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Original article

Long-term effects of culture establishment from shoot-tip explants in micropropagating oak (*Quercus robur* L)

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Summary — This paper describes a method of *in vitro* culture establishment from shoot-tip explants taken from juvenile and mature plant material for oak (table I). The cultures established from shoot-tips were then compared with cultures derived from nodal explants for decontamination, their initial reactivity and their potential for long-term propagation. For the decontamination, the results showed that the use of shoot-tip explants is useful only when culture establishement must be made directly from source-plants growing *in situ* (table II). Otherwise, the use of nodal explants taken from source-plants that are maintained under active growth and controlled sanitary conditions is more advisable due to a better initial reactivity. As regards the potential for long-term propagation, the culture establishment from shoot-tips appeared truly interesting only in the case of recalcitrant clones and/or insufficient optimization of the culture methods (fig 1). However, this positive effect attenuated after a 6–7 month culture period, and the clonal effects and the management of the media became the determining factors of the culture behaviour whatever the initial explant used (fig 2).

shoop-tip explant / decontamination / long-term propagation / *Quercus robur* L / mature plant material / juvenile plant material

Résumé — Effets à long terme de l'introduction *in vitro* à partir de méristèmes sur la micropropagation du chêne (*Quercus robur* L). *L'article décrit chez le chêne les conditions d'obtention d'un clonage* in vitro *à partir de méristèmes prélevés sur du matériel juvénile et sur du matériel mature (tableau I). Il compare ensuite, sur le plan de la décontamination, de la réactivité initiale et de la multiplication à long terme, le comportement de cultures issues de méristèmes à celui de cultures issues de boutures de nœuds. Les résultats montrent que, sur le plan de la décontamination, l'utilisation de méristèmes n'est utile que lorsque le matériel végétal doit être prélevé directement* in situ *(tableau II). Dans le cas contraire, il est préférable d'initier les cultures à partir de nœuds prélevés sur des pieds-*

Abbreviations: AC = activated charcoal; BA = 6-benzylaminopurine; 2iP = 2-isopentenyladenine; Z = zeatine; MS = Murashige and Skoog; GD = Gresshoff and Doy.

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mères maintenus en croissance active dans des conditions sanitaires contrôlées, en raison d'une meilleure réactivité initiale. Sur le plan multiplication à long terme, la culture de méristèmes ne s'avère réellement intéressante que dans le cas de clones récaltritrants, ou lorsque les protocoles de culture sont insuffisamment optimisés (fig 1). Cet effet positif n'est cependant que transitoire. Au-delà des 6-7 premiers mois qui suivent la mise en culture, il s'atténue et ce sont les effets clonaux ainsi que la gestion des milieux qui déterminent le comportement des cultures, quel que soit le type d'explant initial (fig 2).

culture de méristèmes / décontamination / multiplication à long terme /* Quercus robur *L / matériel mature / matériel juvénile

INTRODUCTION

In vitro culture establishment from shoot-tip explants potentially offers 2 kinds of advantages in cloning forest trees. Firstly, *in vitro* propagation of forest trees and other woody plants is often limited by latent internal bacteria or fungi (Bastiaens, 1983). These contaminants make the initial decontamination of the explants difficult. Even in apparently healthy cultures, they may reappear after several transfers causing problems in the cloning (Cornu and Michel, 1987; Fisse *et al*, 1987; McGranaham *et al*, 1988). In the face of these problems, culture establishment from shoot-tip explants, which have a low concentration of contaminants, is an interesting option as demonstrated by numerous examples of recovering virus-free plants (Morel and Martin, 1952; Wang and Hu, 1980), fungi-free plants (Baker and Phillips, 1962), and bacteria-free plants (Knauss, 1976; Theiler, 1977; Moncousin, 1980) from infected stocks. In walnut, data showed that this method is more reliable for definitive decontamination than antibiotic treatments (Meynier and Arnould 1989).

