Micropropagation of *Acacia mangium x A. auriculiformis* hybrids in Sabah

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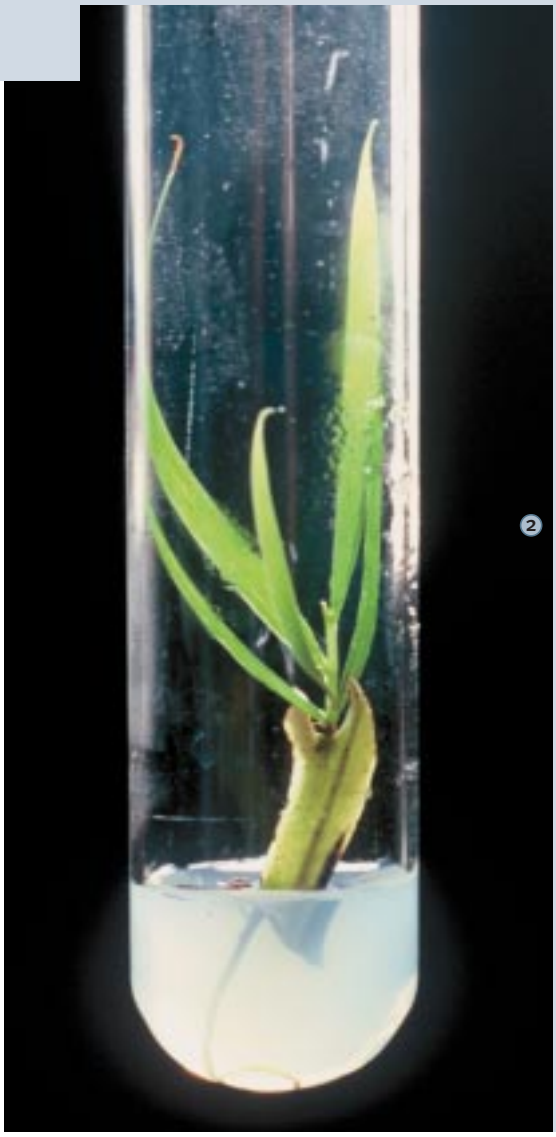
Photo 1.
Two emergent *Acacia mangium x A. auriculiformis* hybrids can be easily distinguished in this 2-year-old stand established with contaminated *Acacia mangium* seedlots (Oumé, Côte d'Ivoire).
Photo A. Galiana.

Acacia mangium x A. auriculiformis **is a promising hybrid for tree plantations. It can, however, only be propagated vegetatively, and there is currently a lack of available plant material and expertise concerning its clonal propagation. The authors combined conventional and *in vitro* propagation methods and demonstrated that large-scale reforestation is possible with this hybrid.**

Framework of the study

Importance and utilization of *Acacia* spp. in reforestation programs

Acacia species, especially *Acacia mangium*, have become major plantation tree species in Southeast Asia in the last two decades, and they are now planted in an area of about 600 000 ha in this region (Turnbull *et al.*, 1998), including 60 000 ha in Sabah (East Malaysia). These species—originating from northern Australia, eastern Indonesia and Papua New Guinea—are fast-growing legume trees that can easily grow on nitrogen-deficient soils due to their ability to fix atmospheric nitrogen in a symbiotic association with soil rhizobia. *A. mangium* is mostly used for pulp production, but its wood can also be used for general construction, furniture making, particle board as well as plywood. In commercial plantations, *A. mangium* often yields 25 $m^3.ha^{-1}.year^{-1}$ on average. *A. mangium x A. auriculiformis* hybrids often occur spontaneously in places where both parental species have been



