DOE/RA/50316--T1
Attachment II

METHANOL PRODUCTION FROM EUCALYPTUS WOOD CHIPS

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Vegetative Propagation of Eucalyptus

April 1982

Prepared by
Biomass Energy Systems, Inc.
Lakeland, Florida

For the
U.S. Department of Energy
Office of Alcohol Fuels
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Attachment II

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METHANOL PRODUCTION FROM EUCALYPTUS WOOD CHIPS

Working Document 2

Vegetative Propagation of Eucalypts

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April 1982

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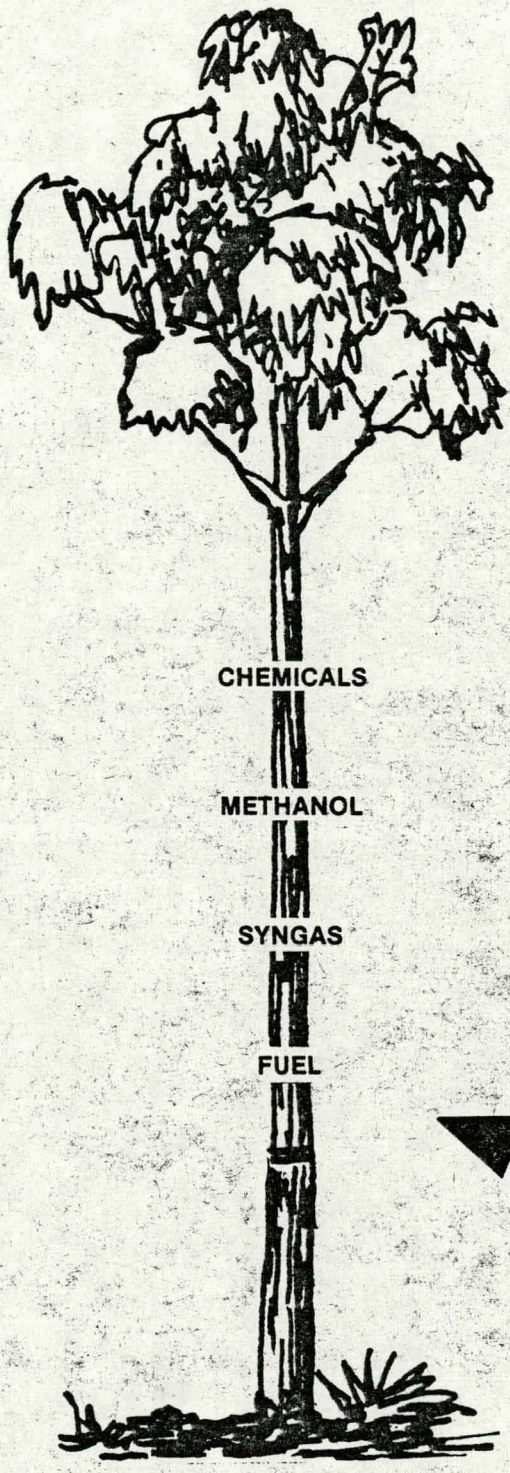
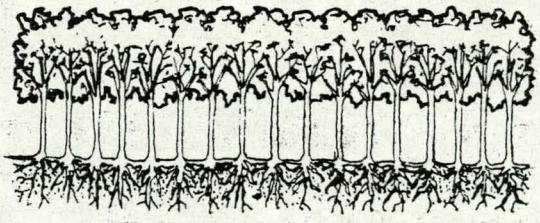
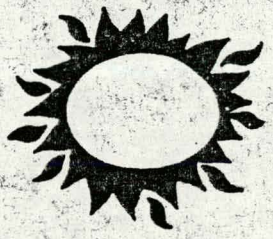


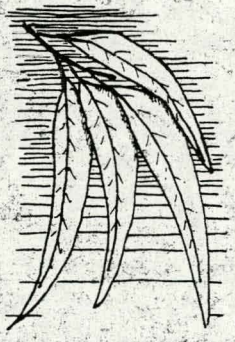


VEGETATIVE PROPAGATION

OF

EUCALYPTS





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1.0 INTRODUCTION

The purpose of this working paper is to consider the various approaches to Eucalyptus propagation, and evaluate the most promising in detail.