Secondly, physiological aging reduces the ability to propagate vegetatively (Martin, 1977; Bonga, 1982; Hackett, 1985). Hence, cloning genetically assessed mature trees is often problematic. Pretreatments of the source-plants, such as pruning, hedging, serial graftings (Franclet, 1981a,b; Copes, 1983; Saint-Clair *et al*, 1985; Bonga, 1987), application of cytokinins (Franclet, 1981b; Bouriquet *et al*, 1985) or fertilization (Barnes and Bengston 1968, Dumas 1987), may improve the physiological state of the explants and make further *in vitro* cloning easier. However, these treatments are awkward and need time. So, direct culture establishment from explants with high organogenetic potential, such as meristems, has been used as a means of improving the reactivity of cultures established from mature source-plants (Rodriguez, 1982; Meynier, 1985; Walker, 1986). Indeed, Monteuuis (1991) reported that culture establishment from shoot-tip explants could restore active growth, rooting ability and juvenile leaf morphology from a 100-year-old tree of *Sequoiadendron giganteum*.

In *Quercus robur*, *in vitro* propagation from stem explants has been achieved (Chalupa, 1984, 1988, 1993; Vieitez *et al*, 1985; Favre and Juncker, 1987; Meier-Dinkel, 1987; San-Jose *et al*, 1988; Meier-Dinkel *et al*, 1993). However, the initial decontamination remains a barrier, and even when successful cloning is obtained, gradual or sudden extinction may occur especially in the case of adult clones (Juncker and Favre, 1989; Slak and Favre, 1990).

We therefore tested methods of shoot-tip culture to improve the initial decontamination and the potential for long-term propagation. We compared the behaviour of several clones established from nodal and shoot-tip explants derived from both juvenile and mature plant materials.

MATERIALS AND METHODS

Source-plants

Three types of source-plants were used.

Actively growing 4-month-old seedlings (28 genotypes) were obtained from acorns collected in NE France and cultured at 26 ± 1°C under continuous lighting in a peat/vermiculite mixture (2:1) fertilized once a month with the Coic and Lesaint solution (1973). They were periodically sprayed with a 0.4 $g.l^{-1}$ benomyl solution. Nodal explants were taken from all the genotypes, and shoot-tip explants from only 14 of them.

One actively growing 3-year-old plant was obtained from seed and cultured under the same conditions as the 4-month-old seedlings. Both nodal and shoot-tip explants were prepared from this plant.

Two- to 6-year-old grafts of mature trees (age 80–100 years) were obtained from one site in the Fontain forest (France), and were grown under the same conditions of active growth as the seedlings (8 genotypes), or in the nursery under natural conditions (12 genotypes).

Shoot-tip explants were collected from 5 out of the 8 genotypes grown in the growth chamber and from the 12 genotypes grown in the nursery. Nodal explants were prepared from all the genotypes grown in the growth chamber, and from 7 out of the 12 genotypes grown in the nursery.

In vitro *culture*

Five-centimetre-long stem explants with swelling buds were cleaned in tap water containing a few drops of a commercial disinfectant (Mercryl laurylé®), and then dipped into ethanol 60% for 10 sec. Shoot-tip explants consisting of the apical dome flanked by 1–2 leaf primordia were excised under a stereomicroscope and planted 3 per Petri dish (55 mm) on the following basic medium (BM):

– half-strength MS macronutrients (Murashige and Skoog, 1962) with 1/4 NH_4NO_3;

– full strength MS micronutrients (Murashige and Skoog, 1962);

– MS vitamin solution (Murashige and Skoog, 1962) complemented with 10 $mg•l^{-1}$ glutamine and 10 $mg•l^{-1}$ asparagine;

– 30 $g•l^{-1}$ sucrose;

– agar (Touzart and Matignon) 7 $g•l^{-1}$.