introduced in the same vicinity (photo 1). The morphological traits of these hybrids (flower colour, pod aspect, leaf shape and size, bark aspect and colour, wood density) are generally intermediate between those of the *A. mangium* and *A. auriculiformis* pure parent species. Although planted to a far lesser extent as compared to *A. mangium*—due to the shortage of plant material and the absence of knowledge and experience concerning the clonal propagation of hybrids—the *A. mangium x A. auriculiformis* hybrid has a promising potential for plantation use since it was shown to be more vigorous and adaptable than both parental species (Chia, 1993). In addition, this hybrid has a higher wood density and cellulose content compared to *A. mangium* and seems to be less prone to heart-rot disease, which may affect a high proportion of logs at the end of the plantation cycle in *A. mangium* (Wong, 1993). Due to increasing demand for genetically superior plant materials by local forest companies, micropropagation studies that have been carried out on *A. mangium* by the Plant Biotechnology Laboratory Project (ICSB/CIRAD-Forêt joint project, Tawau, Sabah, Malaysia) since 1991 have recently been extended to the *A. mangium x A. auriculiformis* hybrid.

Rationale of micropropagating forest trees and *Acacia* spp.

The main advantage of mass vegetative propagation compared to propagation by seeds is that higher yield planting materials with uniform characteristics can be obtained within a minimum time period. Vegetative propagation of superior mature selected trees avoids the long selection cycles associated with genetic improvement strategies using sexual propagation. *Acacia* species are generally propagated by seeds for large-scale reforestation programs. However, in the case of *A. mangium x A. auriculiformis* hybrids, vegetative propagation remains the only means of multiplication because of the very limited capacity for producing interspecific hybrid seeds from bi-specific orchards. Besides, tissue culture technologies and especially micropropagation are faster than horticultural vegetative-propagation methods to rejuvenate and mass-multiply true-to-type adult selected trees. From an economic standpoint, tissue culture technologies could be even more cost-effective than conventional methods for large-scale cloning, since several years could be saved through rapid mobilization of genetically superior materials.

Photo 2.
Elongated axillary shoot with phyllodes emerging from nodal explants collected from a mature *A. mangium x A. auriculiformis* ortet 1 month after *in vitro* introduction.
Photo A. Galiana.

Photo 3.
Flasks containing *A. mangium x A. auriculiformis* hybrid clones under *in vitro* multiplication conditions.
Photo A. Galiana.

Photo 4.
In vitro rooting of *A. mangium x A. auriculiformis* hybrid plantlets 1 month after transfer onto rooting medium.
Photo A. Galiana.

Photo 5.
Acclimatization of micropropagated *A. mangium x A. auriculiformis* hybrid plantlets under a misting system in Brumas nursery (Sabah Softwoods Sdn. Bhd.).
Photo A. Galiana.