Production of the required 3,980 short tons of wood feedstock per day for a methanol refinery will require approximately 68,000 acres in production on a seven-year rotation. Approximately 8,500 acres must be planted with 871 trees per acre each year. In other words, about 7.4-million trees per year will be needed to establish the energy forest.

The biomass productivity of a eucalypt energy plantation is dependent in part, upon a combination of environmental factors, including soil structure and fertility, average sunlight and temperature, precipitation quantity and distribution, vegetative competition, and pathogen impact; but the average genetic quality of the trees is the single most influential factor determining growth potential. The genetic system of Eucalyptus is such that native seed populations include a diversity of genetic types--and consequently, a wide range of environmental adaptability within the species. This diversity is beneficial in providing families adapted to a particular environmental niche (e.g., phosphate mine spoils, native flatwoods soils, high-salt soils). However, it is very difficult to capture desirable genotypes for seedling production. Eucalypts show pronounced "hybrid vigor"; and, conversely, suffer tremendous "inbreeding depression" when seed results from self-pollination (Eldridge, 1978). Commercially available seed is genetically heterogeneous. Planting stock produced from it will invariably yield "aces and spaces" (E. C. Franklin, Pers. Comm.). That is there will be come very good trees and some that do not survive. Once a

series of genetically superior trees have been selected from a seedling plantation, these genotypes can be vegetatively propagated, field-tested and then expanded to provide a uniformly high-yielding planting stock.

As part of the present study, we have examined the feasibility of large-scale plantation establishment by various methods, and have reached the following conclusions.

1. Seedling plantations are limited in potential yield due to genetic variation among the planting stock and often inadequate supplies of appropriate seed.
2. Vegetative propagation by rooted cuttings can provided good genetic uniformity of select hybrid planting stock; however, large-scale production requires establishment and maintenance of extensive cutting orchards. The collection of shoots and preparation of cuttings, although successfully implemented in the Congo and Brazil, would not be economically feasible in Florida for large-scale plantations.
3. Tissue culture propagation of select hybrid eucalypts offers the only opportunity to produce the very large number of trees required to establish the energy plantation. The cost of tissue culture propagation, although higher than seedling production, is more than off-set by the increased productivity of vegetative plantations established from select hybrid Eucalyptus.

2.0 PROPAGATION BY ROOTED CUTTINGS

Vegetative propagation of Eucalyptus spp., and especially hybrids, has been successfully developed to commercial scale in The Congo, Brazil, and New Guinea using the technique of rooted cuttings (cf. Martin and Quillet, 1974a,b,c,d). A clonal plantation established from superior hybrid individuals can easily double the productivity of even "genetically-improved" seedling plantations, and accomplish this in a single step, rather than in a long series of 4-6 year tree generations. Aracruz Celulose, in Brazil, is an excellent example of effective and efficient use of rooted Eucalyptus cuttings to propagate stock for a 17,000 ha plantation.

The rate of genetic improvement for a forest crop is especially important in terms of investment/interest rate considerations. In this regard it is absolutely essential to provide a continuously improving supply of clonal candidates for vegetative propagation. By also having an economical system for large-scale vegetative propagation, we can afford to use relatively expensive breeding techniques (e.g., controlled interspecific crosses, backcrosses and mutation breeding). With the newly introduced clonal candidates, this is true because the expense of the breeding and selection program can then be amortized over a very large number of vegetatively-produced plants from each selected clonal candidate. Major productivity gains have been achieved by combining site-specific genetic selection and vegetative propagation.

2.1 Methodology

Propagation of select Eucalyptus by rooted cuttings has been developed to large-scale commercial application in the Congo (Martin and Quillet, 1974a,b,c,d) and in Brazil (Iemori, 1975). The essential aspects of the technique (Table 1) are (a) use of cuttings from rejuvenated shoots, (b) application of auxin to induce root initiation, (c) intermittent mist to prevent water stress, (d) use of a good draining potting mixture, and (e) high soil temperatures to promote root growth. It is also important to maintain clean nursery conditions.

Juvenile shoots can be induced to develop from the stool of an adult "plus" tree by felling the tree; or the tree can merely be circumscribed with a wound through the vascular cortex. Eucalyptus can easily recover from such wounds, apparently due to the presence of an internal phloem (Wilson and Bacheland, 1975; ref. in Hartney, 1980).

