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# Endogenous cytokinins as biochemical markers of rubber-tree (*Hevea brasiliensis*) clone rejuvenation

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## Abstract

The endogenous levels of isopentenyladenine, isopentenyladenosine, zeatin and zeatin riboside and the ability for *in vitro* axillary shoot organogenesis and rhizogenesis were compared between mature and rejuvenated clones of *Hevea brasiliensis* (Müll. Arg.). Enhancement of the *in vitro* organogenesis ability of rubber-tree clones following somatic embryogenesis or repeated grafting onto juvenile rootstocks was accompanied by an increase of zeatin riboside levels in shoots used as starting material for *in vitro* micropropagation. Furthermore, the zeatin level, in *in vitro* shoots of clones treated by *in vitro* micrografting, and consequently capable of axillary shoot and root organogenesis, was higher than in *in vitro* shoots of non treated mature material incapable of *in vitro* organogenesis. We conclude that the endogenous zeatin-like cytokinin level (free and ribosylated forms) can be considered as a reliable marker for the recovery of *in vitro* shoot and root organogenesis after rejuvenating treatments in rubber-tree clones.

*Abbreviations:* ZR – zeatin riboside; 2iP – isopentenyladenine; iPA – isopentenyladenosine; NAA – α-naphthaleneacetic acid; BA – 6-benzyladenine; IBA – indole-3-butyric acid

## Introduction

Rubber-tree (*Hevea brasiliensis* Müll. Arg.) is a tropical species cultivated for its natural rubber production. Because cutting propagation from selected adult trees is not possible, rubber-tree is usually propagated by grafting clonal axillary buds onto seedling-derived juvenile rootstocks. However, such a grafting method leads to intraclonal heterogeneity and to a decrease in rubber production compared to the mother-tree. Thus, *in vitro* micropropagation is an attractive procedure to clone interesting genotypes on their own roots (Carron et al., 1989). The production of plantlets from juvenile plants by *in vitro* micropropagation was originally described in 1982 (Enjalric & Carron) but the production of plantlets from selected mature plants is usually impossible because they are incapable of *in vitro* axillary shoot outgrowth and/or rhizogenesis (Lardet 1987; Seneviratne 1991). Micropropagation has been made successful in some rubber-tree clones by rejuvenation treatments such as tree pruning, repeated grafting of axillary buds onto juvenile rootstocks (Lardet 1987) or *in vitro* micrografting of apices onto *in vitro* developed seedlings (Perrin et al., 1994). However, the efficiency of these techniques is highly variable among genotypes. This variability can originate either from differences in the initial levels of maturity (Fouret et al., 1988; Dumas et al., 1989) or from the need to adapt *in vitro* culture conditions to each genotype (San Jose et al., 1990; Coleman & Ernst 1990; Das & Mitra 1990). Thus, an estimation of the level of rejuvenation reached after a treatment must not be based only on organogenic criteria since they are partly dependent on culture conditions. Studies on biochemical markers of juvenility have been conducted on different woody species. In the case of rubber-tree, a biochemical study of matu-

ration from young seedlings to adult trees has shown that these markers include hormones, certain minerals and anthocyanins. Many morphogenic modifications related to maturation are accompanied by a decrease in endogenous cytokinin level (Haffner 1991). Thus, we have focused our research on cytokinins as potential markers to evaluate the effects of rejuvenation treatments on rubber-tree clones at the biochemical level. Five genotypes were studied before and after rejuvenation treatments with regard to their cytokinin levels in shoots in culture or out of culture and to their *in vitro* micropropagation performance (i.e. *in vitro* axillary shoot and root organogenesis ability). Rejuvenation treatments included repeated grafting of axillary buds onto juvenile rootstocks, *in vitro* micrografting and somatic embryogenesis.