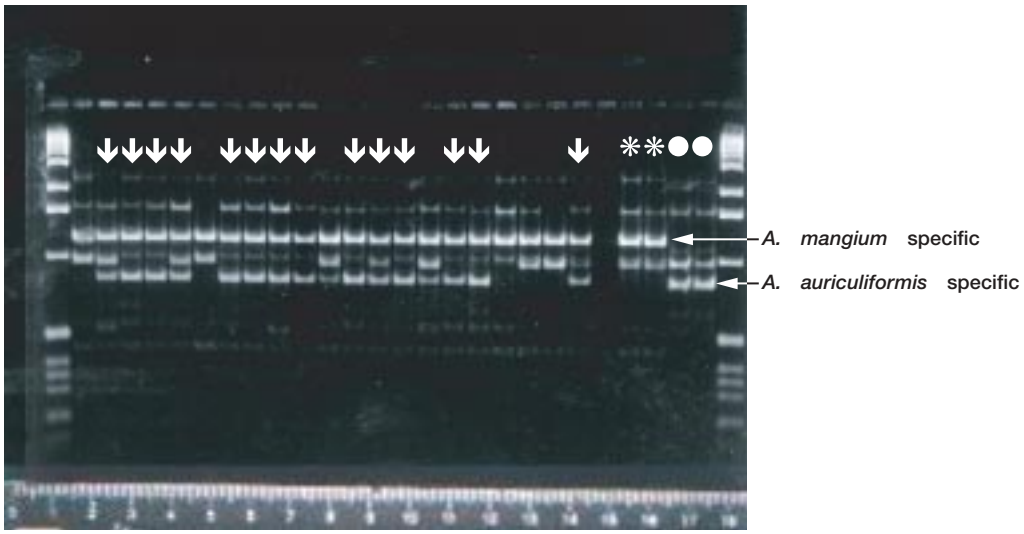


Figure 1.
Molecular identification of different *Acacia mangium x A. auriculiformis* hybrid and *Acacia mangium* clones by RAPD markers using a specific primer.
The second to the twenty-first lanes from left to right correspond to the following clone numbers: T3; 6-15; AH11; 3-21; AH16; 3-19; AH17; AH10; 7-23; AH12; AH15; AH8; 1-1; 5-13; 6-5'; 8-26; AHB; Am24; Am4; Am21; Am15; blank. Clones having both specific fragments of *A. mangium* and *A. auriculiformis*, as indicated by arrows, are confirmed as *A. mangium x A. auriculiformis* hybrids. The two lanes marked by an asterisk correspond to two different *A. mangium* pure seedlots, the two lanes with a white full-circle (O) to two pure *A. auriculiformis* seedlots. The external left and right lanes are ladders.

Development of optimal micropropagation protocols

Origin of plant materials

Optimal culture media were developed using the same *Acacia* origins and clones at each stage of the micropropagation process. Our collection of *A. mangium x A. auriculiformis* hybrid clones originated from seeds collected in Côte d'Ivoire (Oumé, West Africa) on *A. mangium* mother trees located close to an *A. auriculiformis* stand. Seedlings from these progenies were identified as putative hybrids at a young stage according to specific morphological and ontogenetic traits intermediate between those of both parental species (Rufelds, 1988). A large majority of these clones were further genetically confirmed as *A. mangium x A. auriculiformis* hybrids through RAPD analysis (figure 1). In our study, the micropropagation capacity of *Acacia* hybrids was evaluated in comparison with that of *A. mangium* for which specific optimal media have already been developed. *A. mangium* clones originating from 3-year-old selected mature trees as well as unselected juvenile material were used. *Acacia* clones of both species had been maintained under *in vitro* conditions for at least 5 years prior to experimentation.

In vitro multiplication and rooting abilities

Since micropropagation studies on *A. mangium x A. auriculiformis* hybrids had never been previously reported, our experiments were focused on the development of new protocols and optimal culture media for each of the three successive micropropagation stages: *in vitro* introduction, multiplication and rooting (photos 2, 3 and 4). These experiments showed especially that *Acacia* hybrids required a specific multiplication medium which differed from that developed earlier for *A. mangium*. After the addition of suitable growth regulators into the culture medium, shoots were multiplied by stimulating budding and elongation of primary axillary shoots. After 2 months growth on the multiplication medium, the elongated axillary shoots were then sectioned and isolated from the shoot clusters before being transferred onto a fresh multiplication medium or rooting medium. The multiplication rates obtained for *A. mangium x A. auriculiformis* varied greatly between clones but were higher on average than *A. mangium* rates when both species were cultured on their respective optimal multiplication media (table I). Moreover, the *Acacia* hybrids exhibited a far higher rooting ability as well as lower between-clone variability than *A. mangium* (table I). These differences in *in vitro* organogenic capacity between *A. mangium* and *Acacia* hybrids were further confirmed on a larger number of clones from various origins.

Nursery management of micropropagated plantlets

Ex vitro acclimatization of plantlets

Our acclimatization experiments were carried out on both rooted and non-rooted plantlets in order to acclimatize a maximum number of plantlets issued from *in vitro* conditions. After 2 months of culture on the optimal rooting medium, the *Acacia* hybrid plantlets were brought to the nursery and taken out of their culture flasks. They were immediately transplanted into beds of pure coarse sand under a misting system (photo 5). The misting spray frequency was adjusted according to conditions traditionally used for cuttings. One month later, the plantlets were individually transferred to 1-liter polybags containing sandy-clay topsoil and placed in nursery under 50% shade. In a representative acclimatization experiment performed in Taliwas nursery (ICSB research station), the overall percentage of survival did not vary between clones and reached about 95% for pre-rooted plantlets *versus* 75% for non-rooted ones 1 month after *ex vitro* transfer, with the latter type of explants producing neoformed roots at this stage (table II). After 2.5 months of acclimatization, the survival rate dropped to about 55% for pre-rooted plantlets and 40% for non-rooted ones. Under the same conditions, the mean percentage obtained for three different *A. mangium* clones was significantly lower (table II).