2.2 Results and Discussion

Procedures for establishing rooted cuttings were based upon recommendations from commercial sources (Martin and Quillet, 1974a,b,c,d; L. Brandano, Pers. Comm.; A. Brune, Pers. Comm.). Table 2 presents data evaluating potting mixtures and rooting hormone. The best potting mixture found incorporates 1/5 to 1/2 coarse perlite in peat to allow adequate drainage under the automatic mist. Indole butyric acid (IBA) can cause callus formation without root development at supra-optimal dosages. A six-second dip in 0.8% IBA/50% alcohol solution is sufficient to root a proportion of the cuttings; but a 10-second dips results in callus only.

TABLE 1

ROOTED CUTTING PROCEDURE[1]

1. Material collected from stump or wounds of selected trees. Shoots are selected for juvenility. The shoot is normally greater than 0.5 m with at least 8 nodes. Optimum stem diameter is 5-10 mm. To maintain continuous harvesting 1-5 nodes are left on the stump/ wound to insure future growth. Cutting is done with scissor shears.

2. Benlate fungicide is sprayed on stump sprouts 2-3 days before shoots are harvested.

3. Shoots are placed in 1-3 plastic bags and are labeled.

4. Bags are put in cold water for transport to the nursery. Time of transport is 45 minutes to 1 hour. Ocassionally, shoots are left in the bags in water overnight. No adverse effects have been noted from this delay.

5. The shoots are then cut into lengths of 2-4 nodes.

6. The cuttings are placed in Benlate solution (4 tsp/gal) for 20 minutes to 2 hours.

7. Cuttings are dipped either in 8,000 ppm IBA in talc powder, or in a solution of 0.8% IBA in 50% ethyl alcohol for 6 seconds.

8. Cuttings are then planted in Todd #150-5 planter flats (Speedling, Inc.) with a soil medium of peat and perlite (1:1 mix). Charcoal is added to medium by dibbling holes in each tray compartment and pouring the pulverized charcoal in each hole. The soil is then saturated with water before planting occurs. Wetting agents such as "Aqua-Grow" can be used to promote thorough drainage.

9. The trays are then placed under an automatic system which sprays a water mist for about five seconds/ten minutes during daylight hours.

[1]Adapted from techniques observed at Aracruz Celulose (L. Brandano, Pers. Comm.), recommendations (A. Brune, Pers. Comm.) and published data (Martin and Quillet, 1974a,b,c,d).

TABLE 2

SURVIVAL OF EUCALYPTUS CAMALDULENSIS CUTTINGS

		Cuttings Total	Surviving	% Survival
Potting Mixture	• Peat-Perlite	2600	463	18%
	• Peat	5422	246	5%
	• Peat-Sand	1933	150	8%
Hormone	• 0.8% IBA in 50% alcohol--6 sec	6686	768	12%
	• 0.8% IBA/alcohol --10 sec	441	10	2%
	• 8,000 ppm IBA powder	2888	99	3%

After planting, root formation can take as little as 11 days or as long as 4 weeks. Some cuttings never develop roots, but remain green. Shoot development usually correlates with root development. Shoots can form within 7-15 days with no signs of root or callus formation. After surviving the first few weeks, shoots have developed slowly up to a length of about 3 cm. Leaves are almost always pale green in color, linear to elliptical in shape. Many times, the cutting will appear dead after the first few weeks and main stem will turn brown and wither; but, new shoots often form from the lateral nodes.

In some instances, yields of rooted cuttings will be 50, 70, or even 90%; however, the overall success has been much lower. In our hands, the percentage of successfully-established rooted cuttings has been disappointingly low (less than 10% overall); but most of our losses have been attributed to water stress or inadequate drainage of the potting medium. Other sources of variability in results include soil temperature, rooting hormone application and genotypic differences. A gross calculation of labor cost indicates that cuttings could be collected, prepared and planted for 10-20¢ each. Nursery maintenance would require an additional 5-7¢ each, or a total of roughly 15-25¢ per cutting. Although this cost estimate is potentially attractive for vegetative propagules, an efficiency rate of 10% gives a final estimated cost of $1.50 to $2.50 per finished cutting.

A major shortcoming in our rooted cutting technique relates to the need to maintain a constant high soil temperature (25-27^{o}C) for adequate and consistent rooting. Additional expenditures on bed heating is likely to improve yields substantially; but probably not beyond 60-70% overall (Martin and Quillet, 1975d), reducing the cost estimate to 30-45¢

per rooted cutting. Rooted cuttings usually produce multiple shoots, thus requiring the pruning of each individual cutting (L. Brandano, Pers. Comm.). This operation along can add 1-2¢ to the cost of each rooted cutting.

