9. VEGETATIVE PROPAGATION IN VITRO OF CUNNINGHAMIA LANCEOLATA (LAMB.) HOOK.

C. BIGOT and F. ENGELMANN

1. INTRODUCTION

In vitro mass propagation of trees of highest quality and rapid growth probably will become one of the main tools to improve forest productivity in the next 20 years (9). Today's forests are largely plant associations which are only partially accessible to genetic control and agricultural practices. Among species with a great potential for productivity, the gymnosperms are well represented. So far about 20 of them have been studied for their in vitro performance and their capacity to give viable and true-to-type plantlets (14).

In the Taxodiaceae family, only Sequoia sempervirens has been the object of extensive research aimed at inducing mass propagation in vitro (2, 13). However, in vitro propagation of another economically important member of the same family, Cunninghamia lanceolata, has not yet received the attention it deserves. This species is of common occurrence in large natural forests of eastern Asia, especially China, and has been successfully introduced into Africa, Brazil, and Malaysia. In Europe, only a few specimens are available in arboreta. In China it is used extensively for reforestation; it grows fast (up to 25 m high with a trunk diameter of 2 m in 20 years) and is of excellent quality for timber and pulp and paper manufacture.

Nevertheless, some ecological factors such as heliophily, a requirement for high rainfall (1500 mm/year), and siliceous clay soil with good drainage, limit its reforestation potential.

The ability to produce shoots from the base of the trunk is a remarkable characteristic of Cunninghamia, which is only rarely reported for other conifers (i.e., Sequoia sempervirens, Pinus rigida). These stump sprouts can be used in a short rotation

system of clonal propagation similar to that used for some hardwood species (Eucalyptus, poplars).

Because of marked heterozygosity, conventional propagation through seeds gives rise to a heterogeneous population, and in vitro propagation has, therefore, been considered as an alternative to multiply desirable genotypes. In the present study the organogenetic potential, the rooting ability of shoots, and the growth habit after acclimatization of mature and juvenile material are compared. There are two phenomena associated with maturity that have, so far, rendered clonal propagation ineffective, i.e., the loss of rooting ability and the maintenance of plagiotropic growth. In vitro culture could be an experimental means to revert the material to juvenility, i.e., to an orthotropic growth habit, a juvenile morphology, and easy rooting (4, 6).

2. MATERIAL AND METHODS

In vitro culture has been started from 1) mature trees, grown in France for more than 50 years, selected for their resistance to cold (especially clone 78374); 2) the main stem of one 6-month-old tree (clone 78288); 3) seedlings raised from seeds from Brazil and Formosa.

In the case of mature trees, the explants consisted of apical sections or stem pieces without needles taken from stump sprouts. Similarly the explants that were taken from the 6-month-old tree and the seedlings consisted of needle-free apical sections or stem pieces. The explants were disinfected for 1 min in 90% alcohol and then during 20-30 min (15 min for seeds) in calcium hypochlorite (100 g l^{-1}) and Tween 80 (<1‰ w/v), and then were rinsed twice in distilled water.

Murashige and Skoog's (12) mineral medium was used, with vitamins (thiamine-HCl 10 mg l^{-1}, and myo-inositol 100 mg l^{-1}) and chelated iron (5 ml l^{-1} of a solution obtained by dissolving 7.45 g Na_2EDTA and 5.57 g $FeSO_4.7H_2O$ in 1 litre of distilled water). This is called basal medium (BM), to which were added sucrose (30 g l^{-1}), activated charcoal (Merck 2186) in some experiments (2-3 g l^{-1}), a solution of growth regulators, and Bacto-Agar (7 g l^{-1}). The medium was poured into tubes and jars and autoclaved for 20 min at 110°C.

After adding the explants the tubes and jars were placed in a growth chamber where the following conditions prevailed: 16 h light (20 Wm^{-2} provided by a mixture of 40 W Phillips TL, Sylvania Grolux and True-lite Durotest fluorescent tubes), and 26°C ± 1°C day temperature and 22°C ± 1°C night temperature. In different experiments, subcultures were prepared at 4, 6, or 8 week intervals. Acclimatization was carried out at 20°C ± 2°C, 90% relative humidity in small plastic "greenhouses" (miniserres).

