RECENT ADVANCES IN MASS MICROPROPAGATION OF ACACIA SPECIES *

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SUMMARY

Acacia species, and more especially *Acacia mangium*, have become major plantation tree species in Sabah and South-East Asia. These tree species are mostly used for pulp production. Due to the increasing demand of superior plant materials by local forest companies in Sabah, the Plant Biotechnology Laboratory has initiated micropropagation studies on three *Acacia* species : *A. mangium, A. mangium* x *auriculiformis* hybrids and *A. crassicarpa*. The use of this tissue culture technology aims at cloning adult trees selected based on their growth performances in a shorter time than conventional methods of propagation such as production of cuttings. In the case of *A. mangium* x *auriculiformis* hybrids - that are known to have a better growth than the *A. mangium* pure parent species - vegetative propagation is the only mean of propagation since propagation by seeds from bi-specific orchards still remains not feasible at the present time. We recently developed successful micropropagation methods and appropriate culture media on these three *Acacia* species. Our micropropagation experiments were mainly focused on the improvement of the multiplication rate and *in vitro* rooting ability. These protocols firstly developed on juvenile plant material (seedlings) were further applied to mature selected material. The *in vitro* introduction of shoots - directly collected on mature selected trees - as well as further multiplication, rooting and *ex vitro* acclimatization in nursery of the produced plantlets were shown to be successful. Although micropropagation methods can theoretically produce an infinite number of plantlets, the combination of both *in vitro* and conventional methods of propagation will be the most cost-effective strategy to be applied for large-scale afforestation programs.

KEY WORDS : Acacia, hybrids, micropropagation, tissue culture, rooting

INTRODUCTION

Acacia species, and more especially *Acacia mangium*, have become major plantation tree species in Sabah and South-East Asia in the last two decades. These species, originating from 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 the symbiotic association with soil rhizobia. These tree species are mostly used for pulp production but their wood can also be used for general construction, furniture, particle board as well as plywood. In commercial plantations, these species often yields 25 $m^3.ha^{-1}.year^{-1}$ on an average. Due to the increasing demand of genetically superior plant materials by local forest companies, micropropagation studies have been initiated by the Plant Biotechnology Laboratory on the most planted *Acacia* species : *A. mangium, A. mangium* x *auriculiformis* hybrids and *A. crassicarpa*. Our micropropagation experiments described hereafter were more particularly focused on the multiplication and rooting abilities of *A. mangium* and *A. mangium* x *auriculiformis*. Before analyzing the micropropagation process, some general considerations and remarks concerning the rationale of micropropagating forest trees and acacias need to be stressed.

MAIN BENEFITS OF THE MICROPROPAGATION TECHNOLOGY IN FOREST TREES

The main advantage of mass vegetative propagation compared to other propagation methods such as propagation by seeds is to obtain significant gains in the production and quality of planting materials in a minimum amount of time. Vegetative propagation of tree species allows to capture genetic gains rapidly through a true-to-type reproduction and multiplication of superior mature selected trees. It avoids the long selection cycles that are applied in genetic improvement strategies using sexual propagation such as the reciprocal recurrent selection. However, some negative maturation effects, such as plagiotropy (lateral growth), low rooting ability and low multiplication rate, are often observed in many species when conventional methods of propagation are applied to selected adult material. In such a case, rejuvenation of plant material can only be obtained after numbers of multiplication cycles including different combined horticultural techniques such as grafting, marcotting and cutting propagation.

Among the different vegetative propagation methods available, tissue culture technologies and more especially micropropagation are the most promising ones to rejuvenate selected adult trees. As a consequence of the rejuvenation process, the ramets (different copies from the same clone) from a given ortet (original selected genotype) are theoretically more uniform through the use of micropropagation provided that no somaclonal variability or other related

problems due to certain culture medium factors occur. In addition, once the optimum rejuvenation level has been reached, cloning and multiplying trees by *in vitro* methods are faster than conventional methods of propagation such as cutting propagation. Although the production cost of tissue culture technologies is higher than that of the conventional methods of vegetative or sexual propagation for the same number of plantlets produced within a same period of time, the final production costs of *in vitro* propagation might be lower considering the saving of several years for the mass production and fast availability of genetically superior materials.