Although the rooted cutting method is technically possible for Florida (T. Geary, Pers. Comm.) it does not appear to be economically feasible for establishment of large-scale energy plantations here requiring 7.4-million trees per year. The major disadvantage is that the rooted cutting procedure requires about four years to produce fewer than 200,000 clonal plants from a single select tree (Martin and Quillet, 1974d). For large-scale propagation from rooted cuttings, "cutting orchards" must be established and maintained from each clonal line. If five hundred cuttings can be harvested from each select tree stump over a year, 14,800 coppicing stumps would be needed to produce enough cuttings to plant the necessary almost 8,500 acres per year. Approximately four years would be needed to establish these "cutting orchards" before production could begin. It is probable that new selections will be identified much more quickly.

A method which could significantly reduce this time and improve efficiency would afford tremendous economic advantages. Rapid clonal propagation in vitro (i.e., tissue culture[1]) provides this method. The technique is analogous to vegetative propagation of superior trees by rooted cuttings, but is many times more efficient. A single genotype

[1]"Tissue Culture" is a generalized term, referring to all aspects in vitro cultivation of plant cells, tissues and organs. In the strict sense, the in vitro propagation of shoots from stem apices or nodal buds, as described in this chapter, is "organ culture."

(i.e., "plus tree") can be multiplied to a planting stock of millions within a year. The limitations, no longer biological as with cuttings, is set by the scale of the tissue culture operation. In this case, the increase in scale provides advantages in efficiency that can make propagation by tissue culture economically superior to vegetative propagation by rooted cuttings. Further advantages include the rapid production of planting stock from newly-selected elete trees, and the development of specially suited trees (i.e., salt tolerant lines),

3.0 PROPAGATION BY TISSUE CULTURE

3.1 Literature Review

The potential advantages of vegetative propagation to produce Eucalyptus planting stock have been long recognized. Classic approaches to this problem have included grafting, layering and rooting of stem cuttings (reviewed by Hartney, 1980); but these methods are limited in their applicability by high cost and low productivity. As an alternative, workers around the world have studied the potential of Eucalyptus propagation by tissue culture. These techniques are becoming more frequently used in preference to other methods of vegetatively propagation a variety of plants because of high multiplication rates that are possible, and the relative ease of controlling conditions and treatments in the tissue culture laboratory (Hartney, 1980).

A. Rooting Inhibitors and Juvenility

The key to successful vegetative propagation of Eucalyptus species has been the recognition of the requirement for rejuvenation of

the shoot (reviewed by Franclet and Boulay, 1981; Hartney, 1980). One important difference between juvenile (good rooting ability) and adult shoots (poor rooting ability) may be due to the presence of rooting inhibitors. Three chemically distinct rooting inhibitors have been found concentrated in adult leaves of E. grandis, but at much lower concentration in juvenile leaves (Dhawan, Paton and Willing, 1979; see also Paton et al., 1970; Nicholls et al., 1970; Paton and Willing, 1974). Low concentrations of these purified growth regulators, called "G inhibitors" for grandis, have more auxin-like activity than indole-3-acetic acid (IAA) in a Mung bean rooting assay or in the rooting of E. grandis seedling (i.e., juvenile) stem cuttings. The authors also point out other, less compelling observations suggesting a role for "G" as a physiological alternative to the hormone abscisic acid. Abscisic acid, another plant hormone, cannot be detected in adult leaves of E. grandis, but has been shown to affect a number of different responses, including dormancy induction, and resistance to silting. Adult Eucalyptus leaves (high "G" concentration) can withstand water stress, while juvenile leaves wilt (low "G" concentration) (Paton and Willing, 1974).