Table I.
Mean multiplication rates and percentages of rooting obtained in the *Acacia mangium x A. auriculiformis* hybrid and *A. mangium*.

Acacia species	Multiplication rate [a] (mean ± S.D. [c])	% of rooting [b] (mean ± S.D. [c])
A. mangium x A. auriculiformis hybrid	4.4 ± 1.9	73.3 ± 1.4
A. mangium	3.5 ± 0.1	46.2 ± 21.7

[a] The mean multiplication rates were obtained from three clones per *Acacia* species after 2 months of culture on optimal multiplication media, following at least three multiplication subcultures on the same medium. The multiplication rates of the different clones were not significantly different for each species according to the chi-square test at the 5% level (n=7 initial explants per *Acacia* hybrid clone and n=90 initial explants per *A. mangium* clone).
[b] Rooting percentages were established 4 weeks after transfer from optimal multiplication media to optimal rooting media. The mean percentages of rooting were obtained from three clones per species. Significant differences between clones were only found in *A. mangium* according to the chi-square test at the 5% level (n=20 shoots per *Acacia* hybrid clone and n=96 shoots tested per *A. mangium* clone).
[c] S.D.: standard deviation.

Table II.
Percentages of survival obtained in the *Acacia mangium x A. auriculiformis* hybrid and *A. mangium* micropropagated plantlets 4 weeks after *ex vitro* transfer

Acacia species	Type of plantlets	Number of plantlets tested	% of survival *
A. mangium x A. auriculiformis	Pre-rooted	215	94.4
	Non-rooted	115	75.9
A. mangium	Pre-rooted	155	65.4
	Non-rooted	65	59.1

* Mean percentages obtained from three clones per species (same material as that used in table I). The differences in percentages of survival between pre-rooted and non-rooted plantlets were only significant in *Acacia* hybrids according to the chi-square test at the 5% level.

Photo 6.
Acclimatization of 20 000 micropropagated *A. mangium x A. auriculiformis* hybrid plantlets derived from various selected clones in Brumas nursery (Sabah).
Photo A. Galiana.

Rhizobial inoculation

As in most legumes, the soil bacteria rhizobium spontaneously infects the *Acacia* root system and forms root nodules that have the capacity of directly fixing atmospheric nitrogen, thus allowing these species to grow on N-deficient soils. Since the inoculation of *A. mangium* seedlings by selected strains of rhizobium is known to have a positive effect on plant growth (Galiana *et al.*, 1994), an inoculation experiment on the *Acacia* hybrids was performed in Brumas (Sabah Softwoods research nursery).

After 1 month of acclimatization in sand beds, micropropagated plantlets issued from three different clones were inoculated with pure rhizobium cultures just after transfer to polybags. Among the two rhizobium strains tested and regardless of the clone tested, only AH 12c isolated from *Acacia* hybrid nodules showed a positive effect on plant growth, while Aust 13c—isolated from *A. mangium* nodules and previously shown to be the best performing strain on *A. mangium*—had no effect on plant growth (figure 2). The *Acacia mangium x A. auriculiformis* hybrid can be thus considered as a specific host plant species since efficient nitrogen-fixing nodules can only be formed with a strain isolated from the same host plant species. Other *Acacia* hybrid rhizobium strains should be tested in controlled conditions to confirm this specificity.

Mass production of *in vitro* plantlets

The production of 20 000 *Acacia* hybrid plantlets within 1 year in the framework of a service contract between the PBL and Sabah Softwoods Sdn. Bhd. (SSSB) gave us an opportunity to apply the previously developed micropropagation protocols on a large scale and to assess their efficiency in actual conditions. These produced plantlets had to be further used by SSSB as stock plants in a multiplication clonal garden for mass production of macrocuttings.

