DIRECT REGENERATION OF RATTAN SEEDLINGS FROM APICAL MERISTEM AND AXILLARY BUD EXPLANTS

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ABSTRACT. — A study was conducted to develop a protocol for mass-propagation of an economically important rattan species [*Laccosperma secundiflorun* (P. Beauv.) Kuntze] using leaves, axillary bud, and apical meristem explants collected from natural stands. The explants were incubated on basal MS (MURASHIGE & SKOOG 1962) medium containing vitamins (MSV), supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA). Best results (100% vigorous explants) from initiation phase were obtained after four weeks with axillary bud and apical meristem explants cultured in darkness at 28 ± 2°C on MSV supplemented with 30 g L^{-1} sucrose and 0.65 mg L^{-1} BAP. MSV containing 1 mg L^{-1} BAP and 1 mg L^{-1} NAA allowed highest rates of explants forming shoots (> 85%), with a mean of 3 shoots per explant after eight weeks. A high rate (> 95%) of shoots generated well-developed roots after two weeks on MSV containing 0.5 mg L^{-1} 3-in-doleacetic acid (IAA).

KEY WORDS. — Calamoideae, *Laccosperma secundiflorun*, *in vitro* culture, rattan palm, shoot regeneration.

INTRODUCTION

Rattans are spiny climbing palms belonging to the large subfamily Calamoideae. There are around 600 species of rattan with 13 genera solely concentrated in the Old World Tropics. Four genera of rattan palm occur in West and Central Africa (DRANSFIELD 1979, SUNDERLAND 2001), the genus *Calamus* being the most widely distributed in these areas. A total of 22 rattan species have been identified in the lowland tropical forests of Africa, represented by the endemic genera *Laccosperma*, *Eremospatha* and *Oncocalamus* as well as by a single representative of the Asian genus *Calamus* (SUNDERLAND 2002).

Like many other non-timber forest products (NTFPs), rattans, namely the genera *Calamus* (in Asia), and *Laccosperma* and *Eremospatha* (in Tropical Africa) are subjected to various uses (furniture, basket, lampshade, bow string, fish traps, cage for birds, etc.), because of their aesthetic and flexible stems (DRANSFIELD 1979, UMALI-GARCIA 1985, ZORO BI & KOUAKOU 2004a). With 1.2 million persons working in rattan-based industries worldwide, and contributing to the international trade estimated to US$ 6.5

billion per annum (SASTRY 2001), rattans are the most valuable NTFPs (PREBBLE 1997).

In Côte d'Ivoire, rattan is the object of important economical activities at both rural and urban levels, suggesting that this plant should be a NTFP biological model for the implementation of rural forests management and sustainable exploitation programme in the target zone (ZORO BI & KOUAKOU 2004a). Producing annual turnovers that vary from US$ 1 769 for rural peoples to US$ 9 231 for canes wholesalers, the trade of rattan allows annual profits varying from US$ 1 211 (craftsmen) to US$ 4 585 (wholesalers). Nevertheless, many biological, legislative, institutional, and logistic constraints presently threaten the stability and expansion of rattan industry. With respect to resource supply in West and Central Africa, rattan is presently entirely harvested from wild populations (ZORO BI & KOUASSI 2004, SUNDERLAND 2005). The intensity of the exploitation in Côte d'Ivoire has led to over-harvesting and, as a result, the stocks of this important resource are seriously depleted. The depletion is mainly related to extensive and irrational harvesting of canes, observed for economically important species, particularly *Laccosperma secundiflorum* (P. Beauv.) Kuntze, the large-diameter rattan producing canes used in furniture manufacturing. Because at least five to ten years are required for the production of harvestable canes, it was concluded that *L. secundiflorum* was threatened with extinction in Southern Côte d'Ivoire (ZORO BI & KOUASSI 2004). The problem is aggravated by the low rate of the rattan seed viability, coupled with the long germination time (2 to 10 months; MANOKARAN 1978, MORI & RAHMAN 1980, NASI & MONTEU-UIS 1992, OROZCO-SEGOVIA *et al*. 2003). To circumvent difficulties related to the limited supply of planting materials in Côte d'Ivoire, research was carried out with the aim of achieving mass-production of seedlings by conventional vegetative propagation of two species (*Laccosperma leave* Mann and Wendl. and *L. secundiflorum*) using sucker and rhizome (ZORO BI & KOUAKOU 2004b). Despite the fact that promising results were obtained 131 days after planting, with seedling regeneration rates varying from 80 to 93%,

