month interval (see Note 13). As multiplication rate of the cultures increases, the test tubes can be replaced by suitable glass jars for higher mass production and cost efficiency (see Note 14; Fig. 3b, c). Cultures can be sustainably maintained using this protocol for years (see Note 15). Practical multiplication rates by axillary budding range between 3 and 5 at the end of every 2 month-long subculture (see Note 16).

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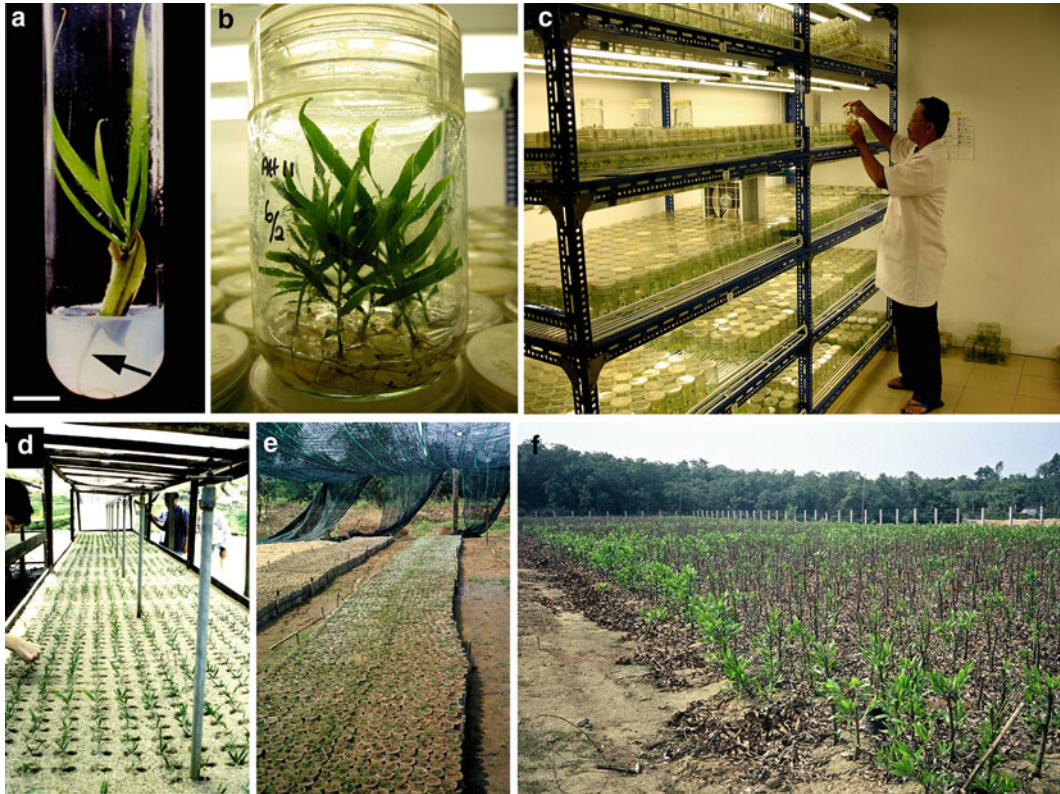

Fig. 3. Micropropagation of mature selected *A. mangium* × *A. auriculiformis* genotypes. (**a**) Primary culture using a mononodal explant, already rooted with an elongating axillary bud (*arrow*, scale bar = 1 cm); (**b**, **c**) mass production in flasks at YSG Biotech, Sabah, Malaysia; (**d**) ex vitro acclimatization under mist system facilities in SSSB, Sabah, Malaysia and (**e**) under shade-cloth in Vietnam; (**f**) in vitro rejuvenated *A. mangium* × *A. auriculiformis* cloned stock plants used for mass producing rooted cuttings at lower cost under appropriate nursery conditions in Vietnam.

3.4. Adventitious Rooting and Acclimatization to Ex Vitro Conditions

1. Almost all *A. mangium* × *A. auriculiformis* shoot explants root on the basal medium and standard light conditions.
2. For *A. mangium*, 2 cm-long shoot tip explants, trimmed from axillary shoots developed on the multiplication medium are used for rooting.
3. Insert the base of these 2 cm-long shoot tip explants into the rooting medium contained in each of the 21 × 150 mm glass culture tubes covered with polypropylene caps (see Note 3).
4. Cultures are maintained in total darkness for 3 weeks before transferring them back to the standard conditions, i.e., 16 h photoperiod (50–60 µmol m^2/s) at 26 ± 2°C.
5. The microshoots, showing newly formed elongating roots after 2–3 weeks (32), are pulled out from the culture tubes and the gelling agent sticking to the roots is carefully removed by washing under running tap water before transferring to ex vitro conditions (see Note 17).

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6. Insert the rooted base of the microshoots in the sand bed (see Notes 18 and 19; Fig. 3d, e) in the nursery under high humidity (see Note 20) and natural light exposure, but avoiding direct sun irradiance by the utilization of shade screens (30–50% of shade) made of various materials (polypropylene cloth, see Fig. 3e).
7. After 3 or 4 weeks, the humidity level is gradually lowered.
8. The developing microshoots are progressively acclimatized to the outdoor conditions. The new roots will become more adapted to natural substrate than those formed in gelled tissue culture media.
9. At this stage, the rooted microshoots can be potted individually in 10 × 15 cm containers filled with clay top soil, mixed with some sand, avoiding hydric stress and direct sun exposure during the first weeks.
10. After 3 months of development under these nursery conditions, the plants reach a suitable size to be field planted (Fig. 3f).

