SEQUOIA SEMPERVIRENS AS AN IN VITRO REJUVENATION MODEL

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ABSTRACT

Rejuvenation modes of two clones of **Sequoia sempervirens** (Endl.) obtained from a 50-year-old tree (Clone 1) and a 500-year-old tree (Clone 2) were studied by **in vitro** culture technique. The progress of the explants towards physiological rejuvenation was assessed by spontaneous rooting, induced rooting, and length of the main shoot. Two criteria (reactivation of isolated apical meristems and ex-flasking stem growth) were used to assess ontogenetical rejuvenation. For Clone 1, frequent subcultures on a medium without hormones effected physiological rejuvenation. Maintenance of explants of both clones on medium with BAP (benzyl-amino-purine) and NAA (naphthalene-acetic-acid) for several months without subculturing was effective to some extent. After frequent subcultures on medium with BAP and NAA the material of Clone 2 was reactivated but later and to a smaller degree than Clone 1 material. The older the original tree, the stronger the treatment needed to obtain physiological rejuvenation. Ontogenetical rejuvenation was more difficult to achieve than physiological rejuvenation.

Keywords: physiological rejuvenation; ontogenetical rejuvenation; **in vitro**; hormonal supply effect; **Sequoia sempervirens**.

INTRODUCTION

One of the most important problems encountered in forestry is mass propagation of adult selected trees. In most species, vegetative propagation by cuttings is slower and more difficult from adult trees than from juvenile ones (Doorenbos 1965; Chaperon 1979; Franclet 1983). These differences have also been observed for *Sequoia sempervirens* clones *in vitro* (Franclet 1983; Bekkaoui *et al.* 1984; Fouret *et al.* 1985). Therefore, rejuvenation of the material is a prerequisite for intensive propagation of selected trees. In this paper, we describe the *in vitro* culture technique applied to *S. sempervirens* explants, used as a "tool" in a study of rejuvenation of propagation material from forest trees.

In vitro culture allows adult material to express juvenile characteristics (Margara 1982). Subcultures improve rooting for different species (Gupta *et al.* 1981; Sriskandarajah *et al.* 1982; Welander 1985; Al Maarri 1986), and seem to be an essential means of rejuvenating clones from adult trees (Mullins *et al.* 1979). According to Franclet (1979) and Chaussat & Courduroux (1980), BAP can be considered an anti-senescence

substance and can be used to facilitate rejuvenation. We examined the rejuvenation of two *S. sempervirens* clones *in vitro:* firstly, the effect of number and frequency of subcultures and, secondly, the effect of adding hormones (BAP and NAA) to the medium. Several criteria were used to assess the degree of rejuvenation during subcultures (Doorenbos 1965; Chaperon 1979; Bekkaoui *et al.* 1984; Fouret *et al.* 1985). This report offers an extension to and comparison with our recent work (Fouret *et al.* 1985).

MATERIAL AND METHODS

Plant Material

Two clones from different geographical origins were used in this study. They were both provided by AFOCEL (Association Forêt-Cellulose, France).

— Clone 1 was from a 50-year-old tree planted in Anjou (France)

— Clone 2 was obtained from the highest tree in the world, about 500 years old and located in California (United States).

Clone 1 was placed *in vitro* in 1977 and Clone 2 in 1978. The treatments applied prior to *in vitro* culture of these clones have been described previously (Franclet 1981; Fouret *et al.* 1985). These clones were maintained successively on two media (MM and EM) as described by Boulay (1978). Explants with three leaves, from *in vitro* stems elongated on EM, were used for this experiment.

In vitro Culture

In vitro culture media

MM	Subculture medium with hormones (BAP/NAA ratio=44)
MO	subculture medium without hormones
EM	elongation medium
RIM	rhizogenesis induction medium
REM	rhizogenesis expression medium
RAM	medium for reactivation of isolated apical meristem