this method is destructive, slow, labour-intensive and few propagules are produced from a single stock plant. Consequently, wildings or suckers are not sufficient to contribute significantly to the implementation of community-based forest ecosystem management strategy using rattans as biological model or the establishment of rattan plantations.

Although a number of studies related to *in vitro* propagation of various rattan species have been published (PATENA *et al*. 1984, UMALI-GARCIA 1985, PADMANADHAN & ILANGOVAN 1989, MULUNG 1992, GUNAWAN 1995, GOH *et al*. 2001a, b), the approach has not been investigated with genera endemic to Tropical Africa (*Laccosperma*, *Eremospatha* and *Oncocalamus*).

The aim of this work was to test some methods for micropropagation of the large-diameter, commercially important, and over-exploited rattan species in Côte d'Ivoire, *Laccosperma secundiflorum*.

MATERIAL AND METHODS

PLANT MATERIAL

Three kinds of explants were used: portions of unopened leaves (0.5 cm × 1 cm on average), axillary buds (0.7 cm in length), and 1 cm long apical meristem fragments. Explants were harvested during the rainy season, in August and September 2005. They were collected from 25 to 50 cm high saplings growing in a natural stand located in Abidjan (5°22'40'' N - 4°40'00'' W, 100 m a.s.l.). Mean annual precipitation and temperature of the sampling site, based on data from 1996 to 2005, were 1 727 mm and 26.2°C, respectively. The explants were rinsed in running tap water before sterilisation.

EXPLANTS STERILISATION

Three protocols were tested for explant surface sterilisation: 1) 1 min in 70% ethanol followed by 30 min immersion in 1.8% sodium hypochlorite containing 2-3 drops of Tween 20; 2) 1 min in 70% ethanol followed by 30 min immersion in 3.6% sodium hypochlorite containing 2-3 drops of Tween 20; 3) 1 min in 70% ethanol followed by 30 min immersion in 3.6% sodium hypochlorite containing 2-3 drops of Tween 20, then 5 min in 0.01% $HgCl_2$. For each proto-

col, the experimental unit consisted of 24 explants in each of 3 culture glass racks (n = 72) for leaves and axillary buds, and 24 explants in each of 2 glass racks (n = 48) for apical meristems. In the three protocols, between two consecutive treatments, explants were rinsed three times with sterile distilled water.

With the aim of reducing the browning of the culture media and of predisposing explants to shoot induction, the explants were inoculated into different media of initiation and incubated in the dark at 28 ± 2°C for 4 weeks.

Two types of initiation media containing macro- and microelement of MS (MURASHIGE & SKOOG 1962) salt supplemented with vitamins (MSV): thiamine (1 mg L^{-1}), nicotinic acid (1 mg L^{-1}), pyridoxine (1 mg L^{-1}) and myo-inositol (100 mg L^{-1}), were tested. 1) MSV supplemented with 30 g L^{-1} sucrose (MI1) and 2) MSV supplemented with 30 g L^{-1} sucrose and 0.65 mg L^{-1} BAP (MI2). The concentrations of vitamins were selected on the basis of investigations carried out on *Calamus merrillii* Becc. and *Calamus subinermis* Wendl. by GOH *et al.* (2001b). Solidification was carried out in culture tubes (55 mL containing 15 mL medium in each) with 2.5 g L^{-1} gelrite and 0.75 g L^{-1} MgCl$_2$, pH adjusted to 5.8 using 0.1 N KOH or HCl, before autoclaving at 121°C for 30 min.