## Materials and methods

### *Plant Material*

#### *Mature material*

Mature material of clones IRCA 18, RRIM 600, PB 260, PB 235 and GT1 were scions of 2 to 3 year-old grafted plants derived from adult mother-trees, selected around 1970 for IRCA 18, 1950 for RRIM 600, PB 260 and PB 235 and 1920 for GT1. Grafted plants were maintained in the greenhouse (60-100% relative humidity; 25–33°C), watered with 2g $l^{-1}$ of a 17N: 7P 22K fertilizer, 3% MgO and pruned every time they reached 5 to 6 foliar whorls (about 2.5 meters high).

#### *Treated material*

Clone IRCA 18 was treated by repeated graftings onto juvenile rootstocks. Axillary buds from mature scions of one foliar whorl were grafted on the stem of 18 month-old seedlings. Two weeks later, rootstocks were pruned to allow for the development of the scion. Five to six weeks later, scions had developed the first foliar whorl and their axillary buds were taken to perform grafts for the next generation. Five successive graftings were performed before evaluation of rejuvenation. Rejuvenated clones RRIM 600 and PB 260 were obtained from somatic embryo-derived plants (further referred as somaplants) developed in the greenhouse for ten and six months respectively. The technique used for somatic embryo production has been described previously (Carron et al., 1994). Before experiments were initiated, axillary buds of somaplants were grafted once, using the same technique as for IRCA 18. Clones PB 235 and GT1 were treated by *in vitro* micrografting. Apices (1–2 mm long) from mature scions were grafted onto three week-old seedlings developed *in vitro*. Three weeks after micrografting, rootstocks were pruned to allow for the development of the scion (Perrin et al., 1994). Clone PB 235 was grafted once. In case of GT1, six successive micrograftings were performed because of the difficulty in restoring its rooting ability. For each micrografting, apices from scions of the previous generation of micrografts were used.

### *Microcutting assays*

For mature and rejuvenated RRIM 600, PB 260 and IRCA 18 and for mature PB 235 and GT1, nodal explants taken from scions of one foliar whorl were placed *in vitro*. For PB 235 and GT1 micrografts, direct microcutting from scions was impossible because they were unable to develop following their isolation from rootstocks. Thus, "mixed" explants were cultured, each composed of the scion and the fragment of rootstock epicotyl (Perrin et al., 1994). Tissue disinfestation and culture conditions are described in Perrin et al., (1994). Explants were cultivated in individual sterile tubes (30 x 150 mm) on solidified mineral medium MB, supplemented with vitamins, 120 mM sucrose and 1.2 µM IBA and 4.4 µM BA. The concentration of growth regulators was reduced to one fifth for the first subculture. Explants were cultured under light (90 µmol $m^{-2}$ $s^{-1}$; 12-h photoperiod), at a constant 27°C and subcultured once a month. At the end of each subculture, shoots developed on explants were induced for rooting: excised shoots of 10 mm or longer were induced by introducing a needle previously soaked in a sterilised aqueous solution 26.8 µM NAA and 24.6 µM IBA into the freshly cut area. The shoots were then immediately cultured on a rooting medium without growth regulators and rooting ability was evaluated 40 days after the induction treatment (Perrin et al., 1994). Basal explants were subcultured to induce the development of new axillary shoots. Capacity for *in vitro* shoot production was evaluated by measuring the proportion of explants still producing axillary shoots after four successive subcultures, compared with the total number of explants placed *in vitro* at the beginning of the experiments. Assays for rooting ability were performed for 3 or 4 subcultures for each clone and data are expressed as the rate of rooted shoots compared

with the total number of shoots tested during the 3 or 4 assays.