3. RESULTS

3.1. In vitro behavior of stem explants with emphasis on shoot production

In all of our experiments, new explants were excised from a stock of mother plants kept under in vitro conditions on BM + activated charcoal and the auxins indoleacetic acid (IAA) (1-2 mg l^{-1}), or indolebutyric acid (IBA) (3 mg l^{-1}). Each mother plant consisted of a small, rooted basal shoot cutting which had developed basal shoots after two months in culture. The explants (1 cm long) were taken from the principal axis of these mother plants; the stock of mother plants was restored every 8 weeks by rooting cuttings taken from the apical zone of the main stem.

Preliminary assays (not reported here) demonstrated that benzylaminopurine (BAP), with or without an auxin, stimulated the growth of axillary meristems of the needles (Fig. 1). However, shoots (Fig. 2) that developed from these meristems did not elongate enough to root. This lack of rooting was subsequently overcome; firstly, by induction of axillary budding by supplying BAP for two months (Table 1); secondly, by elongation of shoots and production of more shoots by 2 months of subculture of BM supplemented with charcoal and IAA 2 mg l^{-1}. Orientation of the explant on the medium was important because when placed horizontally three times more axillary shoots were obtained then when placed vertically (5.9 instead of 2 per primary explant of the mature clone). Thus a sustained production of shoots (Fig. 3) was established and studied over a 1-year period.

Table 1 shows that BAP 1 mg l^{-1}, and α-naphthaleneacetic acid (NAA) 0.03 mg l^{-1}, when used together, stimulate organogenesis and

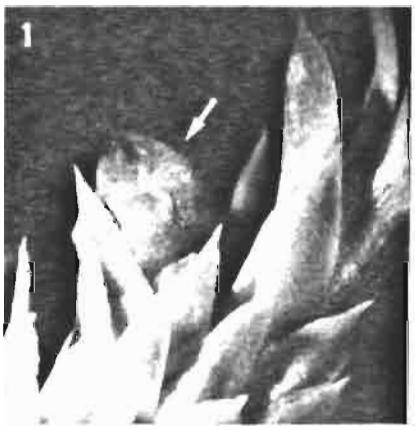


FIGURE 1. Induction of axillary buds (mature clone 78374).
FIGURE 2. Shoots induced by BAP 1, and NAA 0.03 mg l^{-1} at the base of a primary explant placed vertically on the BM.

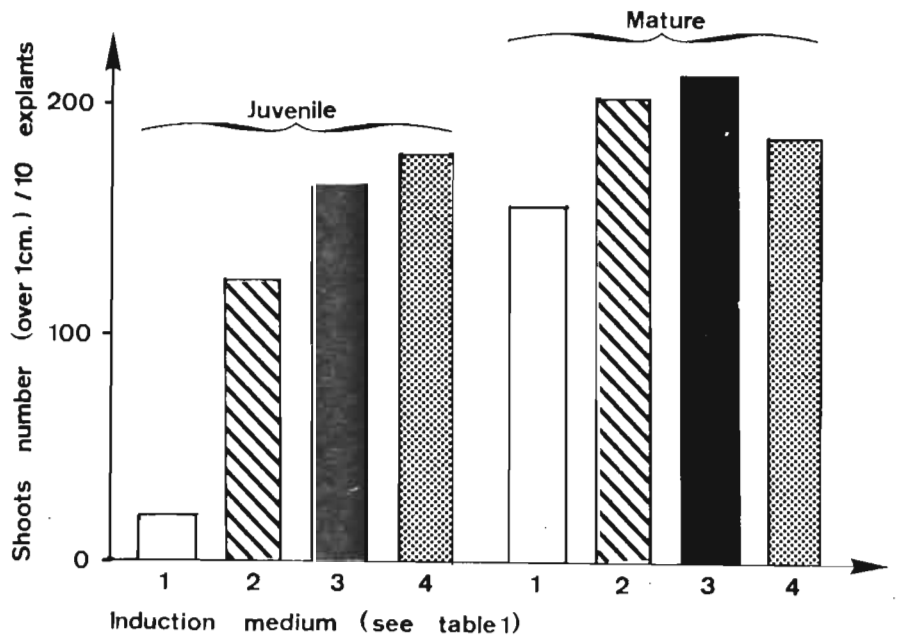


FIGURE 3. Number of shoots (over 1 cm long) produced per 10 explants after 12 subcultures (for composition of induction medium (number 1, 2, 3, and 4) see Table 1).