Induction media were prepared using MSV supplemented with 30 g L^{-1} sucrose, 500 mg L^{-1} casein hydrolysate, 100 mg L^{-1} L-Glutamine (CHUTHAMAS *et al.* 1989, MULUNG 1992), and 10 combinations of 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA) concentrations. Solidification was carried out in 55 mm × 70 mm glass flasks, each containing 20 mL medium with 2.5 g L^{-1} gelrite and 0.75 g L^{-1} MgCl$_2$. The media were sterilised by autoclaving at 121°C for 30 min. The cultures were incubated in a growth chamber at 24 ± 2°C, 75% relative humidity (RH), 14 h photoperiod, and 25 to 30 μmol m^{-2} s^{-1} provided by white fluorescence tubes. Explants were subcultured to fresh medium every two weeks or whenever the medium turned brown.

The percentage of explants inducing buds was estimated after 8 weeks of incubation. After a 12-weeks incubation period, shoots measuring at least 2 cm in length were excised and transferred onto elongation medium (EM) constituted of MSV medium supplemented with 1 mg L^{-1} BAP and NAA. Plantlets were transferred onto rooting medium [MSV containing 0.5 mg L^{-1} of 3-indoleacetic acid (IAA)] after 20 weeks of culture.

The experimental unit consisted of five explants in each culture glass flasks, and there were 12 glass flasks (n = 60) per treatment for leaves and axillary buds, and 9 glass flasks (n = 45) for apical meristems.

The efficiency of the three disinfection protocols was checked by noting the infestation level (% infected explants) after 3 and 7 days of incubation. Percentages of explants bearing shoots in each of the two initiation media (MI1 and MI2), as well as the *in vitro*-grown shoots vigour were recorded. Vigour was determined by visual assessment. For each induction medium, the percentage of explants bearing shoots and the number of shoots per explant were recorded 8 weeks after transfer of explants. One-way analysis of variance (ANOVA1) was performed to test for differences in percentage of explants infected and in number of shoots per explant between the three disinfection protocols and between hormonal combinations (BAP and NAA), respectively. An ANOVA1 was also performed on percentages of explants bearing shoots, but after arcsine transformation of percentages. When the null hypothesis of an ANOVA was rejected, multiple comparisons using the Least Significant Difference (LSD) test were carried out to test for significant pairwise differences between media or protocols of disinfection. All LSD tests were carried out at α = 0.05 significance level. Statistical analyses were performed using the MINITAB for WINDOWS, ver. 13 (MINITAB 2000).

RESULTS

For the leave explants there was no contamination whatever the disinfection protocol used (Table 1). For the two other explant types, infection was observed with the two first protocols but contamination was drastically reduced with the protocol using HgCl$_2$. This protocol affected significantly and positively explants disinfection and after seven days of incubation, the mean percent of explants infected was 0 for leaves, 4.16 ± 5.89 for apical meristems and 6.94 ± 2.41 for axillary buds (Table 1). The protocol with the addition of HgCl$_2$ was, therefore, the most efficient for *L. secundiflorum*.

Explants responded differently to the initiation media. Leaf portions necrosed completely after 4 weeks of initiation, regardless of the

Table 1. Effect of three disinfection protocols on infection of three types of explants from *L. secundiflorum*.

Disinfection protocol	Mean percentage (± SD) of infected explants					
	Three-days incubation			Seven-days incubation		
	Leaf portion	Apical meristem	Axillary bud	Leaf portion	Apical meristem	Axillary bud
1.8% NaOCl	0.0 [a]	12.49 ± 5.89 [c]	27.77 ± 10.49 [c]	0.0 [a]	75.00 ± 17.68 [b]	83.33 ± 11.02 [b]
3.6% NaOCl	0.0 [a]	6.24 ± 2.95 [b]	12.5 ± 8.33 [b]	0.0 [a]	64.58 ± 8.89 [b]	76.39 ± 6.36 [b]
3.6% NaOCl + 0.01% HgCl$_2$	0.0 [a]	0.0 [a]	0.0 [a]	0.0 [a]	4.16 ± 5.89 [a]	6.94 ± 2.41 [a]

Note. Means followed by the same letter are not significantly different at $\alpha = 0.05$.

medium. On the contrary, all the incubated axillary buds and apical meristems generated shoot buds. These explants were most vigorous on MI2, compared to MI1 (Fig. 1A and 1B). Consequently, explants incubated onto MI2 were used for shoot induction.