Depending on the experiment BM was complemented with either AC 2 $g•l^{-1}$ (= BM AC) or cytokinins (= BM Cyt): 0.1 $mg•l^{-1}$, 2iP 0.1 $mg•l^{-1}$, Z, 0.1 and 0.25 $mg•l^{-1}$ BA. The cultures were grown in a growth chamber at 26 + 1°C under a 16 h long photoperiod (40 $\mu E•m^{-2}•s^{-1}$).

Shoots derived from nodal explants and from shoot-tip explants were cloned into test tubes (25 x 200 mm), either on a BM Cyt medium with BA 0.1 $mg•l^{-1}$ in a continuous manner (Juncker and Favre, 1989), or alternately, on the BM Cyt and the GD medium (macronutrients according to Gresshoff and Doy, 1972) with the same concentration of BA. The duration of the culture cycles was 6 weeks.

RESULTS

Shoot growth recovery from shoot-tip explants

Shoot-tip explants were established on BM, BM AC, and BM Cyt. On BM and BM AC growth recovery did not occur. All explants became necrotic within 3 weeks of culture, whatever the type of source-plant.

On BM Cyt, the reactivity was better. Shoot-tip explants enlarged within the first 2 weeks of culture. During the third week, the 1–2 initial leaf primordia of explants expanded. Rosette formation (new formed leaf pieces) occurred during the fourth week and 2 weeks later the rosettes exhibited swelling axillary buds. Two months after the excision, elongation of both main and some axillary buds occurred. The cloning into test tubes could begin.

However, the results varied strongly according to the type and/or the concentration of the cytokinin used (table I). Use of 2iP proved to be ineffective and Z did not allow the culture to initiate elongation; culture evolution stopped at the rosette stage. On BA-containing media, shoot elongation

Table I. Final changes of shoot-tip explants excised from 4-month-old seedlings and cultured on BM media containing 0.1 mg•l^{-1} 2iP, 0.1 mg•l^{-1} Z or 0.1 and 0.25 mg•l^{-1} BA.

	Cytokinin			
	2iP	*Z*	$BA_{0.1}$	$BA_{0.25}$
No of shoot-tip explants	14	12	16	16
Final changes				
No reaction	14	2	1	2
Callogenesis	–	0	1	4
Expansion of the initial leaf primordia of shoot-tip	–	2	0	0
Rosette formation	–	8	0	10[a]
Elongation of main shoot and axillary buds	–	–	14	–

[a] Vitrification.

could be recovered, but at concentrations exceeding 0.1 mg•l^{-1} the rosettes exhibited high levels of vitrification and basal callogenesis that prevented further growth and cloning.

The source-plant also influenced the culture behaviour (table II). Most of the shoot-tip explants derived from the juvenile source-plants gave elongated shoots which could be cloned. In contrast, the reactivity of shoot-

Table II. Final changes of nodal explants and shoot-tip explants derived from seedlings cultured in a growth chamber and grafts cultured in a growth chamber or in the nursery.

	Seedlings [a]		*Grafts* [a]	
	4-month-old	*3-year-old*	*Growth chamber*	*Nursery*
Nodal explants				
Established *in vitro*	96 (28 gt)	24 (1 gt)	183 (8 gt)	35 (7 gt)
Decontaminated	94	24	176	0
Subcultured	88 (28 gt)	22 (1 gt)	102 (8 gt)	0
Shoot-tip explants				
Established *in vitro*	36 (14 gt)	24 (1 gt)	86 (5 gt)	129 (12 gt)
Decontaminated	36	24	82	82
Surviving after 7 d	28 (14 gt)	24 (1 gt)	62 (5 gt)	48 (12 gt)
Final changes:				
No reaction	2	2	36	28
Callogenesis	2	2	0	2
Shoot-tip enlargement	3	2	15	9
Expansion of the 1–2 initial leaf primordia	3	1	11 (4 gt)	4
Rosette formation	1	0	0	0
Swelling of rosettes axillary buds	0	0	0	0
Shoot elongation and subculture	17 (11 gt)	17 (1 gt)	0	5 (1 gt)

[a] gt: genotypes.