*Cytokinin assays*

For mature and rejuvenated RRIM 600, PB 260 and IRCA 18, the most basal nodal explant of each foliar whorl-scion was excised for cytokinin analysis, the other explants were used for microcutting assays. For mature and micrografted PB 235 and GT1, cytokinin analyses were carried out on the *in vitro* shoots developed during the first subculture. Mature and treated materials from each clone were always collected simultaneously. Nodal explants or shoots were immediately frozen in liquid nitrogen, lyophilised and stored at -70°C. Prior to analysis, nodal explants or *in vitro* shoots of each material were pooled and ground together. Ground samples (50 mg dry weight) were homogenised overnight at 4°C, in 5 ml of cold 80% (v/v) aqueous methanol with 18 µM butylhydroxytoluene as an antioxidant. 500 Bq of [$^3$H]isopentenyladenosine dialcohol (d-iPA*; spec. act. 30 GBq mmol$^{-1}$) and of [$^3$H]zeatin riboside dialcohol (d-ZR*; spec. act. 0.25 TBq) were added as internal standards to determine purification losses. Samples were centrifuged at 10000 rpm for 15 min at 4°C and supernatants were purified on C18 Sep-pak cartridges (Millipore) previously equilibrated with 80% aqueous methanol. After sample elution, 5 ml of 80% aqueous methanol was used to rinse the column. Both eluates were pooled, reduced to approximately 400 µl with a centrifuge concentrator under vacuum (Speed-vac, Savant), taken up with 400 µl of acidified water (20 mM acetic acid, 2 mM triethylamine, pH 3.3) and injected into a reverse-phase HPLC column (Lichrospher 5 µm, ODS 100RP18, end-capped, 250 x 4 mm, Merck) thermoregulated at 40°C. The samples were eluted at a 1.2 ml min$^{-1}$ flow rate by a gradient between acidified water (20 mM acetic acid, 2 mM triethylamine, pH 3.3) and acetonitrile as previously described by Imbault et al. (1988). Forty six 1.5 ml fractions were collected for each sample and evaporated to dryness in a Speed-vac concentrator. Fractions were finally taken up in 500 µl of distilled water containing 3 mM sodium azide as a preservative. Aliquots of fractions corresponding to the retention time of d-ZR* and d-iPA* were submitted to liquid scintillation spectrometry (Beckman LS1801, Beckman) to determine the recovery of zeatin-like cytokinins (average of 60%) and iP-like cytokinins (average of 50%) for each sample. Fractions corresponding to the retention time of authentic standards of zeatin, ZR, 2iP and iPA were analysed by ELISA as described by Label et al. (1994), Maldiney et al. (1986) and Sotta et al. (1987). Polystyrene microtitration plates (Nunc) were coated overnight at room temperature with ZR or iPA conjugated with ovalbumin (7 µg ml$^{-1}$ in 50 mM carbonate-bicarbonate buffer, pH 9.6) and subsequently washed four times with distilled water containing 0.1% Photoflo (Kodak). A limited amount of hormone standard or sample, and polyclonal antibody from rabbit (anti-ZR for zeatin and ZR quantification and anti-iPA for 2iP and iPA quantification) were added. Plates were incubated for 2 h at 4°C in darkness to allow competition for anti-ZR or anti-iPA antibodies between ZR or iPA-ovalbumin complex bound to the plate and free hormones (standard or sample) in solution. After washing, antibodies bound to the plate were quantified using the avidin-biotin interaction system. The plate was incubated for 1 h at 40°C with an excess of biotinylated goat anti-rabbit antibody, washed, and then incubated for 1 h at 40°C with an excess of streptavidin-alkaline-phosphatase conjugate. After washing, phosphatase activity bound to the plate was determined with *p*-nitrophenylphosphate (3.8 mM in 1M diethanolamine buffer containing 10 mM $MgCl_2$) and measured at 405 nm with a spectrophotometer (MR 5000, Dynatech). Through cross reactivities of the sera, we were able to quantify zeatin and ZR with ZR antibody (Maldiney et al., 1986) and 2iP and iPA with iPA antibody (Sotta et al., 1987). Calculations were done by reference to a calibration curve established on each microtitration plate by a curvilinear regression to the fourth degree from the average of four experimental standard curves. ELISA data were corrected according to the recovery rates obtained for each sample. Each data point corresponds to the mean of 4 ELISA assays per sample.

*Statistical analysis*

The number of nodal explants used for cytokinin assays and microcutting experiments on RRIM 600, PB 260 and IRCA 18 are shown in table 1 and table 2 respectively. Numbers of shoots used for cytokinin assays and microcutting experiments on PB 235 and GT1 are shown in Table 3. A confidence interval is calculated for each mean. Chi-square test was used to evaluate the significance of differences between percentages.