Table 1. Induction of axillary shoots on primary explants cultured for 2 months on BM

Clones and induction media[a]	Number of explants	Explants with shoots(%)	Shoots per 10 explants	Survival of explants (%)
78288 (juvenile)				
1. Control	12	25	20	25
2. BAP 1 IAA 2	28	78	19	64
3. BAP 1 NAA 0.03	16	81	22	87
4. BAP 2 IAA 2	12	92	21	83
78734 (mature)				
1. Control	12	41	18	41
2. BAP 1 IAA 2	36	83	37	87
3. BAP 1 NAA 0.03	18	88	42	92
4. BAP 2 IAA 2	12	83	25	91

[a]All concentrations in mg l^{-1}.

survival during the shoot induction and production phase. Initially all the shoots originated from pre-established axillary meristems, but after several successive subcultures many of them started to arise from the base of the shoots. In comparison with the control (Fig. 3), addition of growth regulators had a stimulatory effect on the number of shoots produced per year and on the percentage of explants surviving.

3.2. Rhizogenesis

All shoots selected to provide mother plants had rooted spontaneously after 22 weeks of serial subculture on an auxin-free medium, provided that no lateral shoots were excised prior to rooting of the main shoot. Shoots and roots also arose from the more or less callused base of the mother plants, but no apparent vascular connections were established between these shoots and roots.

When shoots (2 cm long) were taken from the primary explant to induce rhizogenesis (Fig. 4) it was found that IBA between 1 to 5 mg l^{-1} or IAA 5 mg l^{-1} in the medium stimulated the formation of short, thick roots without root hairs (Fig. 5 and 6). IAA was more favourable to subsequent root elongation.

In later experiments, IBA (3 mg l^{-1}) was used to test the rooting potential of shoots of the juvenile and mature clones obtained from the 7th subculture of the primary explant. After 6 weeks all

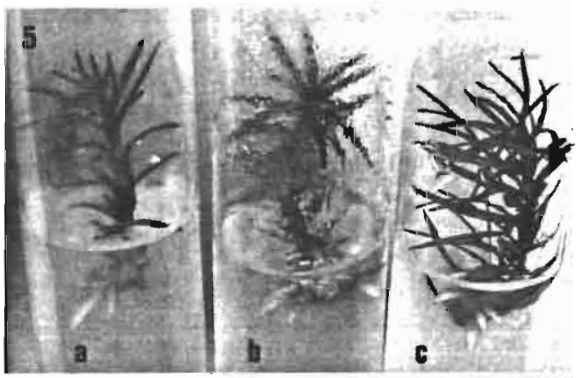


FIGURE 4. Shoots developing from a primary explant (juvenile clone 78288).
FIGURE 5. Shoots rooting in vitro after 4 weeks (IBA concentration: a = 1; b = 2; c = 3 mg l^{-1}).
FIGURE 6. Rooted shoots before acclimatization (juvenile clone 78288).

non-rooted shoots were subjected again to fresh IBA containing rooting medium, and after another 6 weeks shoots that had still not rooted were treated again with IBA. The following results were obtained (Table 2). The rooting percentage varied greatly in the two clones (4 to 76%). Shoot and root formation was not correlated with the number of subcultures. There was extreme variability in

the number of roots per explant (1 to 31) and of the mean number of roots per shoot (1 to 9.5). After three subcultures on rooting medium no differences were observed between juvenile and mature clones (Fig. 7). The total percentage of rooted shoots has reached 70% on average, but 100% was recorded in some experiments.

Table 2. Rhizogenesis of shoots excised from primary explants after 6 weeks on BM + IBA 3 mg l^{-1} (the primary explants have been subcultured 7 times; p = 0.05).