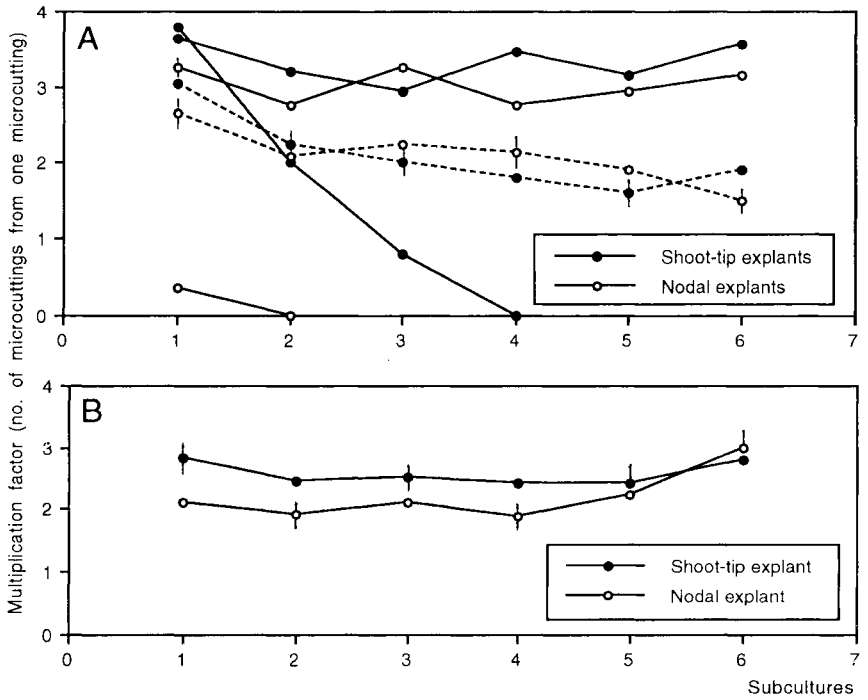

Fig 1. Changes taking place during the 6 first subcultures of the multiplication factor of clones derived from shoot-tip (—●—) or from nodal explants (—O—) taken from juvenile source-plants. **A.** Results of the clones derived from the 4-month-old seedlings; continuous lines: results of the best (top) and worst (bottom) clone; broken line: mean values ± SE. **B.** Results of the clone derived from the 3-year-old plant.

tips was poor from the grafts of mature trees, especially when compared with that of nodal explants of the source-plants cultured in the growth chamber. Thus, elongated shoots could be recovered from only one of the 17 genotypes tested. However, it is worth noting that it came from one of the source-plants growing under natural conditions, while all attempts to establish cultures from nodal explants of these plants failed because of contamination.

Comparison of cloning from shoot-tip and nodal explants

Cultures from shoop-tip and nodal explants, from both the juvenile and the mature tree material, were followed in parallel throughout the multiplication cycles.

Juvenile material

The 4-month-old seedlings were not sufficiently developed to obtain shoot-tip and nodal explants from each of them. Consequently a clone-by-clone comparison could not be made and the overall results were considered according to the type of explants used for culture establishment.

Within the clones derived from nodal explants, different types of behaviour could be recognized. Most showed a continuous multiplication, while some became extinct progressively soon after the culture establishment or later.

