Industrial propagation by rooted cuttings of mature selected clones of *Hevea brasiliensis*

Aurélien Masson
Jean-Marc Julien
Luc Boedt

SoGB, SOCFIN group estate
01BP365
San Pedro
Côte d'Ivoire

Mass production by rooted cuttings of mature selected clones of *Hevea brasiliensis* in SoGB nursery facilities.
Photograph A. Masson.

A. Masson, J.-M. Julien, L. Boedt

RÉSUMÉ

MULTIPLICATION INDUSTRIELLE PAR BOUTURAGE DE CLONES MATURES D'*HEVEA BRASILIENSIS*

L'importance de l'hévéa (caoutchouc), *Hevea brasiliensis*, en tant que culture de rente ne cesse d'augmenter justifiant de s'intéresser à de nouvelles techniques de clonage plus efficaces que le greffage (écussonnage) traditionnellement utilisé pour la production industrielle de matériel de plantation de qualité supérieure. Les bonnes performances sur le terrain (croissance rapide, haut rendement) des hévéas produits par embryogenèse somatique n'ont été constatées jusqu'à présent qu'à l'échelle expérimentale. La propagation de masse *in vitro* par embryogenèse somatique ou microbouturage de clones d'hévéas sur leurs propres racines reste pénalisée par un manque de réactivité de la plupart des génotypes sélectionnés et par des coûts de production prohibitifs. Face à cette situation, la propagation par bouturage de clones matures sélectionnés issus de micropropagation *in vitro* a été tentée par la SoGB en Côte d'Ivoire comme une alternative possible à l'utilisation exclusive des techniques *in vitro*. Les deux clones matures industriels, A (70 ans) et B (53 ans), ont d'abord été rajeunis *in vitro* par embryogenèse somatique puis micropropagés en plus grand nombre par microbouturage. Après acclimatation, les microboutures enracinées *in vitro* ont été rempotées dans des pots individuels pour être gérées de manière intensive comme pieds-mères destinés au bouturage. Après 3 semaines en conditions horticoles adéquates, les taux d'enracinement obtenus pour les boutures des clones A et B ont été respectivement de 74,6 % (1203/1613) et 76,5 % (198/259). Les racines adventices néoformées étaient généralement vigoureuses. A l'issue d'une phase d'acclimatation réussie, les boutures se sont développées de façon conforme pour atteindre 4 mois plus tard une hauteur de 25-30 cm suffisante pour être plantées au champ. En sus d'une plus grande vigueur et conformité sur le terrain, les clones issus de bouturage peuvent être produits plus rapidement, sur des surfaces plus réduites à moindre coût et dans des conditions de travail plus faciles par rapport aux plantes issus d'écussonnage. Des analyses en cours devraient permettre d'établir les avantages comparatifs des boutures par rapport aux plants écussonnés en ce qui concerne d'autres caractères à fort impact économique tels que le rendement de latex.

Mots-clés: *Hevea brasiliensis*, embryogenèse somatique, bouture, clones, rajeunissement, production industrielle , piedmère, rentabilité.

ABSTRACT

INDUSTRIAL PROPAGATION BY ROOTED CUTTINGS OF MATURE SELECTED CLONES OF *HEVEA BRASILIENSIS*

The importance of *Hevea brasiliensis* (rubber tree) as a cash crop keeps increasing warranting the development of new and more efficient techniques than the bud-grafting traditionally used for mass producing superior planting material. *In vitro* production of rubber trees by somatic embryogenesis and the good field performance (fast growth, high yield) of the resulting emblings have been reported for years, but so far only on an experimental scale. *In vitro* mass production of self-rooted rubber clones by somatic embryogenesis or microcuttings is hindered by a lack of responsiveness of most of the selected genotypes and by prohibitive production costs. Given this situation, macro propagation by rooted cuttings following *in vitro* propagation of mature selected clones was attempted by the SoGB estate in Côte d'Ivoire as a possible alternative to *in vitro* techniques only. Two industrial mature clones, clone A (70yrs-old) and clone B (53yrs-old), were first rejuvenated *in vitro* by somatic embryogenesis then micropropagated in greater numbers by microcuttings. After acclimatization, these *in vitro*-rooted microcuttings were potted in individual containers to be managed intensively as stock plants to produce macro cuttings. After 3 weeks under suitable rooting conditions in the nursery, rooting rates of 74.6% (1203/1613) for clone A and 76.5% (198/259) for clone B macrocuttings were obtained. The cuttings produced vigorous and taproot-like adventitious roots. After successful acclimatization, all the rooted cuttings developed true-to-type and 4 months later reached a sufficient height of 25-30 cm to be field planted. In addition to higher field vigor and true-to-typeness, cutting-derived rubber tree clones can be produced more quickly, on less nursery space at lower cost and in easier working conditions than traditionally bud-grafted plants. Further investigations are underway for assessing at the field level the comparative advantages of rooted cuttings *versus* grafted clonal offspring with respect to traits of major economic importance such as latex yield.