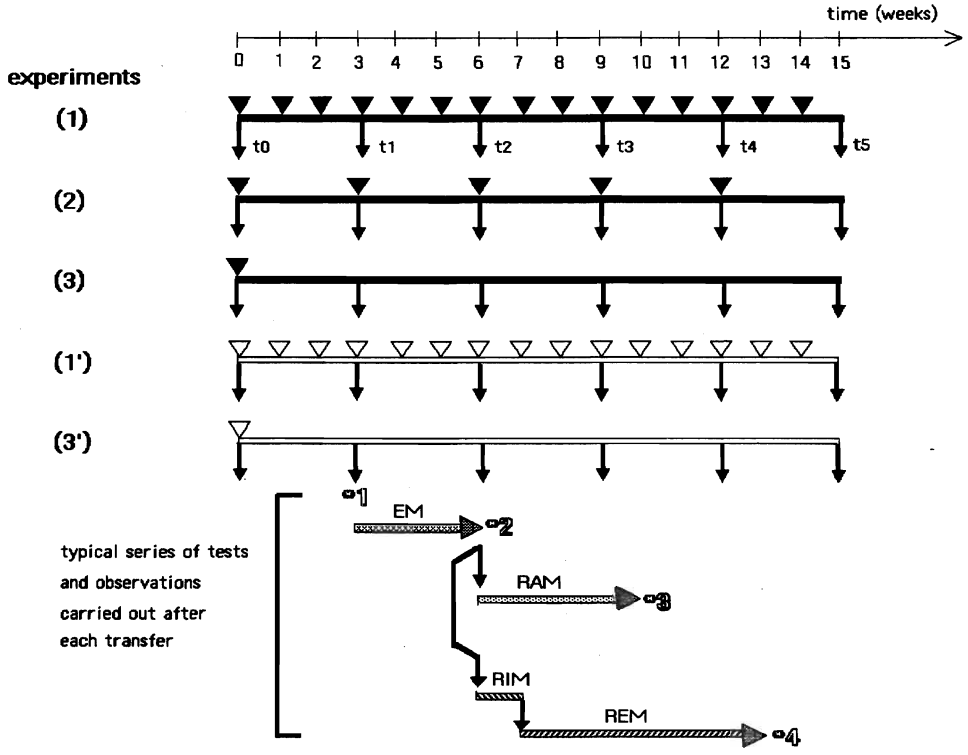
Media MM and MO were used for the rejuvenation treatments; media EM, RIM, REM, and RAM were used to follow the rejuvenation process (Fouret *et al.* 1985). For all media, the basal medium contained the macro- and micro-elements of Murashige & Skoog (1962) and two organic constituents – thiamine HCl (0.1 mg/*l*) and meso-inositol (100 mg/*l*) (Boulay 1978); it was supplemented with purified Bacto-agar DIFCO (7 g/*l*) and sucrose (30 g/*l*). The pH of all media was adjusted to 5.6±0.1 before autoclaving (112°C for 30 min).

MM and MO contained the diluted (d=2) basal medium; MM was supplemented with BAP ($2.2 \cdot 10^{-6}$ M) and NAA ($5 \cdot 10^{-8}$ M; BAP/NAA ratio=44); MO did not contain any plant growth regulator. EM was used by Boulay (1978): the half-diluted basal medium was supplemented with activated charcoal (20 g/*l*). The rooting technique was according to Bekkaoui *et al.* (1984): for the two media RIM and REM the basal medium was diluted (d=3); rhizogenesis was induced for 7 days on RIM containing NAA ($5 \cdot 10^{-5}$ M); then the explants were transferred on REM without any plant growth regulator. Apical stem meristems were reactivated on RAM (basal medium half-diluted). The culture conditions were 22°±3°C under 8 h photoperiod with a light intensity of 26 W/m² provided by 40 INC Mazdafluor Incandia fluorescent tubes.

Experimental

Details are given in Fig 1.

On MM the explants were subcultured every 7 days in Exp. 1 and every 21 days in Exp. 2; no subculture was made in the control Exp. 3. These experiments were carried out with Clone 1 and Clone 2 explants. Exp. 1 and 3 were repeated once with Clone 1.



KEY

: subcultures on MM : subcultures on MO

t_0 ..... t_5 : time of transfer to EM for further observations and tests

*1 : observation of spontaneous rooting before the transfer to EM

*2 : observation of spontaneous rooting 3 weeks after the transfer to EM and morphological observations

*3 : observation of isolated meristems 4 weeks after the transfer to RAM

*4 : observation of induced rooting after the transfers to RIM (1 week), then to REM (6 weeks)

FIG. 1—Experimental schema. Explants with three leaves were placed on a fresh medium at the start of each experiment. They were then variously subcultured on medium with hormones (MM Exp. 1, 2) or on medium without hormones (MO Exp. 1') or maintained on MM (Exp. 3) or on MO (Exp. 3') without subcultures. Regularly, 12 explants with axillary buds were transferred to elongation medium (EM) for 3 weeks and they were then tested to determine the development of rejuvenation. A typical series of observations and tests is represented.