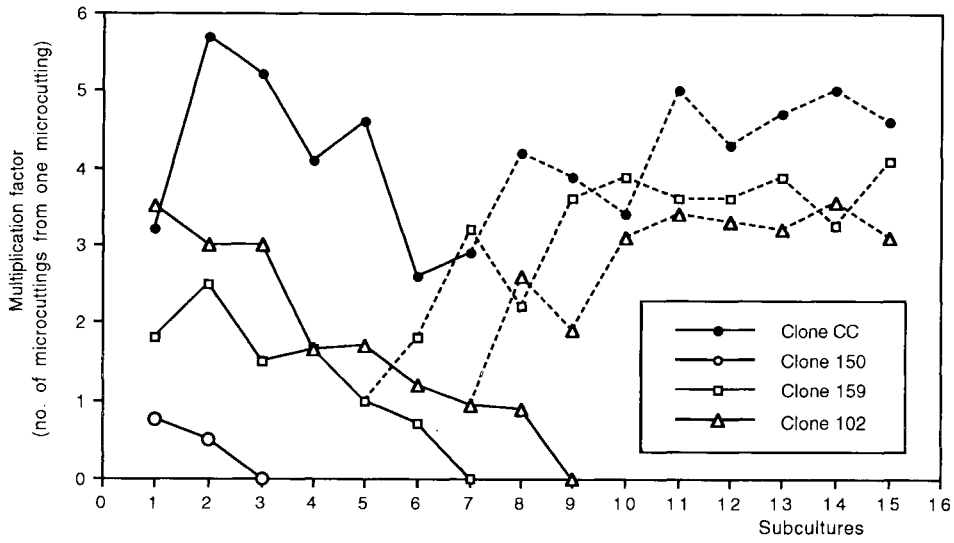

Fig 2. Changes taking place during the 15 first subcultures of the multiplication factor of clones derived from shoot-tip (clone CC (—●—)) or from nodal explants (clones 102 (—O—), 150 (—□—), 159 (—Δ—)) taken from mature source-plants. Continuous lines: cultured on BM Cyt with BA 0.1 mg•l^{-1}; broken lines: cultured alternately on BM Cyt with BA 0.1 mg•l^{-1} and GD with BA 0.1 mg•l^{-1}.

The clones derived from shoot-tip explants exhibited the same fundamental behaviour (fig 1A). Differences could be noted only when considering the best and the worst clones. Compared with the equivalent clones established from nodal explants, the former had greater multiplication factors, and the latter became extinct later.

However, these indications needed to be considered with care because of possible interference of clonal effects (Juncker and Favre, 1989). The information obtained from the 3-year-old seedling was more instructive. Indeed, from this source-plant, it was possible to establish both shoot-tip and nodal explant cultures. The results recorded in figure 1B definitely show that, for a single clone, the shoot-tip-derived plant material has better initial growth potential than that established from the nodal explant. However the difference was small and tended to disappear after the 4th subculture.

Mature tree materials

On BM Cyt with 0.1 mg•l^{-1} BA the clones derived from nodal explants exhibited highly variable reactivity with multiplication factors that ranged between 0 and 3 at the end of subculture 1, and declined thereafter (fig 2). By subculture 8, only clone 159 still remained. In contrast the only clone obtained from the shoot-tip explant propagated well, showing multiplication factors of 3–6 with, however, a decrease after subculture 5.

When subcultures were made alternately on BM Cyt and GD media with 0.1 mg•l^{-1} BA, the differences between nodal and shoot-tip derived clones reduced. For 2 out of the 3 clones tested, multiplication could

be maintained with multiplication factors approaching that of the shoot-tip clones.

DISCUSSION

These results generally confirm the potential advantages of shoot-tip explants in the decontamination of infected stocks and in the stimulation of the growth capacity of plant material for oak.

However this general conclusion has to be qualified carefully. Firstly, when considering the culture establishment phase, the utilization of shoot-tip explants appears preferable only when the culture must be made directly from shoots taken in the forest or in the nursery, due to a better decontamination efficiency. Otherwise, the use of nodal explants taken from source-plants maintained in active growth under controlled sanitary conditions in a growth chamber is more advisable because of an improved initial reactivity, especially with mature material.

Secondly, as regards the potential for long-term propagation, the advantage of culture initiation *via* shoot-tip explants was only obvious in difficult situations, such as badly propagating clones, which would otherwise become extinct, and/or in the case of insufficient optimization of the culture method.