The relevant micropropagated plant materials were composed of *A. mangium x A. auriculiformis* clones selected by SSSB and from two distinct origins: *i)* 15 clones from Ulu Kukut, Brumas and Silam (Sabah) that were maintained for several years in the Brumas nursery through successive cycles of propagation by cuttings, and *ii)* 15 other clones corresponding to "plus" trees selected from a clonal test and stored in the nursery in the form of marcottes. Applying the introduction protocol developed for teak (Monteuuis *et al.*, 1998) to these materials, 52% out of 550 single nodes of the former origin introduced *in vitro* were responsive (*i.e.* developing axillary shoots) and free of bacterial and fungal contaminants, *versus* 33% of 1 000 nodes in the latter, which could be explained by the higher degree of lignification of shoots from marcottes. One to two months after introduction, the developed axillary shoots were dissected and transferred onto the optimal multiplication medium developed for hybrids. The number of plantlets produced increased exponentially thereafter, with a mean multiplication rate of 2.8 every 2 months. The number of multiplied plantlets varied markedly between clones (figure 3) although the rates were biased by high bacterial contamination that affected all clones at different levels. After the multiplication stage, the plantlets were transferred onto an original multiplication-rooting medium that promoted both elongation/multiplication by axillary budding and rooting. One month after transfer, 100% of rooting was obtained for all clones and successive batches of rooted plantlets were gradually delivered to the SSSB nursery (photo 6). The overall acclimatization success calculated from the 20 000 plantlets delivered reached 94% after 1 month under a misting system and 85% in polybags 3 months after transfer, just before field transplanting.

This contract with SSSB demonstrated the efficiency of our micropropagation technique with respect to setting up a multiplication clonal garden in a minimum amount of time.