Clones	Subculture	Number of shoots	Rooted shoots (%)	Mean number of roots per shoot
Juvenile (78288)	I	19	10	1.00
	II	24	30	3.57 ± 2.72
	III	33	57	3.95 ± 1.81
	IV	12	25	3.33 ± 1.05
	V	24	4	2.00
	VI	31	22	2.71 ± 2.19
	VII	28	75	5.57 ± 2.78
Mature (78374)	I	30	50	3.27 ± 1.84
	II	65	65	5.50 ± 0.92
	III	119	47	3.91 ± 0.75
	IV	63	65	3.30 ± 0.64
	V	116	21	4.75 ± 1.33
	VI	87	35	4.29 ± 1.33
	VII	135	76	9.51 ± 1.27

In subsequent attempts to obtain more uniform rooting, shoots were pretreated with an auxin solution and then cultured on auxin-free medium or subjected to a dark period of 15 days. Both methods failed to induce uniform rooting of the shoots.

On the other hand, direct rooting by dipping the base of the shoots for 5 hours in a sterile solution of IBA (3 mg l^{-1}) followed by storage in a moist chamber for one day before planting has been effective. All shoots of juvenile clone 78288 rooted after three months in rooting medium in the greenhouse under mist (5 1.4 roots per shoot, p = 0.05), but the method failed with the mature clone 78374 because of callusing.

3.3. Acclimatization

After several tests, the following acclimatization method was established. The plantlets were maintained in a closed environment

(20°C ± 2°C, 90% R.H.) on a freely draining medium made up of pine bark (5 mm chips), perlite, and enriched TKS_2-type peat (3:1:1 v/v) for 6 weeks, under low intensity natural light complemented by artificial light (8 hours per day, Phytoclaude 400 W Lamps, 10 Wm^{-2}). Liquid fertilizer (3) was applied at weekly intervals.

In all experiments combined, about 23% of the plantlets of the juvenile clone 78288 and 19% of the plantlets of the mature clone 78374 were lost during the acclimatization phase. No correlation was found between the initial root number and the successful establishment of the plantlet. At the end of the acclimatization period the plantlets of the mature clone showed a high variability in growth rate (Table 3).

After acclimatization, plantlets were planted in pots with the earlier described pine bark, perlite, peat (3:1:1) mix and transferred to a greenhouse. Complementary artificial lighting (10 Wm^{-2}) was supplied during 6 hours every day. Liquid fertilizer was applied as before. After 4-5 months the plants formed basal shoots (1 to 3 per plant) and subapical branches (1 to 2 per plant) (Fig. 8). The plants were then planted in containers (3 liters or more) with the pine bark, perlite, peat mix to which

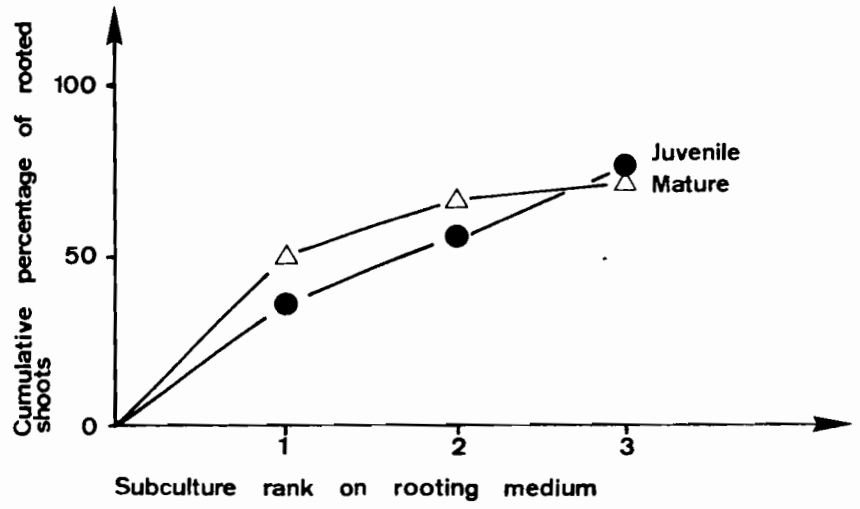


FIGURE 7. Rooting percentage after three subcultures on BM with IBA 3 mg l^{-1}.

a slow release fertilizer ('Osmocote' 15-12-15) had been added. These plants as well as those obtained from seeds (from Sao Paulo, Brazil) were transferred from the greenhouse to outdoors. Table 4 shows the growth characteristics of plants propagated *in vitro* from the juvenile clone (Fig. 9) and plants derived from seeds after two years of growth. The micropropagated plants had grown more in height and had produced more basal shoots than the plants obtained from seed. Variability within the two groups was similar.