Keywords: *Hevea brasiliensis*, somatic embryogenesis, rooted cuttings, clone, rejuvenation, mass production, stock plant, cost efficiency.

RESUMEN

PROPAGACIÓN INDUSTRIAL POR ESTAQUILLAS DE CLONES MADUROS DE *HEVEA BRASILIENSIS*

La importancia de la hevea (árbol del caucho), *Hevea brasiliensis*, como cultivo comercial no cesa de aumentar, lo que justifica el interés de nuevas técnicas más eficientes que el injerto (en escudo) tradicionalmente empleado para la producción industrial de material de cultivo de calidad superior. El buen desempeño en el campo (crecimiento rápido y alto rendimiento) de las heveas producidas por embriogénesis somática, llevan años observándose, pero hasta ahora sólo a escala experimental. La propagación masiva *in vitro* por embriogénesis somática o microestaquillas de clones de hevea de sus propias raíces, está obstaculizada por falta de reacción positiva de parte de la mayoría de los genotipos seleccionados y por costos de producción prohibitivos. Ante esta situación, la propagación de estaquillas de clones maduros seleccionados procediendo de micopropagación *in vitro*, ha sido llevada a cabo por la compañía SOGB de Côte d'Ivoire, en tanto que posible alternativa al uso de técnicas exclusivamente *in vitro*. Los dos clones maduros industriales, A (70 años de edad) y B (53 años de edad), fueron primero rejuvenecidos *in vitro* por embriogénesis somática y luego micropropagados en mayor número por microestaquillas. Una vez aclimatadas, las microestaquillas enraízadas *in vitro* se trasplantaron en macetas individuales para su manejo intensivo como plantas madres destinadas a producir estaquillas. Al cabo de tres semanas en condiciones adecuadas de cultivo, las tasas de enraizamiento logradas fueron: 74,6 % (1203/1613) para el clon A y 76,5 % (198 /259) para el B. Las raíces adventicias neoformadas resultaron generalmente vigorosas. Tras el éxito de su aclimatación, todas las estaquillas tuvieron un desarrollo favorable y cuatro meses después alcanzaron una altura de 25-30 centímetros, lo suficiente para su trasplante en campo. Además de mayor vigor y mejor conformación en plantación, los clones reproducidos por estaquillas pueden multiplicarse en vivero con mayor rapidez, menor espacio, fáciles condiciones de trabajo y costos inferiores a lo que requieren las plantas procedentes de injerto. Estudios en curso deberian poder establecer ventajas comparativas de plantas procedentes de estaquillas frente a las producidas por injerto en lo que se refiere a factores de gran importancia económica tales como el rendimiento en látex.

Palabras clave: *Hevea brasiliensis*, embriogénesis somática, estaquillas, clones, rejuvenecimiento, producción industrial, planta madre, rentabilidad.

Introduction

Hevea brasiliensis Muëll. Arg. (rubber tree) is a species belonging to the *Euphorbiaceae* family and originated from the South American Amazonian forest. Being the main source of natural rubber, it has been domesticated and introduced in many countries of Africa and South East Asia to be exploited in large estates since the end of the 19th century. This expansion was warranted by an increasing need for tyres and the development of new ways of transportation. Rubber plantations were originally established with unselected seedlings resulting in high heterogeneity and very low yield (300 to 600 kg of dry rubber (DR) per ha per year). Since the 1920s, bud-grafted clones have been used in breeding programs and have contributed to a dramatic yield increase (2,500 to 3,500 kg of DR/ha/y). The "Wickham" population exported from Brazil to Asia via Kew Gardens in 1876 has been the first and only gene pool used in *H. brasiliensis* breeding, until recent genetic enrichment by other sources (CLEMENT-DEMANGE *et al.*, 2007). Genetic improvement through conventional breeding is hampered by a long immature period, an insufficient fruit production and a high level of heterozygosity. Development of superior *H. brasiliensis* clones remains a long term and high-investment process: RRIM600 which is currently the most widespread clone worldwide was obtained by manual pollinations (TJIR1 x PB86) in 1937 at the Rubber Research Institute of Malaysia.