RATIONALE OF MICROPROPAGATING ACACIAS

Acacia species are generally propagated by seeds in large scale afforestation programs. However, this type of propagation can be sometimes limited due to the shortage of seeds or even good-quality seeds. In addition, a relatively high variability in the tree performances is sometimes observed between or within the progenies as in the case of *A. crassicarpa* (another *Acacia* species closely related to *A. mangium* phylogenetically). In the case of *A. mangium* x *auriculiformis* hybrids - that are known to have a better growth than the *A. mangium* pure parental species (Chia, 1993) - vegetative propagation is the only mean of propagation since propagation by seeds from bi-specific orchards still remains not feasible at the present time. The main interest of *Acacia* micropropagation is to rejuvenate and multiply mature selected plus trees. In *A. mangium* and *Acacia* hybrids, which are mature and generally exploited at 5 to 8 years-old, the negative maturation effects occur very early compared to many other tree species as attested by their early ages of flowering and fruiting that generally appear as soon as two years after germination. Considering the high cost of the tissue culture technologies, the genetic value of clonal materials needs to be significantly higher than that of the best seed sources available before micropropagating clones.

So far, very few micropropagation studies or reports have been published on *Acacia* hybrids. Conversely, micropropagation of *A. mangium* has been widely reported (Darus, 1991 ; Galiana, 1991 ; Bon *et al.*, 1998). Our experiments on *Acacia hybrids* were mainly focused on the development of suitable protocols and methods for the three major micropropagation stages : *in vitro* introduction, multiplication and rooting. Our experiments carried out on *A. mangium* mainly concerned *in vitro* rooting improvement as its low rooting ability has always been found as the major limitant factor for micropropagation, especially when using initially mature plant material. Some *ex vitro* acclimatization experiments of the micropropagated materials were also performed in nursery and are described hereafter.

THE MICROPROPAGATION PROCESS

In Acacias, the micropropagation process consists in transferring axillary shoots onto successive multiplication subcultures in an appropriate medium before transferring all the multiplicated shoots onto a rooting medium at the end of the production cycle. The multiplication of shoots is produced by stimulating budding, branching and elongation of the primary axillary shoots due to growth regulators added to the basal multiplication medium. After six to eight weeks of growing on the multiplication medium, the elongated axillary shoots are then dissected from the shoot clusters and transferred onto fresh multiplication media.

1. *In vitro* introduction of mature material

In order to test the cloning ability of adult trees through *in vitro* culture, the *in vitro* introduction stage was studied and performed on six *A. mangium* and *Acacia* hybrids mature trees.

The collection of *A. mangium* shoots was performed on three ten-year-old genotypes, clones nò. T2, T3 and T4, from a stand located in Taliwas forestry center (Lahad Datu area, Sabah). Clones T2 and T4 were identified as plus trees according to their growth performances and their oustanding bole straightness with no branches up to a minimum of 15 m height. Shoot collection of *Acacia* hybrid clones was performed on three 5-year-old ortets, clones no. A, B and C, located in Luasong Forestry Center (Sabah). The shoots were collected on low branches at 2 to 3 meters height from the ground for clones T3, A, B and C or at the top of the trees for clones T2 and T4.

The shoots were divided into single nodal segments, disinfected with 0.25% (w/v) of mercuric chloride for 20 minutes before rinsing them in sterile distilled water and introducting into test tubes that contained the basal introduction medium for the primary culture. The tubes were placed under light with a daily photoperiod of 16 h in alternance with 8 h of darkness for two months before observation and data collection.

As shown in Table 1, the percentage of contaminated plantlets obtained two months after transfer was very variable and generally high according to the clone and the *Acacia* species. However, all the different genotypes tested were successfully introduced and successfully propagated further with about 5 to 60% of survival. Only a low proportion (0 to 15% according to the genotype) of contaminant-free explants did not exhibit any response whereas the successfully introduced explants responded positively by developing axillary shoots.

Table 1. Introduction success and percentage of contaminations obtained two months after *in vitro* introduction of monodonal explants of *Acacia* hybrids and *A. mangium* collected from matu trees

Clone no.	Species	Origin	Number of explants introduced	% of contamination		% of mortality	% of successfully introduced explants
				fungal	bacterial		
T2	*mangium*	Taliwas	48	27.1	0	14.6	58.3
T3	"	"	85	45.9	20.0	14.1	20.0
T4	"	"	102	35.3	0	8.8	55.9
A	Hybrid	Luasong	48	89.6	2.1	4.2	4.1
B	"	"	38	52.6	5.3	0	42.1
C	"	"	34	58.8	20.6	2.9	17.7

2. Micropropagation experiments

- Plant material and culture conditions

a) *Acacia mangium*

All the multiplication and rooting experiments described hereafter were performed on three different clones. Two of these clones, no. 15 and 21, were selected in Ivory Coast from mature plus trees whereas clone no. 24 originated from non-selected juvenile material (*i.e.* seedlings from Rex Range, an australian origin). These materials had been maintained under *in vitro* conditions and multiplication since 1990. In our *in vitro* experiments, plants were grown in lighted culture rooms under a daily photoperiod of 16 h in alternance with 8 h of darkness for two months. Twenty tubes per treatment were tested.