## Results

*Impact of rejuvenation treatments on the endogenous cytokinin status of clones*

Results show that for each clone, nodal explants from rejuvenated plants contain higher amounts of ZR (approximately two fold) than nodal explants from mature plants (Table 1). However, there is no difference of zeatin levels between mature and rejuvenated materials. Clones RRIM 600 and IRCA 18 show similar levels of Z-like cytokinins whereas PB 260 shows slightly lower levels than the other two clones. Neither 2iP nor iPA was detected in the mature or rejuvenated plants except for mature IRCA 18 and rejuvenated PB 260.

*Impact of rejuvenation treatments on the microcutting ability of clones*

For RRIM 600, PB 260 and IRCA 18, the proportion of explants still producing axillary shoots after four subcultures was significantly higher for the rejuvenated material than for the mature material (Table 2). Most of the mature explants lost their shoot proliferation ability prior to the fourth subculture. The rejuvenated material also produced more shoots per explant during the four subcultures. There was a mean of two shoots per explant for the treated material whereas the mature material produced only one shoot per explant (data not shown). Rooting ability was high for shoots derived from rejuvenated plants (40% to 70% rooting) but nearly absent for shoots derived from mature plants (Table 2).

*Differences in endogenous cytokinin levels between shoots unable and able to root*

*In vitro* shoots produced from mature PB 235 and GT1 were incapable of rooting (Table 3). In contrast, shoots produced from micrografted PB 235 and GT1 rooted at 60% and 35% respectively. The zeatin level was higher in shoots derived from micrografts than in shoots derived from mature material for PB 235 (Table 3). The difference in zeatin level was less for GT1. In contrast, iPA levels were higher in mature shoots than in micrografted ones (Table 3). ZR and 2iP levels were low and similar in all the samples (Table 3).

## Discussion

For all the genotypes tested, treated materials (i.e. somaplants and grafts) had a higher ability for *in vitro* shoot proliferation and rhizogenesis than non-treated materials. Somatic embryogenesis from maternal tissues of mature plants has been obtained from only a limited number of species (Robbins 1957; Mullins & Srinivasan 1976; Rohr et al., 1989; Carron et al., 1994; Rugini & Caricato 1995). Morphological observations showed that ontogenic development of zygotic embryo-derived seedlings and somatic embryo-derived plantlets are similar, however rejuvenation by somatic embryogenesis was not described with respect to organogenic criteria. Repeated grafting of plants *ex vitro* is efficient in rejuvenating clone IRCA 18. Likewise, *in vitro* micrografting clones of PB 235 and GT1 enhance their rooting ability, as well as their shoot proliferation (data not shown). *Ex vitro* and *in vitro* grafting procedures have been effective in the recovery of micropropagation ability of several woody species (Franclet 1983; Monteuuis 1986; Pliego-Alfaro & Murashige 1987; Dumas et al., 1989; Huang et al., 1992 a,b). Clone GT1 still shows a low rooting ability even after six micrografting. Considering the gradual enhancement of this ability during the successive micrografting (data not shown), the difficulty of GT1 to recover its rhizogenesis ability appears to be related to a difficulty to rejuvenate it. Clone GT1 was originally selected around 1920 and is thus probably composed of ontogenetically older trees than PB 235 which originated from a hybrid around 1950. Data from hormonal assays show that endogenous cytokinins may be used as markers of rejuvenation. ZR levels were higher in somaplants than in mature plants for clone RRIM 600 and PB 260. The ZR level was also higher in grafted plants than in mature plants for IRCA 18. An increase in ZR level could therefore constitute a reliable marker of rejuvenation and micropropagation ability regardless of the rejuvenation method used. The zeatin level increased in *in vitro* shoots of micrografted PB 235, as compared to shoots derived from mature material whereas it did not increase between mature and micrografted shoots of the poorly rooting GT1. Analysing zeatin and ZR together, it appears that the zeatin-like cytokinin (zeatin + ZR) ratio compared to the total cytokinin level (Z + ZR + 2iP + iPA) increases in *in vitro* shoots at the same time as rooting ability is recovered. Indeed, mature shoots that are incapable of rooting have a lower ratio of zeatin-like cytokinins (17% for PB 235 and 21% for GT1) than