Table 3. Effects of 6 weeks of acclimatization on plantlets of juvenile and mature clones (samples from 5 subcultures of primary explants, $p = 0.05$).

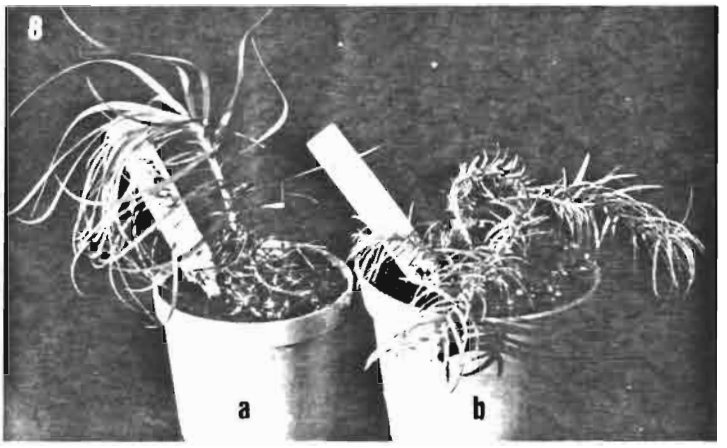
Condition / Clone	Number of plantlets	Roots number per plantlet	Mean length of stem (cm)	Variance (stem)
Not acclimatized				
Juvenile	200	6.51 ± 0.88	5.03 ± 0.24	2.8
Mature	490	4.75 ± 0.33	5.14 ± 0.15	2.6
Acclimatized				
Juvenile	154	8.61 ± 1.09	6.83 ± 0.43	7.07
Mature	394	6.63 ± 0.41	7.44 ± 1.89	353.44

3.4. Plagiotropic behavior

All 265 plants derived from mature clone 78374 were plagiotropic (Fig. 8b), whereas of the 156 plants obtained from juvenile clone 78288, 93% showed orthotropic growth (Figs. 8a and 9) (similar percentages of orthotropic plants were obtained *in vitro* from other juvenile clones).

During the culture of shoots successively sampled (subculture I-VII in Table 2) from the mature clone, no reversion in needle morphology from the mature pattern (short, spiny) to the juvenile pattern (long, flexible), nor in the pattern of needle implant from asymmetrical to symmetrical occurred.

Attempts were made to modify the plagiotropic growth by decapitation of the shoot, by grafting on seedlings, by cold treatment (3°C, 8 h/day at low light intensity), by frequent subculture



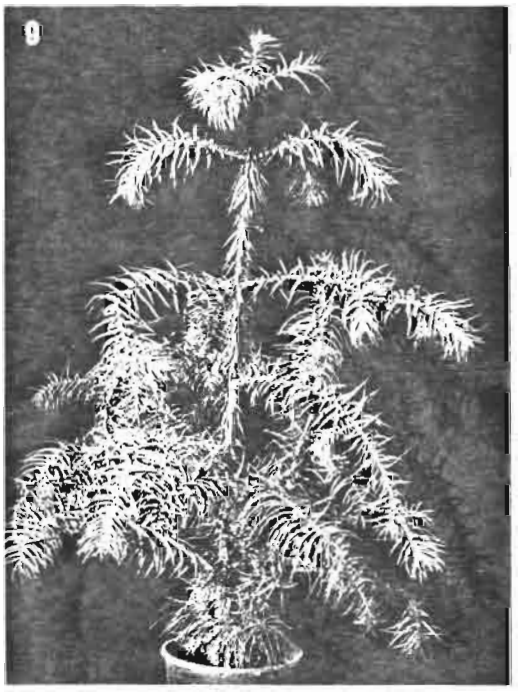


FIGURE 8. Young plants after 3 months acclimatization in the greenhouse (a = juvenile clone; b = mature clone).
FIGURE 9. Plant obtained from juvenile clone 78288 after two years in the greenhouse.