The percentage of explants producing shoots was significantly influenced by hormonal combination, and ranged from $11.67 \pm 10.30\%$ to $83.33 \pm 14.35\%$ for axillary buds ($F = 38.46$, $P < 0.001$), and from $11.11 \pm 14.35\%$ to $86.67 \pm 10.00\%$ for apical meristem fragments ($F = 27.70$, $P < 0.001$) (Table 2). The mean number of shoots per explant was also significantly influenced by hormonal combinations, and ranged from 0.71 ± 0.11 to 3.71 ± 1.96 for axillary buds ($F = 29.39$, $P < 0.001$), and from 0.61 ± 0.16 to 3.08 ± 1.46 for apical meristem fragments ($F = 22.67$, $P < 0.001$). With a fixed BAP concentration of 1 mg L^{-1}, the percentage of explants inducing shoots as well as the mean number of shoots per explant increased with increasing concentrations of NAA. Further increase in BAP concentration (> 1.5 mg L^{-1}) induced hypertrophy of explants, coupled with low rates of shoot induction (Fig. 1C). Media containing 2 mg L^{-1} BAP and 0.5 mg L^{-1} NAA or 0.65 mg L^{-1} BAP without NAA were particularly different from the other media because they markedly induced callus formation. Medium with 1 mg L^{-1} BAP and 1 mg L^{-1} NAA yielded highest percentages of explants forming shoots ($> 85\%$, Fig. 1D and 1E), followed by media containing 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA and 1.5 mg L^{-1} BAP and 1 mg L^{-1} NAA (from 55% to 75% of living explants). When the formed shoots were subcultured onto rooting medium (MSV containing 0.5 mg L^{-1} IAA), 1-2 roots were observed after 2 weeks on 96% of living explants (Fig. 1F).

DISCUSSION

The addition of HgCl$_2$ to the sterilisation solution significantly improved explant disinfection. In contrast to sodium hypochlorite, a surface disinfectant that has a restricted action spectrum, HgCl$_2$ has a double surface- and internal-sterilisation property (ZHANG & TYERMAN 1999). The efficiency of HgCl$_2$ in the sterilisation of palm explants has been reported by RAJESH *et al*. (2003). Their results suggested that rattan explants are characterised by a high load of external- and surface-infecting microbes.