*Table 1.* Levels of isopentenyladenine (2iP), isopentenyladenosine (iPA), zeatin (Z) and zeatin riboside (ZR) (fmoles $mg^{-1}$ dry weight) in nodal explants of mature plants and somaplants of RRIM 600 and PB 260 and of mature and grafted IRCA 18.

| Genotype | Origin | n[1] | ZR level* | Z level* | 2iP level* | iPA level* |
|---|---|---|---|---|---|---|
| RRIM 600 | Mature | 11 | 196±22 | 564±127 | 0 | 0 |
| | Somaplants | 26 | 393±51 | 636±145 | 0 | 0 |
| PB 260 | Mature | 13 | 92±31 | 333±105 | 0 | 0 |
| | Somaplants | 12 | 196±46 | 209± 36 | 27±9 | 0 |
| IRCA 18 | Mature | 31 | 152± 27 | 564±109 | 155±70 | 0 |
| | Grafted | 24 | 467±33 | 709±154 | 0 | 0 |

[1]Number of explants per analysed sample
*Mean of 4 ELISA assays per sample ± confidence interval

*Table 2.* Rates of explants still producing axillary shoots (explants with shoots) after four subcultures and rooting rate (rooting) obtained with mature plants and somaplants of RRIM 600 and PB 260 and with mature and grafted IRCA 18.

| Genotype | Origin | n[1] | Explants with shoots (%) | n[2] | Rooting (%) |
|---|---|---|---|---|---|
| RRIM 600 | Mature | 26 | 38.5 a* | 18 | 0 a* |
| | Somaplants | 111 | 82 b | 179 | 69.8 b |
| PB 260 | Mature | 42 | 0 a | 13 | 0 a |
| | Somaplants | 55 | 76.4 b | 101 | 42.6 b |
| IRCA 18 | Mature | 168 | 0.6 a | 43 | 2.3 a |
| | Grafted | 111 | 45.9 b | 36 | 72 b |

[1]Total number of tested explants
[2]Total number of tested shoots
*Percentages within a genotype followed by a different letter are significantly different at 0.1% level according to the chi-square test

shoots from micrografted PB 235 (74%) which are able to root. Furthermore, shoots from micrografted GT1, which have an intermediate rooting ability, have an intermediate ratio of zeatin- like cytokinins (53%). Therefore, in addition to an increase in ZR levels in *in vivo* shoots, an increase in zeatin levels and zeatin-like cytokinin ratio in *in vitro* shoots appears to be a marker of rejuvenation and of a recovery of rooting ability. An increase in cytokinin levels during the rejuvenation process can be related to other studies that show that a high cytokinin level is one of the characteristics of the juvenile state of trees in some species (Hendry et al., 1982; Galoch 1985; Haffner 1991). In contrast, 2iP and iPA cannot be used as markers of rejuvenation since in most cases their levels in *in vivo* material is too low to be detected by the ELISA assay. The higher amount of 2iP-like cytokinins in *in vitro* material compared to *in vivo* material agrees with other studies (Letham & Palni 1983; Kuiper et al., 1989; Haffner 1991). This observation could be correlated to the presence of exogenous BA (0.9 µm) in the culture medium during the first subculture since a supply of exogenous cytokinins can trigger the production of endogenous cytokinins (Thomas & Katterman 1986; Vankora et al., 1991). The iPA levels in PB 235 and GT1 shoots decreases between mature and micrografted materials at the same time as a great enhancement of the ability for *in vitro* shoot proliferation (data not shown). However, low levels of iPA cannot be correlated to an increase in rooting ability since the decrease in iPA level was measured since the first micrografting for GT1 when shoots were still incapable of rooting (data not shown). The decrease of iPA level in *in vitro* shoots could thus only be used as a marker for a first step of rejuvenation allowing the recovery of shoot proliferation.