B. Organ Culture of Eucalyptus Nodes

Nodal segments, or shoot apices containing already organized centers of growth, have been used to multiply Eucalyptus grandis seedlings (i.e., juvenile tissue) in culture by development of axillary buds and subsequent rooting of the propagated shoots (Cresswell and deFossard, 1974). Cresswell and Nitch (1975) then reported that presence of the leaf blade on adult nodal explants appears to inhibit rooting in culture; but that the presence of part of the petiole was

necessary for axillary bud development and rooting in a medium containing 1 μM indole-butyric acid to promode root initiation. These workers obtained plantlets from about 30% of the adult nodes tested. Plants that developed in culture from as high as the 50th node were successfully established in the field. The applicability of this technique was extended to include E. camaldulensis and E. robusta, as well as E. grandis, using a medium with 7 μM indole-butyric acid (Goncalves, 1975; cf. Goncalves, 1979). However, this particular procedure did not work with E. alba, E. saligna or with juvenile E. grandis, pointing out the existence of significant distinct cultural requirements for adult nodal culture of different species, and between juvenile and adult tissue in a single species.

Baker, deFossard and Bourne (1977) have reported on the successful multiplication of seedling (juvenile) nodes from E. ficifolia and also nodes from 25 year-old trees. In the latter case, the single shoot that had been tested on rooting medium (containing 4 μM indole-butyric acid) produced a root after two weeks. These workers also reported that juvenile nodes of E. grandis from seedlings, coppice and young trees formed plants when cultured directly on rooting medium. Buds developed into shoots by one to two weeks and formed over the next two weeks. They had no success with adult E. grandis nodal cultures due to microbial contamination which they could not eliminate from the explants. Limited success was also reported with E. regnans nodes from seedlings and young trees (50-150 cm high), but all nodes from adult forest trees were lost to contamination. The fourth species examined in this study, E. polybractea, yielded small shoots and multiple buds from adult nodes. No results on rooting attempts were mentioned.

Cultures of E. citriodora seedling shoots (about 5 mm with or without cotyledons) on media containing benzylamino purine at 0.5, 1.0 and 2.0 mg/ℓ produced multiple shoots as early as within 15 days; and up to a hundred shoots each after four months (Lakshmi Sita and Vaidyanathan, 1979). As in earlier works, the axillary shoots can be multiplied by further subculture and roots form when shoots are transferred to medium without the hormone benzylamino purine. deFossard and associates (deFossard, et al., 1978) have attempted to refine the procedures and media for axillary shoot propagation of E. ficifolia. They used nodal cultures from seedling (juvenile and 25 year old (adult) trees, which had been multiplied in culture over three years and one and one half years, respectively. The small amount of data presented shows a generally accepted negative effect of light intensity on rooting in seedling cultures. (Adult cultures did not repond efficiently to the many different rooting media tested.)

More recently, techniques have been reported for the efficient organ culture of Eucalyptus shoots from juvenile or rejuvenated nodes (Hartney and Barker, 1980; Hartney, 1981; Destremau et al., 1980; Franclet and Boulay, 1981) (see Section 3.2. B(4)).

C. Callus Cultures of Eucalyptus

Although hundreds of combinations and concentrations of hormones had been tested (e.g., Lee and DeFossard, 1974; Gonclaves, 1975), differentiation from callus cultures of Eucalyptus was limited to occasional root development. In 1969, Aneja and Atal reported that lignotuber callus of E. citriodora produced several buds (1 mg/ℓ 2-naphthoxacetic acid) which developed into complete plantlets with roots.

Although there are legitimate questions about this report, other work has followed to support the observation. Shoot formation has also been reported from hypocotyl callus of E. alba on media containing 1 mg/ℓ indole-3-acetic acid (Kitahara and Caldas, 1975). Lakshmi Sita (1979) found that cultures of E. citrodora cotyledonary callus on media containing 1 mg/ℓ indole-acetic acid produced shoots from tissue not previously organized as shoots. (Note the contrast with nodal culture, cited above.) When excised, these shoots readily rooted in media containing 1 mg/ℓ naphthalene acetic acid or indole-butyric acid, but only three plants survived loss to contamination. The key to these results is whether or not shoots arose in the absence of any pre-formed axillary buds in the initial plant.

3.2 Methodology for Tissue Culture Propagation

Commercial application of in vitro propagation to production of clonal eucalypts involves four distinct culture "Stages," each requiring specific manipulations, culture media and growth conditions. The major problems to be addressed and overcome are (1) the successful establishment of material in axenic (i.e., bacterial- and fungal-free) culture, (2) the optimization of the multiplication media to give reliable, high plant yields, and (3) the rooting of the propagules, and (4) their subsequent acclimation to a nursery condition.