4. Notes

1. For seed germination, the medium composition is half-strength MS macro- and micronutrients and 20 g/L sucrose. Suitable media for the subsequent steps of micropropagation and rooting from in vitro germinated seedlings are detailed in the literature (28). For producing seedlings to be used as in vitro rootstocks for micrografting, pour 5 mL of this liquid medium into the 21 × 150 mm glass test tubes, containing the plugs used as supports (30).
2. Culture media are prepared using the following concentrated stock solutions: macronutrient (×10), iron (×20), micronutrients (×1,000), vitamins (×1,000), myo-inositol (×100), growth regulators (×1,000). These stock solutions are stored at 2–4°C in darkness for up to 6 months. For more details, see ref. (33).
3. Once the cultures are successfully established, replace test tubes with glass jars (300–800 mL as total volume) and cover them with appropriate caps for higher mass production efficiency.
4. After sterilization, store culture media for a limited period preferably in the darkness at low temperature (2–4°C).
5. Addition of a few drops of a wetting agent (dishwashing soap more easily accessible and cheaper than Tween 20 or 80) increases the efficiency of the disinfecting solution.
6. As for a lot of arborescent species, seed germination rate in *Acacia mangium* may vary, usually between 60 and 70%.

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7. Epicormic shoots can be produced by low branch portions of 60–70 cm long, collected from the selected trees, and placed horizontally on sand bed under mist system. Felling the selected trees in order to expect the development of sprouting shoots from the stump is another option (21, 34) but more destructive and risky because the stump may fail to sprout, and as a result, the genotype may be lost.
8. Another option for initiating *Acacia* spp. tissue culture is to use shoot apices (0.2–0.4 mm) further to their in vitro micrografting onto 2- to 3-month-old rootstocks produced from seedlings germinated and tissue cultured in vitro on plugs saturated with suitable liquid medium (see Fig. 1). This innovative technique (35) is useful for avoiding contaminations, particularly the endogenous ones (*Acacia* spp. develop naturally and synergically with *Bradyrhizobium* bacteria), and for inducing varying degrees of physiological rejuvenation (30). In vitro micro-grafted materials can beneficially be used for further mass micropropagation or for outdoor plant production. For *A. mangium* mature selected genotypes, success rate is far higher for in vitro micrografting than for more conventional in vivo grafting (35). On the other hand, shoot apex (0.1–0.2 mm as overall size) cultures from mature outdoor selected *A. mangium* trees on gelled media have been attempted but failed to develop into microshoots (Monteuuis, unpublished results). Despite being reported for *A. mearnsii* (34), meristem culture per se seems not possible due to the minute size of *Acacia* spp. shoot apical meristem (30 µm in height for 90 µm in diameter as average measurements for *A. mangium*).
9. The identity and origin of the selected plant material to be micropropagated must be recorded and labeled properly and with great care using practical and reliable means, such as permanent markers and pencil, thereby avoiding risks of mixing-up distinct origins.
10. Placing the primary explants in the darkness for the first 4–7 days following the in vitro introduction is advisable for excised shoot apex explants.
11. Contaminated explants are checked and promptly removed on a daily basis.
12. Trimming and transfer of explants is done in contamination-free conditions under laminar flow hood, on sterilized absorbing filter paper, or newspapers to save costs, using scalpels and forceps which are flamed after dipping in 95% ethanol.
13. The explants, especially taken from mature trees, gradually become more organogenic; thus the multiplication rate increases. The morphology may revert to juvenile-like form with compound leaves instead of the phyllode type that characterizes the mature stage (see Fig. 2b, c). Spontaneous rooting can even be observed at this stage.

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14. Test tubes containing only one explant are preferably used during the first stages of micropropagation in order to limit the expansion of contaminations that may arise from the introduction in vitro of primary explants taken from the greenhouse or field-grown plant material.
15. These protocols have permitted to micropropagate sustainedly *A. mangium* and *A. mangium* × *A. auriculiformis* mature clones for more than 20 years up to now, which is more than the usual life span of such trees in natural conditions, and these materials are still being micropropagated with the same responsiveness.
16. These figures, quite conservative (9), are based on records established during several years for different *A. mangium* mature genotypes. They include possible contamination or physiological mortality losses, quantitatively quite limited overall. We deliberately chose to use moderate concentration of BA to ensure sustainable long-term micropropagation using a sole culture medium suitable for shoot elongation and multiplication by axillary budding. This seems more natural and safer for preserving the true-to-typeness of the materials, while reducing the risks of somaclonal variations.
17. Rooting rates are usually higher than 80% for juvenile materials and can reach 70% for mature selected genotypes (18, 29, 32).
18. River sand, more or less chemically neutral and pathogen-free, is recommended as a potting substrate. It is advisable to boil it before use and to apply efficient soil fungicides in aqueous solutions. This sand must be fine enough, but not overly for ensuring a good drainage.
19. Since *Acacia* spp. roots are very fragile, shorter roots reduce the risks of damage and breakage. Usually a pre-hole is made for the insertion of the rooted microshoots into the sand, thereby reducing the risks of breaking the newly formed roots.
20. High relative humidity (more than 80%) is usually provided by a reliable automatic mist-system.

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