Bud grafting was used for clonal mass propagation of selected clones as an alternative to propagation by rooted cuttings. Experiments were conducted in 1952 at the Firestone estate, Liberia (LEVANDOWSKY, 1959) on thousands of cuttings collected from bud-grafted clonal stock plants regularly rejuvenated by intensive hedging practices. The clones originated from the 1920s breeding programs and were about 30 yr-old from seed germination. These cuttings were set for rooting at different dates between 1952 and 1954 and after 75 days on average, rooting success varied from 10 to 75% according to the clones. Among the various factors tested *i.e.* application of exogenous rooting substances, urea sprays, use of various rooting substrates, the genotype had the greater influence on the rooting ability of the cuttings. Despite these encouraging results, experiments on clonal propagation of rubber trees by rooted cuttings stopped prematurely and emphasis was given to tissue culture for attempting to producing clones on their own roots. Noteworthy success has been obtained by *in vitro* somatic embryogenesis but, although well demonstrated (MONTORO *et al.,* 2012), this technique is still restricted to a few genotypes produced in limited numbers. Moreover prohibitive cost remains a major hindrance to its large-scale utilization despite a strong and persisting interest from the planters. Recent findings have confirmed that industrial clones, when produced after *in vitro* rejuvenation on their own roots, grow faster and yield more latex than those derived from budding (XIONTING *et al.,* 2001; NAYANAKANTHA & SENEVIRATNE, 2007; DIBI *et al.,* 2010; MONTORO *et al.*, 2012).

This situation, the increasing pressure on available land and demand for higher and earlier returns have prompted SOCFIN group to reconsider the possibilities of mass production of *Hevea* clones by rooted cuttings. Research was done in SoGB estate facilities, Ivory Coast, and the method and results constitute the purpose of this paper.

Photograph 1.
Acclimatization of the *in vitro* plantlets in SoGB nursery facilities.
Photograph A. Masson.

Rejuvenating the mature selected genotypes by somatic embryogenesis

Direct or primary somatic embryogenesis (CARRON & ENJALRIC, 1985) was first used for rejuvenating the mature genotypes: the emblings obtained from the integuments of the fruits have the same genotype as the mother tree (grafted selected clone) and are by definition ontogenetically juvenile (LARDET *et al.*, 2009; MONTEUUIS *et al.,* 2011).

Immature fruits were harvested from 4 yrs-old bud-grafted trees of the industrial mature clones A and B – respectively 70 and 53 yrs-old since seed germination – at SoGB estate in 2008, then sent to the Laboratory for Applied *In Vitro* Plant Biotechnology of University Ghent in Belgium. Applying the CARRON and ENJALRIC (1985) somatic embryogenesis protocol, 153 emblings were obtained for clone A and 91 for clone B. These emblings were further micropropagated by axillary budding to produce microcuttings. These were rooted *in vitro* with average success rates of 63.3% (752/1,188) for clone A and 64.5% (178/275) for clone B, then shipped to SoGB estate facilities for acclimatization to ex-vitro conditions in 2012.

Getting responsive stock plants

After a 3 day-long airfreight shipment, the *in vitro* rooted microcuttings arrived in SoGB nursery facilities in Grand Bereby, Ivory Coast, and were immediately potted individually in 200 cm^3 (50mm wide x 150 mm high) containers filled with coconut peat to be acclimatized during 6 weeks in a greenhouse equipped with a shade and mist-system giving nearly 95% relative humidity (RH) and 75% shade. These conditions were progressively reduced to reach ambient humidity and 50% shade at the end of the acclimatization process (photograph 1). After acclimatization, 77% (581/752) of the microcuttings of clone A and 78% (139/178) of clone B survived

Photograph 2.
Acclimatized microcutting-derived plant of clone A ready to be managed as stockplant.
Photograph A. Masson.

Once acclimatized, the rooted microcuttings were hardened outdoors under a suitable watering regime and sprayed with appropriate foliar fertilization and phytosanitary treatments. They were initially protected from direct sun exposure by a 50% shade net that was removed after 1 month. Plants were ready to be suitably managed as stock plants as soon as the bark at the base of the trunk was lignified (photograph 2), roughly 6 months after the acclimatization process started.