b) *Acacia mangium x auriculiformis* hybrids

Three different *A. mangium x auriculiformis* hybrid clones were used in the following multiplication and rooting experiments : no. 3-21, 5-13 and 7-23. These materials had been maintained under *in vitro* conditions and multiplication since 1993. These three clones originated from seeds collected in Ivory Coast (Oumé, West Africa). The seeds were collected in 1992 on five-year-old *A. mangium* parent trees of unknown origin close to an *A. auriculiformis* plantation. Several plots established in 1990 at Oumé with seeds originating from the same mother trees and seedlot contained a quite high proportion of superior trees (about 20%) compared to *A. mangium* exhibiting all phenotypic traits of *A. mangium* x *auriculiformis* hybrids. These morphologic characters are generally intermediate between *A. mangium* and *A. auriculiformis* pure parent species (flower colour, pod aspect, leaf shape and size, bark aspect and colour). From the one thousand seeds germinated in 1993 and grown for seven months in greenhouse (Nogent-sur-Marne, France), forty genotypes were selected. This early selection of hybrids was based on two criteria : *i)* the seedlings were identified as putative hybrids according to their specific morphological traits as determined by the Rufeld's identification method (Rufelds, 1988) and *ii)* their high nitrogen-fixing potential. This potential was expressed by growing the plants in jars on a nitrogen-free nutrient solution after they were inoculated with selected rhizobium strains. Recently, these three clones were genetically identified and confirmed as *A. mangium x auriculiformis* hybrids through the use of two methods : electrophoresis of isozymes and RAPD analysis.

- *In vitro* multiplication

a) ***Acacia mangium***

The plants were grown on a basal multiplication medium containing 0.5 $mg.l^{-1}$ of benzyladenine (BA) and were cultured for two successive multiplication subcultures at 2 month-intervals. Before the first subculture of this experiment, the plantlets were grown on the basal multiplication medium with 0.5 $mg.l^{-1}$ BA for at least three multiplication subcultures. The multiplication rate was recorded before dissecting and transferring the explants onto fresh culture media. It was calculated according to the mean number of explants obtained per initial one after transfer of the dissected shoots onto new media. These data were measured after two successive subcultures of multiplication.

Table 2. Multiplication rates obtained in three different *A. mangium* clones after 2 months of culture and for two successive multiplication subcultures

Clone no.	Multiplication rate 1st subculture	Multiplication rate 2d subculture
15	3.7	2.6
21	3.5	2.6
24	3.4	2.8

As reported in Table 2, there was no significant difference between the multiplication rates of the three different *A. mangium* clones in each of the two subcultures. The heights of the main shoots (results not described here) - that were shown to be correlated with the multiplication rates - were not significantly different according to the different clones in each subculture.

b) ***Acacia mangium x auriculiformis* hybrids**

The main shoot height and multiplication rate were recorded on the three selected clones (no. 3-21, 5-13, and 7-23) at the end of a 2 month-interval multiplication subculture. Before this subculture, the plantlets were also grown on the same basal multiplication medium for at least three multiplication subcultures. The multiplication rate was calculated as described above for *A. mangium*.

As described in Table 3, significant differences (according to the t-test) were observed between the multiplication rates and main shoot height of the three different Acacia hybrid clones, the multiplication rates being correlated to the main shoot heights. The multiplication rate of clone 3-21 was much more higher (about 2 to 3 times higher) than that of the two other tested clones, as well as its main shoot height (about 2 times higher) whereas the main shoot heights and multiplication rates measured on clones 5-13 and 7-23 were not significantly different.

Table 3. Main shoot heights and multiplication rates obtained in three different *A. mangium x auriculiformis* hybrid clones after 2 months of culture and for two successive multiplication subcultures

Clone no.	Main Shoot Height (mm)	Multiplication rate
3-21	34.2	7.0
5-13	16.3	2.5
7-23	16.4	3.8

- *In vitro* rooting

For *A. mangium*, the experiment was performed after the two subsequent multiplication subcultures described above while for *A. mangium x auriculiformis* hybrids, the rooting experiments were performed after four multiplication subcultures. The axillary shoots from the different clones were dissected and then transferred individually on a same basal rooting medium containing an auxin. The basal rooting medium was the same for both species whereas different and optimal auxin concentrations were used for each species. These optimal concentrations were determined according to previous rooting experiments that we performed on juvenile material. The percentages of rooting were recorded 4 weeks after transfer onto rooting medium from 20 tubes per single treament.