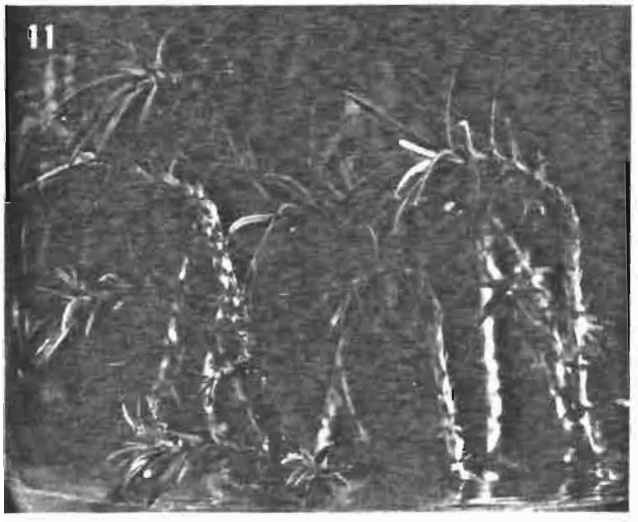


FIGURE 10. Orthotropic shoots obtained from the mature clone after 6 weeks in darkness on BM + IBA 3 mg l^{-1}.
FIGURE 11. Reversion to plagiotropy after 8 weeks exposure to light following 8 weeks in the dark (mature clone).

FIGURE 12. Control shoots of the mature clone maintained in continuous light (8 weeks of culture).

Table 4. Comparison between plants obtained by in vitro propagation (juvenile clone 78288) and from seeds after two years growth (variance is shown in brackets, p = 0.05).

Origin	Number of plants	Mean of stem height(cm)	Mean number of layers	Mean number of basal shoots
In vitro 78288	13	61.4 ± 9.4 (240.56)	5.54 ± 0.73 (1.44)	4.08 ± 0.97 (2.59)
From seeds	9	42.4 ± 7.18 (87.04)	5.33 ± 0.94 (1.48)	2.33 ± 1.22 (5.42)

(every 15 days) and by culture in darkness. Only culture in darkness led to a temporary modification of growth of plants derived from the mature clone. As is shown in Fig. 10, plants obtained in vitro from isolated shoots or basal shoots of mother plants were orthotropic at the end of the dark period. However, after exposure to light, they quickly (after one month) reverted to plagiotropic growth (Fig. 11), similar to that of the control (Fig. 12). It appears that in plants derived from the mature clone plagiotropic behaviour is induced by light.

4. CONCLUSIONS

The in vitro propagation of Cunninghamia lanceolata is feasible as evidenced by this study and this species can now be added to the list of conifers cultured in vitro compiled by Thorpe and Biondi (14). Regeneration through axillary budding was performed several times during a one year period. However, viable plantlets maintained the behaviour of the original clone, i.e., there was no obvious rejuvenation when mature clones were used. Plants derived from juvenile clones in vitro were compared with those obtained from seeds in a 2-year field test. The micropropagated plants were somewhat more vigorous than the seed plants. This could be a reflection of the clone being a selected superior genotype and the seed plants having varying genotypes due to the heterozygosity of the species.

Unlike Sequoia sempervirens, which rejuvenated in vitro (7), the mature clone of Cunninghamia that we used failed to do so. Therefore, other methods such as micrografting on seedlings (5, 8), pruning (10, 11), etc., will have to be applied. However, no

difference in rooting ability was observed between shoots obtained from explants from mature and juvenile clones (mother plants) maintained in vitro.

We have observed orthotropy in some of our mature Cunninghamia clones. In 1985, several 4-year-old container grown plants each produced one vigorous dominant orthotropic shoot with axial symmetry, but bearing mature needles. Black (1) observed such orthotropy in rooted cuttings of 3-8 year old Douglas fir. In addition, we found that shoots in darkness become orthotropic, but revert to plagiotropic growth when light is applied. This environmental factor could be a determining one for the maintenance of plagiotropy, at least in Cunninghamia. Studies should be started to determine if phytochrome or specific metabolic modifications are linked to these changes in tropism.

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