In normal situations the positive effect of using shoot-tip explants appears only temporarily, during the 6–7 month period following the culture establishment. After this time the behaviour of both shoot-tip and node-derived clones tends to become comparable.

Finally, while the type of source explants may have some influence during the first steps of the cloning, in the long term, the management of the culture media, together with the clonal effects (Juncker and Favre, 1989), appeared to be the main factors determining the culture growth capacities and potential for propagation.

In oak, BA and the macronutrient composition of the media, and especially the nitrogen source, have already been noted as playing an important role in the *in vitro* control of the expression of the episodic growth pattern of the species, these factors therefore have a major influence (Favre and Juncker, 1989). An alternation of culture on a high nitrogen content media, such as BM Cyt, and a lower nitrogen content media, such as GD, is of crucial importance, whatever the initial explant used.

REFERENCES

Baker PK, Phillips DJ (1962) Obtaining pathogen-free stock by shoot-tip culture. *Phytopathol* 52, 1242-1244

Barnes RL, Bengston GW (1968) Effect of fertilization, irrigation and cover cropping on flowering and on nitrogen and soluble sugar composition in slash pine. *For Sci* 14, 172-180

Bastiaens L (1983) Endogenous bacteria in plants and their implications in tissue culture. A review. *Meded Facult Landbouwwet Rijksuniv Gent* 48, 1-11

Bonga JM (1982) Vegetative propagation in relation to juvenility, maturity and rejuvenation. *In: Tissue Culture in Foresty* (JM Bonga, DJ Durzan, eds), Martinus Niijhoff, Dr W Jing, The Hague, The Netherlands, 384-413

Bonga JM (1987) Clonal propagation of mature trees: problems and possible solutions. *In: Cell and Tissue Culture in Forestry* (JM Bonga, DJ Durzan, eds), Martinus Nijhoff, Boston, USA, vol 1, 249-271

Bouriquet R, Tsogas M, Blaselle A (1985) Essais de rajeunissement de l'Epicéa par les cytokinines. *Ann AFOCEL* 1984, 173-186

Chalupa V (1984) *In vitro* propagation of oak (*Quercus robur* L) and linden (*Tilia cordata* Mill). *Biol Plant* 26, 374-377

Chalupa V (1988) Large-scale micropropagation of *Quercus robur* L using adenine-type cytokinins and thidiazuron to stimulate shoot proliferation. *Biol Plant* 30, 414-421

Chalupa V (1993) Vegetative propagation of oak (*Quercus robur* and *Q petraea*) by cutting and tissue culture. *Ann Sci For* 50 Suppl, 295-307

Coic Y, Lesaint C (1973) La nutrition minérale en horticulture avancée. *La Revue Horticole* 2316, 29-34

Copes DL (1983) Effects of annual crown pruning and serial propagation an rooting of stem cuttings from Douglas fir. *Can J For Res* 13, 419-424

Cornu D, Michel MF (1987) Bacteria contaminants in shoot cultures of *Prunus avium* L. Choice and phytotoxicity of antibiotics. *Acta Hort* 212, 83-86

Dumas E (1987) Micropropagation d'un clone âgé de Pin maritime en vue de l'obtention de pieds-mères. *Ann AFOCEL* 1986, 95-107

Favre JM, Juncker B (1987) *In vitro* growth of buds taken from seedlings and adult plant material *in Quercus robur* L. *Plant Cell, Tissue Organ Culture* 8, 49-60

Favre JM, Juncker B (1989) Variation in expression of episodic growth by *in vitro* cultured shoot of oak (*Quercus robur* L). *Ann Sci For* 46 Suppl, 206-208

Fisse J, Batalle A, Pera J (1987) Endogeneous bacteria elimination in ornemental plants. *Acta Hort* 212, 83-86

Franclet A (1981a) Rajeunissement et propagation végétative des ligneux. *Ann AFOCEL* 1980, 11-40

Franclet A (1981b) Rajeunissement et micropropagation des ligneux. *Colloque International sur la culture* in vitro *des essences forestières*, IUFRO Fontainebleau, France (AFOCEL ed), 55-63