For stock plant establishment, 500 microcutting-derived plants of clone A and 100 of clone B were individually potted in 3000 cm^3 containers (156 mm wide x 200 mm high) filled with coconut fiber and placed in lines on benches for easy maintenance, sanitation treatments and harvesting of macro cuttings. Spacing of the stock plants was 30 cm x 30 cm giving 10 plants per m². The objective was to produce the more shoots that can be rooted per stock plant. These consisted of newly produced soft shoots with 2 to 4 fully developed leaves and a resting terminal bud in their upper part, as illustrated in photograph 3. Fifteen shoots of this type to be used as cuttings on average could be produced monthly per m² of stock plants. These shoots were harvested every 6 days, during the 4 months from February to May 2013 so that the rooted cuttings could be planted from June to September, at the beginning of the rainy season (from end of May to October with average rainfall of 1 150 mm for this period *vs*1 600 mm per annum on the plantation site).

Rooting and development of cuttings

Immediately after collection from the stock plants, the leafy shoots were trimmed to 15 to 20 cm long terminal cuttings with half of the leaf surface cut in order to lower evapotranspiration (photograph 3). The cuttings were then inserted into 200 cm^3 (50mm wide x 150 mm high) containers filled with coconut peat and maintained under the same greenhouse conditions as detailed previously for the acclimatization of the microcuttings. The first adventitious roots appeared 15 days later and after 15 more days the cuttings were hardened off in the same conditions as described above for the microcuttings.

Of the cuttings initially set for clone A, 74.6% (1 203/1 613) and 76.5% (198/259) for clone B were successfully rooted and acclimatized applying these protocols. The adventitious roots formed were vigorous and showed a strong positive geotropism (photograph 4). After a 2.5 month-long raising period under standard nursery conditions, the rooted cuttings resumed new growth, developing new leaves to reach a sufficient height of 25 to 30cm to be field planted (photograph 5).

Discussion

For the present, this work is the first demonstration that industrial clones of *Hevea brasiliensis* can be mass propagated by rooted cuttings with success rates exceeding 70%. This can be considered as a real breakthrough for this highly economically important species.

Until now, bud grafting has been the only means of mass clonally propagating mature selected rubber tree genotypes. For rubber, as with other tree species, grafting is liable to induce graft incompatibility symptoms such as: within clone variability, reduction of vigor and of latex production, anticipated mortality and bark necrosis syndrome (HARTMANN *et al.,* 1997; CHRESTIN *et al.,* 2004). The failure of the few experiments conducted during the 1950's on the propagation of industrial mature clones by rooted cuttings (NAYANAKANTHA & SENEVIRATNE, 2007) was very likely due, similarly to other tree species, to the inhibitory effect of ageing on adventitious rooting ability (BORCHERT, 1976; HACKETT, 1985). This was supported by the observation that rubber tree cuttings root more easily when collected from juvenile than from mature donor plants (MUZIC, 1958; OLIVER & MONTEUUIS, 2009; MONTEUUIS, personal communication). The determining factor of our success was the capacity first, to physiologically rejuvenate the mature selected genotypes by somatic embryogenesis. This was made possible by *Hevea brasiliens* is being one of the very few trees species for which somatic embryos can be obtained from maternal tissues of mature genotypes. However, direct somatic embryogenesis was preferred to longer protocols in order to reduce the risks of somaclonal variations, or even mutations. These can be increased by prolonged exposure to non-optimal culture media, which

Photograph 3.
Suitable shoot to be used as a cutting.
Photograph A. Masson.

Photograph 4.
Cutting rooted after 1 month.
Photograph A. Masson.