A. The Tissue Culture Laboratory

A laboratory for plant tissue culture must accommodate a variety of distinct functions, some of which can be conducted in the same room (e.g., glassware cleaning, media preparation and

sterilization) and others which should be conducted in separate areas (e.g., transfer of explants, culture incubation). These areas are listed by function:

(1) Glassware cleaning area (equipped with sinks, purified water supply, and washing machine);

(2) Media preparation and sterilization area (equipped with balances, pH meter, mixers, refrigerator and autoclaves);

(3) Storage (for glassware, media supplies, etc.);

(4) Transfer room (equipped with laminar flow hoods);

(5) Culture room (equipped with shelves and lights for incubation of cultures);

(6) Office space; and

(7) Toilets.

In addition, it is desirable to have a lunch room so that employees do not leave the sanitary conditions of the laboratory during their work breaks. Laboratory requirements are discussed in more detail in Section 3.3.D.

B. Techniques

(1) Selection of Clonal Candidates

Selections must be done with great care, since the vegetatively propagated plants are only as good as the original genetic stock placed in culture. Criteria for identifying select trees can include stem volume, form, wood density, disease resistance, drought or flood tolerance, and unique site adaptability. It is evident that most of these characteristics can only be evaluated in adult trees. The minimum 3-6 years required for expression of these qualities argues for

the establishment of provenance test plantings as soon as possible, to provide the clonal stocks for propagation in future years.

Current selections have been made in 6-1/3 year-old plantations of E. camaldulensis on two 10 acre phosphate, mine, soil, sites: one on overburden soil and the other on sand tailings. Table 3 lists the height and diameter data for these select trees.

TABLE 3

SELECT EUCALYPTUS CAMALDULENSIS CLONAL CANDIDATES

BESI #	HEIGHT		DIAMETER AT BREAST HEIGHT		MINE SPOIL SITE
	m	(ft)	cm	(in)	
158	17.0	(56)	28.2	(11.1)	overburden
159	19.3	(63)	24.6	(9.7)	sand tailings
160	19.7	(64)	26.2	(10.3)	sand tailings
161	18.7	(61)	23.1	(9.1)	sand tailings
162	19.0	(62)	27.2	(10.7)	sand tailings
163	18.3	(60)	24.1	(9.5)	sand tailings
164	19.3	(63)	23.9	(9.4)	sand tailings
165	19.3	(63)	23.4	(9.2)	sand tailings
166	18.7	(61)	27.7	(10.9)	sand tailings
167	19.3	(63)	35.8	(14.1)	sand tailings
168	22.0	(72)	24.4	(9.6)	sand tailings
169	18.7	(61)	30.7	(12.1)	sand tailings
170	16.7	(55)	25.1	(9.9)	sand tailings
171	17.3	(57)	26.9	(10.6)	sand tailings
172	16.7	(54)	25.9	(10.2)	sand tailings
173	19.0	(62)	24.6	(9.7)	overburden
174	19.3	(63)	26.7	(10.5)	overburden
175	21.3	(70)	27.7	(10.9)	overburden
176	21.3	(70)	31.2	(12.3)	overburden
177	19.0	(62)	23.9	(9.4)	overburden
178	22.3	(73)	25.4	(10.0)	overburden

Note: Selected 6 1/2 year-old trees were unforked and straight, with wood volumes substantially greater than the average for the strands.

(2) Induction of Rejuvenated Shoots

In order to capture the genotypes of adult trees which have demonstrated superior growth and/or special environmental adaption, it is necessary to induce shoots in cuttings which have reverted to juvenile growth physiology. This is necessary in order to achieve rooting of the shoots following multiplication in culture, or in cuttings to be established directly in the nursery.

Juvenile shoots (coppice) develop on the stump following felling of an adult tree, or in the region directly below a wound through the bark which circumscribes the bole. Epicormic or water sprouts appear on adult trees following certain types of trauma (e.g., fire or freeze damage, ref. Hartney, 1980). In addition, when an adult branch is grafted to juvenile rootstock, juvenile sprouts will emerge (Destremau et al., 1980). Reversion to juvenility can also be encouraged by spraying the developing coppice sprouts with a cytokinin solution (A. Franclet, Pers. Comm.).