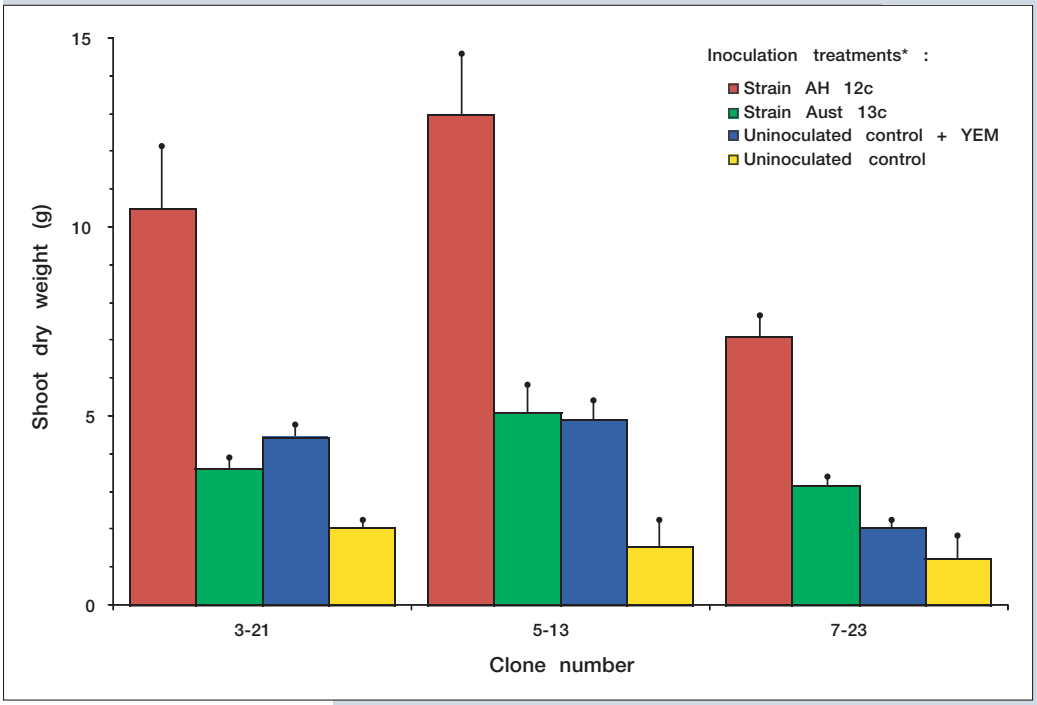


Figure 2
Effect of different rhizobium inoculation treatments on shoot dry weight (means and standard errors) of three *A. mangium x A. auriculiformis* hybrid clones after 9 months of plant growth in nursery.
* AH 12c is a *Bradyrhizobium* sp. strain isolated from *A. mangium x A. auriculiformis* hybrids and Aust 13c, a *Bradyrhizobium* sp. strain isolated from *A. mangium*; YEM: yeast extract mannitol medium (culture medium for rhizobium without bacteria).
A two-way analysis of variance showed that both the clone and inoculation treatment factors had highly significant effects on shoot growth at $P \leq 0.001$ (n=6 replicates per clone x inoculation treatment combination). Inoculation with strain AH 12c was the best treatment according to the Newman and Keuls multiple range test.

Photo 7.
Tissue culture-issued *A. mangium x A. auriculiformis* hybrids ready for planting in Taliwas nursery (Innoprise Corporation Sdn. Bhd., Sabah). Photo A. Galiana.

Conclusion

The field behavior and growth performances of the micropropagated plantlets produced remain to be assessed in local conditions (photos 7 and 8). In this respect, about 5 000 additional *Acacia* hybrid plantlets issued from the CIRAD and SSSB clonal collections were multiplied to set up clonal tests in Taliwas and Brumas, with both trials involving all *Acacia* hybrid clones from our collections and different *A. mangium* clones and progenies used as reference materials.

Micropropagation of *A. mangium x A. auriculiformis* hybrids was shown to be very efficient at all steps of the process, while preliminary studies on the production of macrocuttings not reported here showed their high rooting potential. Moreover, the coppicing ability of *Acacia* hybrids is known to be high and more than 100 rooted macrocuttings per stock plant can be expected each year from this production system. Since vegetative propagation is currently the only way to multiply *Acacia* hybrids, the combination of both *in vitro* and conventional propagation methods appear to be very well adapted to this plant material in the framework of large-scale reforestation programs, provided that, as in Sabah, the laboratory is not too far from the production area.

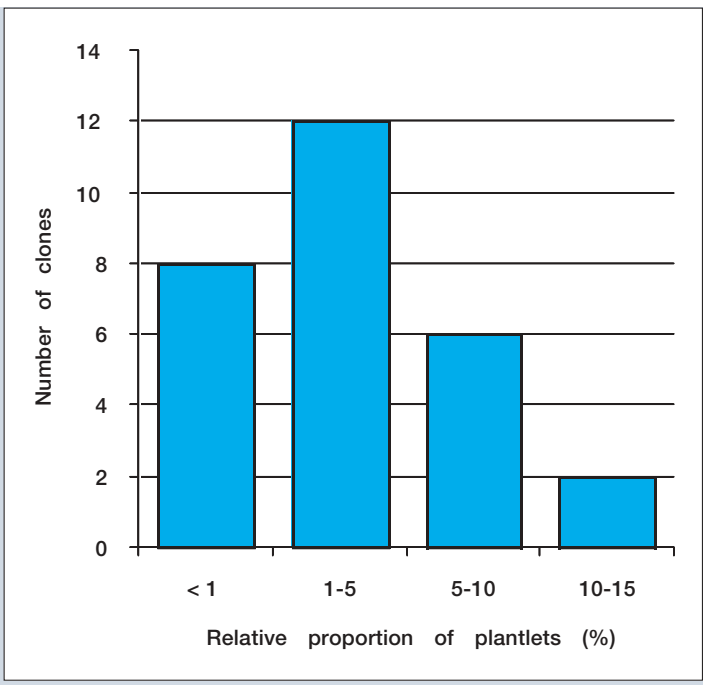


Figure 3
Distribution of the relative proportions of plantlets produced by 28 *A. mangium x A. auriculiformis* hybrid clones in the framework of the *in vitro* plantlet production project for Sabah Softwoods at the end of the multiplication cycles (total number of plantlets = 13 500).

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Photo 8.
In vitro A. mangium x A. auriculiformis hybrids 6 months after transfer to the field in Brumas (Sabah Softwoods Sdn. Bhd.). Photo A. Galiana.