On MO Clone 1 explants were subcultured every 7 days in Exp. 1′; no subculturing was done in the control (Exp. 3′).

The basal part of each explant was excised at every subculture. At 21-day intervals, 12 explants of each experiment were transferred on to elongation medium (EM). Three weeks later, morphological observations and physiological tests were made on each explant to characterise the stage of rejuvenation reached during subculturing. The whole experiment lasted about 6 months.

In this paper various criteria of juvenile state were considered among those previously defined (Fouret *et al.* 1985):

— Spontaneous and induced rooting;

— Average length of the main shoot (the longest shoot issued from axillary buds of the explants);

— Reactivation of the apical meristem, isolated from the main shoot; this criterion was the most sensitive and the best one for rapid assessment of the development of rejuvenation (Fouret *et al.* 1985).

RESULTS

Spontaneous Rooting

During the experiment, explants from Clone 1 formed roots spontaneously on MM and MO (Fig. 1, *1). The number of roots increased gradually with successive subcultures (from t_0 to t_5). BAP limited spontaneous rooting (Fig. 2; compare Exp. 3 and 3′, Exp. 1 and 1′). Spontaneous rooting appeared first on medium without hormones (MO) and successive subcultures on MO improved rooting (compare Exp. 1′ and 3′). The rooting percentage was highest at t_5 in Exp. 1′ (after 14 subcultures on MO).

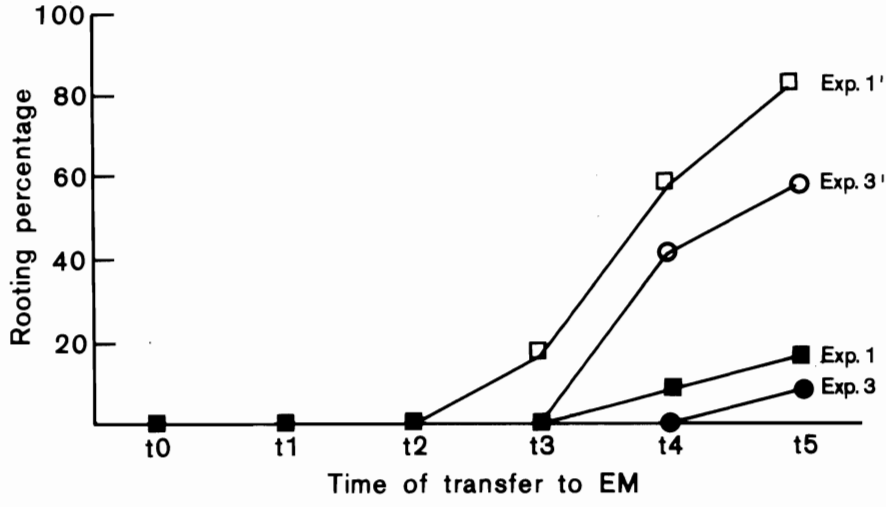


FIG. 2—Spontaneous rooting percentage on MM or MO (Clone 1) observed at the time of transfer to EM (*1). For the explanation of t_0 . . . t_5, *1, and the different experiments, see Fig. 1.

When explants were transferred to EM, the basal part was excised and root initials were cut off. After a 3-week period on EM (see Fig. 1), the presence of spontaneous rooted explants was noted. For Clone 1, spontaneous rooting percentages on EM were similar to those previously observed on MM or MO: at t_5, rooting reached 100% in Exp. 1′ and only 50% in Exp. 3′ (data not presented); but the rooting percentage did not exceed 17% in Exp. 1, 2, and 3 (Table 1, *2). For Clone 2, after successive subcultures on MM (Exp. 1), spontaneous rooting on EM appeared only at t_5 and the percentage of rooted explants was very low (Table 1, *2).

Spontaneous rooting of explants was maximal for Clone 1 when there were a number of weekly subcultures on a medium without hormones. Only a few explants of Clone 2 started to form roots spontaneously and only if they had been frequently subcultured on a medium with BAP and NAA.