(3) Stage I--Isolation of Axenic Cultures

The collection of explants from field-grown plants usually results in very high rates of Stage I contamination losses. By spraying the coppice shoots with a BENLATE or BENOMYL fungicide solution one to two days before collection of material (M. Boulay, Pers. Comm.; Franclet and Boulay, 1981) we have increased successful disinfestation of field-grown material from an expected 1-5% to more than 25% on average (Table 4). Material thus treated can be more easily established in culture free of associated microorganisms.

Coppice shoots are collected, held in water, and brought to the laboratory. Shoots are trimmed to approximately 3-6 cm above and below each node; and the leaves are removed, leaving the petioles attached. Twenty to thirty cuttings can be disinfested in approximately 500 ml of 100% laundry bleach (5.25% sodium hypochlorite) plus several drops of Tween-20 detergent as a wetting agent. Exposure times of 3-7 minutes, depending upon the diameter of the cuttings, have yielded successfully disinfested cuttings without extensive tissue damage.

TABLE 4

COPPICE NOTE CULTURES (*E. camaldulensis*)[1]

BESI #	Total Nodes Cultured	Dead or Contam. Cultures	Nodes with Developing Shoots		
			n	% of Total Cultures	% of Surviving Cultures
158	740	35	404	55	57
159	1279	150	369	29	33
160	431	91	106	25	31
161	224	81	23	10	16
162[2]	228	182	3	1	7
163	308	148	34	11	21
164	238	65	35	15	20
165	251	121	40	16	31

[1]Data incomplete for clones #166-178.

[2]Disingestation procedure for #162 modified, with resulting increase in contamination loss.

Following several rinses in sterile water, the cuttings are aseptically trimmed further to a final size of about 10 mm above and below the node and the petiole is cut to one-half its original length. The nodes are then planted in culture tubes containing Stage I medium (Tables 5 A,B). The charcoal is especially important to absorb phenolic compounds which are produced by the cuttings and released into the medium. An alternate medium (Franclet and Boulay, 1981) is composed of the minimal organic medium (Table 5 A) with naphthalene acetic acid (0.01 ppm), and benzyladenine (1 ppm). Axillary and proventive buds begin to develop after one week incubation in the dark (25-27°C). The culture tubes are then transferred to light (100-200 foot-candles, 16 hr/day) for further growth. These newly-developed shoots can then be cut and established on Stage II multiplication medium (next section).

Alternatively, cultures can readily be established from select seedlots (e.g., seed from controlled pollination or very rare seed) by disinfestation in 70% ethanol (30 seconds), 10% laundry bleach plus 1-2 drops Tween-20 (10 minutes), followed by 3-4 rinses in sterile deionized water. The disinfested seed is innoculated onto the minimal organic medium and allowed to germinate. In most cases, contamination microorganisms present will proliferate on this medium, thus revealing their presence; contaminated cultures are always autoclaved and discarded. Single-seedling clonal lines have been established from ten "premier mother tree" seedlots each of Eucalyptus grandis and E. robusta (T. Geary and G. Meskimen, USFS-Lehigh Acres, FL). These seedlots are listed in Table 6. Seedlings which are genetically deficient (e.g., albino, developmental mutants) are readily identified and eliminated at this stage (Table 7). The shoots of normal, healthy seadlings are used

to initiate State II cultures for multiplication. Note that the probability of obtaining a "select" or truly "elite" clone from seedlings is far lower than with clones isolated from select adult trees, and is dependent on the frequency of desirable genotypes in the seedlot used to establish the cultures.

TABLE 5A

MINIMAL ORGANIC MEDIUM COMPOSITION

Mineral Salts; modified from Murashige and Skoog (1962)

NH_4NO_3	20.6 mM	KI	5.0 µM
KNO_3	18.8 mM	H_3BO_3	100 µM
KH_2PO_4	1.25 mM	$MnSO_4$	100 µM
$CaCl_2$	1.0 mM	$ZnSO_4$	30 µM
$MgSO_4$	1.5 mM	$CuSO_4$	0.1 µM
		$CoCl_2$	0.1 µM
		Na_2MoO_4	1.0 µM
		$FeSO_4$	100 µM
		Na_2EDTA	100 µM

Organic Supplement

Nicotinic acid	1.0 mg/ℓ	Agar	6 g/ℓ
Pyridoxine HCl	1.0 mg/ℓ		
Thiamine HCl	10.0 mg/ℓ		
Myo-Inositio	100 mg/ℓ		
Sucrose	30 mg/ℓ		

pH adusted to 5.8

TABLE 5B

MEDIA FORMUALTIONS

Component	I	II[1]	IIB[1]	III
A. Mineral Salts	½x	1x	1x	½x
B. Organic Supplement	1x	1x	1x	1x
C. Hormones				
Naphthalene acetic acid	--	0.01ppm	0.01ppm	--
Indole butyric acid	--	--	--	1-2ppm
Benzylamino purine	--	0.01-1ppm	0.01 ppm	--
Bibberellic acid	--	--	1 ppm[2]	--
D. CharcoaL	1%	--	1.5%	--

[1]Franclet and Boulay (1981).