*Table 3. In vitro* rooting rates and zeatin (Z), zeatin ribosine (ZR), isopentenyladenine (2iP) and isopentenyladenosine (iPA) levels (fmole $mg^{-1}$ dry weight) of shoots from mature and micrografted PB 235 and GT1.

| Genotype | Origin | n[1] | Rooting rate (%) | n[2] | Z level** | ZR level** | 2iP level** | iPA level** |
|---|---|---|---|---|---|---|---|---|
| PB 235 | Mature | 26 | 0 a* | 51 | 64± 26 | 32±11 | 77±25 | 393± 82 |
| | Micrografted | 45 | 60 b | 86 | 326±107 | 49±15 | 43± 7 | 87± 28 |
| GT1 | Mature | 16 | 0 a | 21 | 64±18 | 36±13 | 24± 4 | 347±109 |
| | Micrografted | 52 | 34.6 b | 22 | 103±30 | 13± 6 | 44±12 | 59± 14 |

[1]Total number of tested shoots
[2]Number of shoots per analysed sample
*Percentages within a genotype followed by a different letter are significantly different at 0.1% level according to the chi-square test
** Mean of 4 ELISA assays per sample ± confidence interval

## References


Carron MP, Enjalric F, Lardet L & Deschamps A (1989) Rubber (*Hevea brasiliensis* Müll. Arg.). In: Bajaj YPS (Ed) Biotechnology in Agriculture and Forestry, Vol 5 (pp 222–245). Springer-Verlag, Berlin

Carron MP, Etienne H, Lardet L, Campagna S, Perrin Y, Leconte A & Chaine C (1994) Somatic embryogenesis in Rubber (*Hevea brasiliensis* Müll. Arg.): expression features yield of the technique, field growth and juvenility of the somaplants. In: Jain SM, Gupta PK & Newton RJ (Eds) Somatic embryogenesis in Woody Plants (pp 117–136). Kluwer Academic, The Netherlands

Coleman GD & Ernst SG (1990) Axillary shoot proliferation and growth of *Populus deltoïdes* shoot cultures. Plant Cell Rep. 9: 165–167

Das T & Mitra GC (1990) Micropopagation of *Eucalyptus teraticornis* Smith.. Plant Cell Tiss. Org. Cult. 22: 95–103

Dumas E, Franclet A & Monteuuis O (1989) Microgreffage de méristèmes primaires caulinaires de pins maritimes (*Pinus pinaster* Ait.) âgés sur de jeunes semis cultivés *in vitro*. C. R. Acad. Sci. Ser.2, 309: 723–728

Enjalric F & Carron MP (1982) Microbouturage *in vitro* de jeunes plants d'*Hevea brasiliensis* (Kunth) Müll. Arg.. C. R. Acad. Sci. Ser. III 295: 259–264

Fourret Y, Larrieu C & Arnaud Y (1988) Rajeunissement *in vitro* chez le *Sequoia sempervirens* (Endl.). Ann. AFOCEL 1988: 55–82

Franclet A (1983) Rajeunissement, culture *in vitro* et pratique sylvicole. Bull. Soc. bot. Fr. 130: 87–98

Galoch E (1985) Comparison of the content of growth regulators in juvenile and adult plants of Birch. Acta Physiol. Plant. 7: 205–215

Haffner V (1991) Recherches de marqueurs de la juvenilité/maturité et de la réactivité *in vitro* lors du microbouturage chez *Hevea brasiliensis*; relation entre la juvénilité et la réactivité *in vitro*. Ph.D. Thesis, University Paris VI, France, 184 p.