may also induce maturation symptoms (DUNSTAN *et al.*, 1995; VON ADERKAS & BONGA, 2000; MONTORO *et al.*, 2010). The possibility of further amplifying by micropropagation the limited number of emblings obtained per genotype resulted in greater numbers of *in vitro* rejuvenated plants produced which could then be used as stock plants. This is a real asset as the ability of *Hevea brasiliensis* to be *in vitro* propagated by somatic embryogenesis and micropropagation is strongly genotype dependent (NAYANAKANTHA & SENEVIRATNE, 2007). Such genotype-related efficiency of the *in vitro* methods and the constraints associated with the operational use of these methods themselves should not be underestimated. In this regard, the possibility to physiologically rejuvenate mature selected genotypes to produce stock plants with a high rooting capacity without resorting to tissue culture needs to be seriously investigated. An encouraging indication is that the cuttings rooted in nursery conditions could be used as new stock plants with similar responsiveness as the *in vitro* ones from which they were derived. Mass clonal propagation by rooted cuttings of mature selected genotypes of teak and of some eucalypt species and hybrids has been proven to be successful using nursery techniques only, even for physiologically rejuvenating the mature material, which remains the fundamental requisite (MONTEUUIS *et al.*, 1995; SAYA *et al.*, 2008). Rooting rates of more than 75% for cuttings from mature *Hevea brasiliensis* genotypes were unexpected based on previous experiments and must therefore be considered as very encouraging. This demonstrates the impact of suitable techniques – and not necessarily the most expensive and sophisticated ones – on successful true-to-type propagation by rooted cuttings of a plant material considered till then as strongly recalcitrant. This high responsiveness for adventitious rooting allowed for rooting the cuttings directly in the containers saving manipulations and rooting substrate resulting in higher cost efficiency.

Mass production of rooted cuttings in nursery conditions required more equipment, with special mention of a reliable and automatic mist system, than for grafting. But the higher cost of the facilities needed is offset by numerous advantages associated with the use of rooted cuttings compared with grafted plants, as described below.

Photograph 5.
Four-month old rooted cutting of clone A ready to be field planted.
Photograph A. Masson.

Photograph 6.
Container-grown rooted cuttings are more convenient to handle than grafted plants in poly bags.
Photograph A. Masson.

The propagation process is much shorter

Industrial clones can be produced by rooted cuttings for field planting within 4 months *vs* 10 months for bud-grafts: 7 months are needed to get the sufficiently developed rootstocks from seed, then 3 more months for the grafted plants to be ready for field planting.

More plants can be produced on a much smaller nursery area

Projections in our site conditions based on an activity period of 4 months indicate a requirement of 100-150 m^2 for stock plants and 50 m^2 for rooting, acclimatization and nursery raising to produce enough cuttings to plant 10 ha at a density of 550 trees/ha (ratio 1:1,000) *vs* 1,200 to 1,400 m^2 of budwood garden and a nursery raising area of 800 m^2, *i.e.* 8 times more for planting the same area with grafted plants (that are raised in bigger 50 x 15 cm containers).

The differences between the two systems are likely to be more important under heavier rainfall in absence of dry season as rooted cuttings can be produced and field planted year round.

Cuttings-derived plants require less manpower than bud-grafted ones

Two rounds of grafting are required to get similar propagation success rates (74-75%) as by rooted cuttings. Also cuttings rooted directly in small and reusable containers placed in racks of 60 units are definitely easier to handle than grafted plants, as illustrated in photograph 6. Despite the relatively small volume of medium in the rooted cuttings containers, growth in the field is rapid (photograph 7).Grafted plants are produced in much bigger (60 x 15 cm) and heavier plastic bags, requiring larger planting holes. These plastic bags cannot be re-used after planting in contrast with the containers used for the cuttings.

Cuttings-derived *Hevea brasiliensis* clones are expected to be more vigorous and produce more latex than the same bud-grafted clones

XIONTING *et al.* (2001) reported superiority in average stem girth and dry rubber yield of 9.1 to 35.2 % and of 29.9 to 46.3 % respectively from microcutting-derived clones compared to the same clones produced by bud-grafting. More specifically, RRIM600 rooted microcuttings trees had an average trunk volume 14.36 % bigger and produced 32.5 % more dry rubber than bud-grafted trees of the same clone at the same age. These results show that self-rooted juvenile clones *i.e* microcuttings and cuttings-derived clones, are considered to be superior in growth and production.

Further investigations on mass propagation by rooted cuttings of *H. brasiliensis* industrial clones must be pursued, focusing more particularly on the following issues:

- The applicability of the protocols developed to a wider range of clones and of environments, especially under higher levels of precipitation and in areas with no distinct dry season.
- The possibility to succeed using nursery techniques exclusively, even for physiologically rejuvenating the mature clones without resorting to *in vitro* methods.
- Advanced field observations of the rooted-cuttings-derived clones compared with the same clones traditionally produced by bud-grafting, with special consideration for economically important traits such as vigor, within clone variation and latex yield.

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Photograph 7.
Rooted cutting of clone A one month after planting at SoGB estate.
Photograph A. Masson.

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