Lower concentrations of NAA (0.5 mg L^{-1}) and BAP (2 mg L^{-1}) or both combined or BAP (0.65 mg L^{-1}) alone resulted in callus formation. The presence of callus on explants cultured in media supplemented or not with auxin has already been reported for other palm species (WANG *et al*. 2003). These authors observed important callus formation on shoots of *Areca catechu* L. cultured on MS supplemented with 0.2 mg L^{-1} BAP and 0.2 mg L^{-1} Thidiazuron (TDZ). Using root explants from *in vitro* plant of *Calamus manan* Miq. cultured in MS containing 0.15 mg L^{-1} BAP, YUSOFF & MANOKARAN (1985) obtained somatic embryos.
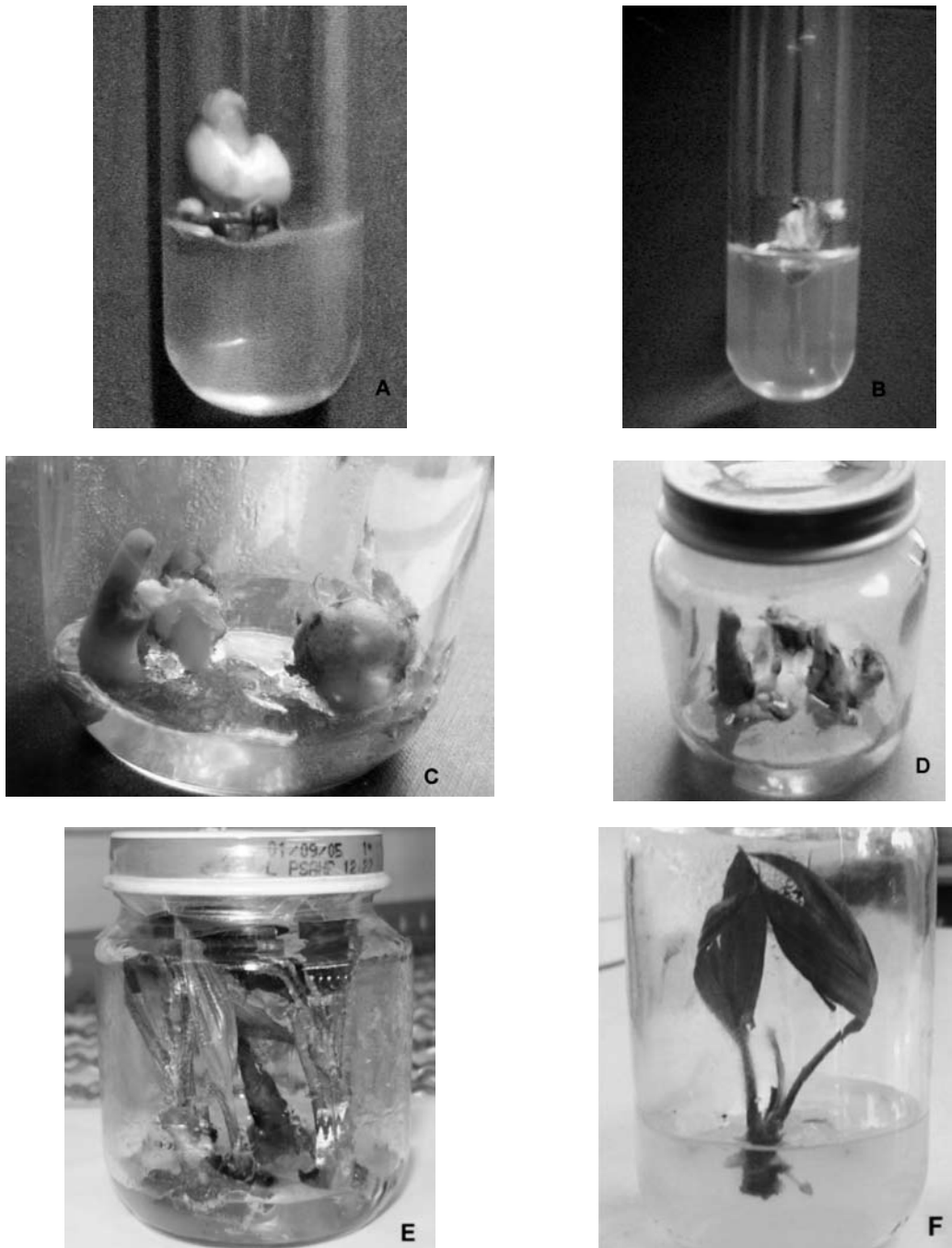
Fig. 1. Plant regeneration from axillary bud and apical meristem explants collected from seedlings of *L. secundiflo-rum* growing in natural stand. A: axillary bud cultured 3 weeks on initiation medium; B: apical meristem cultured 3 weeks on initiation medium; C: hypertrophied axillary bud incubated on medium containing high concentration of BAP (> 1.5 mg L^{-1}); D: multiple shoots formation on the collar of explants; E: elongation of shoot with well-developed leaves; F: established plant.

Table 2. Effect of BAP and NAA on direct formation of shoots from two types of explants of *L. secundiflorum* after an eight-week incubation period.