Induced Rooting

At the time of transfer to EM and then to RIM, the basal part of the explants, including possible root initials, was excised; then rhizogenesis was induced on RIM (Fig. 1). Most of the explants of Clone 1 (Table 1, *4) were able to form roots *in vitro* at t_0, but the explants of Clone 2 did not form roots. At t_5, 100% of the explants of Clone 1 bore roots (Table 1, *4). For Clone 2, the induced rooting was considerably improved when the explants had been maintained on MM (Exp. 3; Table 1, *4); the maximal induced rooting percentage was obtained when the explants had been submitted

TABLE 1—Spontaneous and induced rooting of explants after subcultures (Exp. 1, 2) or maintained without subcultures (Exp. 3) on MM and then:
*2 — transferred to EM; spontaneous rooting percentage observed after 3 weeks on EM
*4 — transferred to EM and then to RIM and REM; induced rooting percentage observed after 6 weeks on REM.
(For an explanation of *2 and *4 see Fig. 1; t_0 and t_5 are respectively the start and end of the experiment)

Experiments		Clones 1	Clones 2
***2 Spontaneous rooting (%)**			
t_0		0	0
t_5	Exp 1	8	9
	Exp. 2	17	0
	Exp. 3	8	0
***4 Induced rooting (%)**			
t_0		82	0
t_5	Exp. 1	100	90
	Exp. 2	100	90
	Exp. 3	100	66

to successive subcultures on MM (Table 1, *4; Exp. 1 and 2). Therefore, root induction was highest after successive subcultures on MM. Moreover, when the explants of Clone 2 were subcultured or maintained on MM (Exp. 1, 2, and 3), the rooting time reduced from 29 days at t_1 to 13 days at t_5. For Clone 1, the same tendency was observed, but the rooting times (16 days at t_0 and 9 days at t_5) were always lower (Fouret *et al.* 1985). However, the rooting time decreased more rapidly for Clone 1 when the explants were subcultured on MO (Exp. 1′ – 16 days at t_0 and 7 days at t_3).

Comparison Between Induced Rooting and Spontaneous Rooting

For Clone 1 in Exp. 1′, we compared the percentages of spontaneous rooting (observed after 3 weeks on EM) and induced rooting (observed after 6 weeks on REM) (Table 2). The rooting percentage of induced explants remained at 100% until t_4; at t_5, it decreased to 77%. Spontaneous rooting increased to 100% at t_5. Therefore, a root induction treatment did not seem to be necessary for Clone 1, when explants have been subcultured several times on MO.

When explants were submitted to numerous weekly subcultures, the rooting percentage reached about 100% for Clone 1 (spontaneous and induced rooting) and Clone 2 (induced rooting only); furthermore, the rooting time decreased appreciably. Thus, a certain degree of rejuvenation was obtained.

TABLE 2—Comparison of the spontaneous rooting percentage (observed after 3 weeks on EM) and the induced rooting percentage (observed after 6 weeks on REM) in explants of Clone 1 subcultured on MO (Exp. 1′).

Rooting	Time of transfer to EM				
	t_1	t_2	t_3	t_4	t_5
Spontaneous	0	18	8	41	100
Induced	100	100	100	100	77

Length of the Main Shoot

The length of the main axillary shoot, formed during 3 weeks on EM (Fig. 1), increased progressively for Clone 1 (Fig. 3). In Exp. 1 and 3, the shoot length reached 3 or 4 cm at t_5. However, the average shoot length was 6.5 cm when explants were maintained on MO (Exp. 3′). When explants were subcultured on MO (Exp. 1′), the average shoot length was 14 cm. This "subculture effect" had already been reported for Clone 2 when cultured on MM (Fouret *et al.* 1985).

Reactivation of Isolated Apical Meristem

Isolated meristems from main shoot were considered to be reactivated when they were green on RAM. The time necessary for the reactivation of apical meristem was steeply reduced at the beginning of the experiment; for example, for Clone 1, it was 7 days at t_0 and decreased to 3 days at t_1 (Exp. 1′).