As reported in Table 4, the percentages of rooting varied significantly (t-test) according to the different *A. mangium* clones tested. By contrast with *A. mangium*, there was no significant differences between the percentages of rooting of the three *Acacia* hybrid clones. In addition, the mean percentage of rooting is much more higher in Acacia hybrids than in *A. mangium* except for one of the three *A. mangium* clones. These data confirmed the quite low *in vitro* rooting ability of *A. mangium* whereas *Acacia* hybrids were shown to be easily rootable, although more clones should be tested for confirmation.

Table 4. Percentages of rooting obtained in three *A. mangium* clones and three *A. mangium x auriculiformis* hybrid clones 4 weeks after transfer onto rooting medium

Species	Clone no.	% of rooting
Acacia mangium	15	76.0
"	21	25.0
"	24	37.5
Acacia hybrids	3-21	74.3
"	5-13	71.4
"	7-23	74.3

3. Acclimatization phase

After two months of culture in the rooting medium, the plantlets from the above experiments were removed from the tubes and transferred to the nursery in sand beds under a misting system and shade in Taliwas forestry center. The survival rate of the plantlets was recorded after one month of weaning under mist and just after transfer to 1-liter polybags. The plantlets were separated into two distinct groups for both species : the pre-rooted plantlets that were already rooted before *ex vitro* transfer and the non-rooted ones.

On the three *A. mangium* clones tested (no.15, 21 and 24), the overall mean percentage reached 65.4 % from 156 pre-rooted plantlets tested *versus* 59.1 % from 66 non-rooted ones. We did not observed any significant clonal effect on survival rate. On the two *Acacia* hybrid clones tested (clones no. 3-21 and 5-13), the mean percentage reached 95.2 % from 83 rooted plantlets tested *versus* 80.5 % from 87 non-rooted ones. As in *A. mangium*, the survival rates of both *Acacia* hybrid clones were not significantly different.

Many other acclimatization experiments confirmed these very high percentages of weaning success obtained with *Acacia* hybrids.

CONCLUSION AND PROSPECTS

The data exposed in this paper showed that *A. mangium* and *A. mangium x auriculiformis* hybrids have been successfully micropropagated from the introduction to the acclimatization stages. The relatively high rate of contamination that we obtained in the introduction phase can be a limitant factor for further propagation although most of the contaminant-free nodal explants were reactive in producing elongated shoots from the axillary buds. Contrary to the fungal infections that are easily controllable through the successive multiplication subcultures, the bacterial infections remain a constant problem in reducing the multiplication rate and require more specific studies to be overcome. We observed an uniform multiplication rate in *A. mangium* according to the clone whereas that of *Acacia* hybrids was highly variable. Although only three *Acacia* hybrids clones were tested in the experiments described above, we have often observed this high variability among the other *Acacia* hybrid clones from our collection. We obtained opposite results in the rooting experiments as the three *Acacia* hybrid clones tested had similar rooting abilities with about 70-75% of rooted explants after one month in the rooting medium whereas the rooting success of the three *A. mangium* clones was very variable from 25 to 75% after one month. This variability confirms the low-rooting ability of *A. mangium* although the clones with low percentages of rooting had more than 75% of rooted explants after two months in the rooting medium (results not shown). However, the rooting success often remains unpredictable in *A. mangium* and depends on the previous conditions of culture and more especially the composition of the multiplication medium. Although the subsequent phase of acclimatization was shown to be successfull in nursery, the *in vitro*-produced plantlets still remain to be tested in the field in order to assess the levels of rejuvenation and physiological age of the introduced mature material.

As it has been stated earlier, the main interest of micropropagating Acacias is to rejuvenate and multiply mature selected plus trees. Considering the high cost of tissue culture techniques, the superior genetic value of *A. mangium* clonal materials compared to the best seed sources available needs to be demonstrated before micropropagating these clones since this species is known to have a low level of genetic diversity (Butcher *et al.*, 1996). Conversely to *A. mangium*, vegetative propagation is so far the only way of multiplication for the *Acacia* hybrids. Although the micropropagation methods can theoretically produce an infinite number of plantlets, the combination of both *in vitro* and conventional methods of propagation remain the most cost-effective strategy to be applied for these hybrids in large-scale afforestation programs. The saving of time - probably several years - that can be obtained in setting up

clonal multiplication gardens through micropropagation could strongly reduce the cost of plant production.

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Objet : Proceedings of the Malaysian Science & Technology Congress '98

Cher Bernard,

Pour ton information, tu trouveras ci-joint deux copies (une pour toi et l'autre pour Olivier Monteuuis) d'un papier sur la culture *in vitro* des Acacias que j'ai envoyé il y trois semaines au comité d'organisation du Congrès MSTC '98 qui s'est tenu à KK le 25 Novembre dernier. Il paraîtra dans les proceedings de ce congrès au cours duquel j'avais fait une communication orale.

Très cordialement,

Antoine GALIANA