Hormone (mg L⁻¹)		Mean percentage (± SD) of explants bearing shoots		Mean number (± SD) of shoots per explant	
BAP	NAA	Axillay bud	Apical meristem	Axillay bud	Apical meristem
0.65	0	13.33 ± 13.03 [a]	11.11 ± 14.53 [a]	0.71 ± 0.11 [a]	0.61 ± 0.16 [a]
1	0.25	43.33 ± 14.35 [b]	37.78 ± 15.63 [b]	1.31 ± 1.07 [bc]	1.31 ± 0.71 [bc]
1	0.50	53.33 ± 9.85 [b]	46.67 ± 10.00 [bc]	1.77 ± 1.35 [cd]	1.47 ± 0.91 [cd]
1	1	85.03 ± 14.35 [c]	86.67 ± 10.00 [e]	3.71 ± 1.96 [e]	3.10 ± 1.46 [e]
1.5	0.25	51.67 ± 10.30 [b]	24.44 ± 8.82 [a]	1.52 ± 0.82 [c]	1.00 ± 0.67 [ab]
1.5	0.50	55.00 ± 15.08 [b]	71.11 ± 14.53 [d]	1.33 ± 1.11 [bc]	1.78 ± 0.90 [d]
1.5	1	75.00 ± 12.43 [c]	57.78 ± 23.33 [cd]	2.06 ± 1.21 [d]	1.81 ± 1.43 [d]
2	0.25	23.33 ± 11.55 [a]	15.56 ± 8.82 [a]	0.83 ± 0.75 [a]	0.72 ± 0.70 [a]
2	0.50	16.67 ± 7.78 [a]	17.78 ± 12.02 [a]	0.94 ± 0.86 [ab]	0.83 ± 0.65 [a]
2	1	11.67 ± 10.30 [a]	15.56 ± 8.82 [a]	0.78 ± 0.75 [a]	0.69 ± 0.60 [a]

Note. Means followed by same letter are not significantly different at α = 0.05

The high vigour of explants in MI2 (MSV supplemented with 0.65 g L⁻¹ BAP) could be attributed to the presence of BAP in this medium. The beneficial role of exogenous BAP in explant micropropagation or organogenesis has been reported for *Ceropegia candelabrum* L. (BEENA *et al*. 2003).

Our study highlights the higher potential of axillary bud and apical meristem explants to produce shoots in *L. secundiflorum* and the non-responsiveness of foliar explants. Similar tendencies have been observed for other rattan species. Indeed, GOH *et al*. (1999, 2001a) showed that foliar explants collected from *in vitro*-grown plantlets of Asian *Calamus* species were useless for micropropagation. In contrast, meristem portions from *in vitro*-grown plantlets of *C. manan* (YUSOFF 1989) and *Calamus manillensis* H. Wendl. (PATENA *et al*. 1984) allowed the regeneration of 3-6 shoots per explant, after a three-month period. Nevertheless, explants from *L. secundiflorum* responded weakly in most of the media used, compared to *Calamus* species (PATENA *et al*. 1984). Such a difference could be explained by the fact that the serial immersions of explants in disinfection solutions could subsequently decrease their reactivity. Such treatments were not required for PATENA *et al*. (1984), who used explants from seedlings grown in vitro.

To our knowledge, tissue culture of African rattans has never been documented. First investigations were aimed at conventional vegetative propagation of two species (*L. leave* and *L. secundiflorum*) using suckers and rhizomes (ZORO BI & KOUAKOU 2004b). Therefore, this study constitutes the first basis on which programmes for the conservation and large-scale *in vitro* propagation of desired genotypes of *L. secundiflorum* can be developed. Based on the selected media and incubation conditions, three plantlets may be expected per explant after two months, resulting in about 4 100 plantlets per explant after six culture cycles.

In spite of the seemingly successful regeneration of plantlets from apical meristem and axillary bud explants, more studies are needed to improve the reliability and efficiency of the protocol implemented, especially by starting from zygotic embryo explants. The use of zygotic embryo explants should avoid a sophisticated disinfection phase that is likely to influence explant responsiveness. Also, improvement of the *in vitro* growth medium, as well as incubation conditions, is necessary for better and faster regeneration of uniform plants from desired genotypes. Studies on the survival and growth of the plantlets under nursery and field conditions should be considered once a better medium is established for mass-production of *L. secundiflorum*.

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