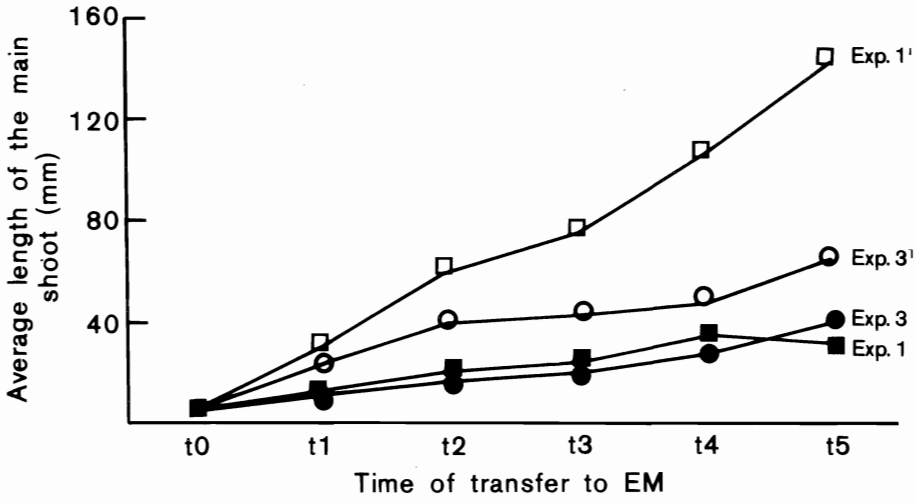


FIG. 3—Average length (mm) of the main shoot (Clone 1) observed 3 weeks after the transfer to EM (*2). For the explanation of t_0 . . . t_5, *2, and the different experiments, see Fig. 1.

Furthermore, when explants of Clone 1 were subcultured or maintained on MO, the minimum time to reactivation was 3 days (Exp. 1′) or 4 days (Exp. 3′); when explants were subcultured or maintained on MM, the reactivation time was only 1 day (Exp. 1) or 2 days (Exp. 3). It appears that the addition of BAP and NAA to the medium improves the further reactivation of apical shoot meristem. This "hormonal effect" had also been noticed for Clone 2 (Fouret *et al.* 1985).

Therefore, with clones from 50- and 500-year-old trees, the reactivation of isolated apical meristems is promoted if the material is previously maintained on medium with BAP and NAA. After numerous weekly subcultures of the material on this medium, the phenomenon is enhanced.

CONCLUSION

Several criteria must be considered in describing the phenomenon of *in vitro* rejuvenation of woody plants.

Physiological rejuvenation (Fortanier & Jonkers 1976) of clones of *Sequoia sempervirens* was determined according to spontaneous rooting percentage, induced rooting percentage, rooting time, length of the main shoot. The main shoot was longer for explants of Clone 1 frequently subcultured on medium without hormones than for those not subcultured. Spontaneous and induced rooting were higher and rooting times lower. Successive subcultures enhanced physiological "rejuvenation" of Clone 1. As the explants were trimmed at each subculture, we cannot distinguish the "subculture effect" from the "injury effect". Maintaining explants of both clones for several months on

medium supplemented with BAP and NAA had some effect. Clone 1 was more sensitive than Clone 2. After numerous weekly subcultures on medium with BAP and NAA, Clone 2 was "rejuvenated", but more slowly or later than Clone 1. Physiological "rejuvenation" of a clone originating from a 500-year-old tree was more difficult to obtain than that of a clone from a 50-year-old tree.

We tried to assess **ontogenetical rejuvenation** (Fortanier & Jonkers 1976) with the test "reactivation of isolated apical meristems". Material of both clones needed numerous and frequent subcultures on medium with BAP and NAA; it seems that ontogenetical rejuvenation is more difficult to obtain than physiological rejuvenation. The *in vitro* study was followed by observation of growth of plants in soil (ex-flasking). Clone 1 plants which had been subcultured frequently on medium with BAP and NAA and then transplanted developed orthotropic stems after 1 year of culture in a greenhouse. After the same treatment, stem growth of Clone 2 material was as plagiotropic as that of control plants. Either ontogenetical rejuvenation of Clone 2 had not been completely obtained *in vitro,* or the plants had partially lost it when transplanted. Further studies are necessary on the reacquisition of orthotropic habit. Plants treated on the medium without hormones (Clone 1) were transplanted and stem growth is being studied in the greenhouse. Work is now in progress on biochemical aspects of this problem, such as hormonal levels and peroxidase activities. Nevertheless, the results here presented show that *Sequoia sempervirens* can be considered a good experimental model for *in vitro* rejuvenation study of woody plants.

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