Hendry NS, Van Staden J & Allan P (1982) Cytokinins in Citrus. II. Fluctuations during growth in juvenile and adult plant. Sci. Hort. 17: 247–256

Huang LC, Liu S, Huang BL, Murashige T, Mahdi FM & Van Gundy R (1992a) Rejuvenation of *Sequoia sempervirens* by repeated grafting of shoot tips onto juvenile rootstocks *in vitro*. Plant Physiol. 98: 166–173

Huang LC, Hsiao CK, Lee SH, Huang BL & Murashige T (1992b) Restoration of vigor and rooting competence in stem tissues of mature *Citrus* by repeated grafting of their shoot apices onto freshly germinated seedlings *in vitro*. In Vitro Cell Dev. Biol. 28: 30–32

Imbault N, Tardieu I, Joseph C, Zaerr JB & Bonnet- Masimbert M (1988) Possible role of isopentenyladenine and isopentenyladenosine in flowering of *Pseudotsuga menziesii*: endogenous variations and exogenous applications. Plant Physiol. Biochem. 26(3): 289–295

Kuiper D, Kuiper PJC, Lambers H, Schuit J & Staal M (1989) Cytokinin concentration in relation to mineral nutrition and benzyladenine treatement in *Plantago major* ssp. pleiosperma. Physiol. Plant. 75: 511–517

Label P, Imbault N & Villar M (1994) ELISA Quantification and GC-MS identification of abscisic acid in stigma, ovary and pedicel of pollinated poplar flowers (*Populus nigra* L.). Tree Physiol. 14: 521–530.

Lardet L (1987) Amélioration des conditions du microbouturage de l'*Hevea brasiliensis* Müll. Arg.. Ph.D. Thesis, University of Sciences et Techniques du Languedoc, Montpellier France, 242 p.

Letham DS & Palni LMS (1983) The biosynthesis and metabolism of cytokinins. Ann. Rev. Plant Physiol. 34: 163–197

Maldiney R, Pelèse F, Pilate g, Sotta B, Sossountzof L & Miginiac E (1986) Endogenous levels of abscissic acid, zeatin and zeatin-riboside during the course of adventitious root formation in cuttings of Craigella and Craigella lateral suppressor tomatoes. Physiol. Plant. 68: 426–430

Monteuuis O (1986) Microgreffage du sequoia géant. Ann. AFOCEL 1986: 39–61

Mullins Mg & Srinivasan C (1976) Somatic embryos and plantlets from ancient clone of the grapevine (cv Cabernet-Sauvignon) by apomixis *in vitro*. J. Exp. Bot. 27: 1022–1030

Perrin Y, Lardet L, Enjalric F & Carron MP (1994) Rajeunissement de clones matures d'*Hevea brasiliensis* (Müll. Arg.) par microgreffage *in vitro*. Can. J. Plant Sci. 74: 623–630

Pliego-Alfaro F & Murashige T (1987) Possible rejuvenation of adult avocado by graftage onto juvenile rootstock *in vitro*. Hort. Sci. 22: 1321–1324

Robbins WJ (1957) Physiological aspects of ageing in plants. Am. J. Bot. 44: 289–294

Rohr R, Von Aderkas P & Bonga JM (1989) Ultrastructural changes in haploid embryoids of *Larix decidua* during early embryogenesis. Am. J. Bot. 76: 1460–1467

Rugini E & Caricato G (1995) Somatic embyogenesis and plant recovery from mature tissues of olive cultivars (*Olea europea* L.) "Canino" and "Moraiolo". Plant Cell Rep. 14: 257–260

San-Jose MC, Vieitez AM & Ballester A (1990) Clonal propagation of juvenile and adult trees of sessile oak by tissue culture techniques. Sylvae Genet. 39: 50–55

Seneviratne P (1991) Micropropagation of juvenile and mature *Hevea brasiliensis*. Ph. D. Thesis, University of Bath, UK, 278 p.

Sotta B, Pilate G, Pelèse F, Sabbagh I, Bonnet-Massimbert M & Maldiney R (1987) An avidin-biotin solid phase ELISA for femtomol isopentenyladenine and isopentenyladenosine measurements in HPLC purified plant extracts. Plant Physiol. 84: 571–573

Thomas JC & Katterman FR (1986) Cytokinin activity induced by thidiazuron. Plant Physiol. 81: 681-683

Vankora R, Hsiao KC, Bornman CH & Gaudinova A (1991) Effect of synthetic cytokinins on levels of endogenous cytokinins and respiration patterns of *Beta vulgaris* cells in suspension. J Plant Growth Regul. 10: 197–199