Gresshoff OM, Doy CH (1972) Development and differentiation of haploid *Lycopersicum esculentum* (tomato). *Planta* 107, 161-170

Hackett WP (1985) Juvenility, maturation and rejuvenation in woody plants. *Hortic Rev* 7, 109-155

Juncker B, Favre JM (1989) Clonal effects in propagating Oak trees *via in vitro* culture. *Plant Cell, Tissue and Organ Culture* 19, 267-276

Knauss JF (1976) A tissue culture method for producing *Diffenbachia picta* cv Perfection free of fungi and bacteria. *Proc Florida State Hort Sco* 89, 293-296

Martin B (1977) Le bouturage des arbres forestiers: progrès récents, perspectives de développement. *Rev For Fr* 29, 245-262

Mc Granaham GH, Driver JA, Tulecke W (1988) Tissue culture of Juglans. *In: Cell and Tissue Culture in Forestry* (JM Bonga, DJ Durzan, eds), Martinus Nijhoff, Boston, USA, vol 3, 261-271

Meier-Dinkel A (1987) Vermehrung und Weiterkultur von Stieleiche (*Quercus robur* L) und Traubeneiche (*Quercus petraea* (Matt) Liebl). *Allg Forst u J Ztg* 158, 199-204

Meieir-Dinkel A, Becker B, Duckstein D (1993) Micropropagation and *ex vitro* rooting of several clones of late flushing *Quercus robur* L. *Ann Sci For* 50 Suppl, 319-322

Meynier V (1985) Mise en culture *in vitro* de méristèmes de Noyers hybrides. *CR Acad Sci Paris 301 Série III* 5, 261-264

Meynier V, Arnould MF (1989) Compared effectiveness of antibiotic treatments and shoot-tip culture on bacterial decontamination of an *in vitro* propagated clone of hybrid walnut (*Juglans nigra* x *J regia*). *Biol Plant* 31, 269-275

Moncousin Ch (1980) Micropropagation *in vitro* de *Cynara scolymus*: conséquences bactériologiques. *In: Application de la culture* in vitro *à l'amélioration des plantes fourragères*, Eucarpia, Versailles, France, 8-13

Monteuuis O (1991) Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through *in vitro* meristem culture. I. Organogenic and morphological arguments. *Physiol Plant* 81, 111-115

Morel GM, Martin C (1952) Guérison de Dahlias atteints d'une maladie à virus. *CR Acad Sci Paris* 235, 1324-1325

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15, 473-497

Rodriguez R (1982) *In vitro* propagation of *Castanea sativa* Mill through meristem tip culture. *Hortscience* 17, 888-889

Saint-Clair JB, Kleinschmitt J, Svolba J (1985) Juvenility and serial vegetative propagation of Norway spruce clones *Picea abies* (Karst). *Silv Genet* 34, 42-48

San-José MC, Ballester A, Vieitez AM (1988) Factors affecting *in vitro* propagation of *Quercus robur* L. *Tree Physiol* 4, 281-290

Slak MF, Favre JM (1990) Possibilités actuelles de la multiplication végétative chez les Chênes. *Rev For Fr* 42, 220-226

Theiler R (1977) *In vitro* culture of shoot tips of *Pelargonium* species. *Acta Hort* 78, 403-414

Vieitez AM, San-José MC,Vieitez E (1985) *In vitro* plantlet regeneration from juvenile and mature *Quercus robur* L. *J Hort Sci* 60, 99-106

Walker N (1986) *Sequoia sempervirens* : rejuvénilisation et culture de méristèmes en cascade. *Ann AFOCEL* 1985, 25-47

Wang PJ, Hu CY (1980) Regeneration of virus-free plants through *in vitro* culture. *In: Advances in Biochemical Engineering* (A Fiechter, ed) Springer-Verlag, New York, USA, vol 8, 61-99