[2]Added prior to sterilization of the medium by autoclaving.

TABLE 6

EUCALYPTUS SEEDLOTS

BESI I.D. #	Eucalyptus Species	USFS-Lehigh Acres Designation
36	grandis	G90 '79
37	grandis	G905 '80
38	grandis	G987 '80
39	grandis	G999 '80
40	grandis	G1001 '80
41	grandis	G1002 '80
42	grandis	G1003 '80
43	grandis	G1010 '80
44	grandis	G1012 '80
45	grandis	G1020 '80
46	robusta	R192 '79
47	robusta	R397 '79
48	robusta	R437 '79
49	robusta	R400 '79
50	robusta	R416 '79
51	robusta	R458 '79
52	robusta	R464 '79
53	robusta	R2129 '79
54	robusta	R2246 '79
55	robusta	R2453 '79

TABLE 7

FREQUENCIES OF MUTANT PHENOTYPES IN SEEDLOTS OF *EUCALYPTUS* spp.

BESI #	Eucalyptus Species	n	ungermi-nated	Mutant Phenotypes		% deficient seed detected
				Chlorophyll	develop-mental*	
36	grandis	40	0	0	6	15
37	grandis	47	0	4	11	32
38	grandis	47	7	--	3	6
40	grandis	50	5	--	2	4
41	grandis	48	19	0	3	6
42	grandis	50	9	0	1	2
43	grandis	52	9	0	2	4
45	grandis	48	2	1	15	33
46	robusta	42	0	1	1	5
47	robusta	39	3	--	7	18
48	robusta	43	3	6	6	28
49	grandis	45	0	0	11	24
50	robusta	56	5	0	4	7
51	robusta	49	0	0	8	16
52	robusta	53	2	1	5	11
53	robusta	53	0	3	--	6
54	robusta	55	11	0	1	2
68	dalrympheana	48	14	--	--	0
69	gunnii	53	11	--	--	0

*Note: Development deficiences include incomplete germination, lack of root (surprisingly frequent), stunted or abnormal shoot growth.

(4) Stage II--Rapid Multiplication of Shoots in Tissue Culture

Media for proliferation of eucalypts by enhanced axillary bud multiplication have been developed in Australia by Hartney and Barker (1980) and by Franclet and Boulay (1981; Destremau, et al., 1980) at AFOCEL in France. A.N. Gonclaves (1979), and Cresswell and Nitsch (1975; Durand-Cresswell and Nitsch, 1977) have also described shoot multiplication media. In our hands, the AFOCEL media and procedures described by Dr. Michel Boulay (Pers. Comm.) have given high rates of shoot multiplication *in vitro*.

The Stage II multiplication medium is described in Table 5B. The concentration of cytokinin, benzylamino purine, is adjusted between 0.1 and 1 ppm to suit the individual clone. At this time it is advisable to index each line for the presence of latent microbial contaminants within the tissue (cf. Knauss, 1976). Failure to detect and eliminate contaminants at this Stage could lead to unwitting multiplication of the contaminant with the tissue until the microbe reaches a population density in the tissue sufficient to reduce multiplication rates and eventually result in collapse of the line.

Once the explants have been established in Stage II, free of associated fungal or bacterial contamination, shoot nodes are planted flat on the surface of the medium. If the shoots are stuck, basal end down into the medium, the base develops a callus and apparently does not transport water and nutrients to the leaves, resulting in defoliation of the explant. This is avoided by laying the small shoot flat on the surface or slightly embedded into the medium. Since contamination should be eliminated at this point, larger and more efficient culture vessels can be employed (e.g., baby food jars).

If the explant causes "browning" of the medium (phenolic production), the culture must be transferred to fresh medium. Incubation in the dark will reduce phenolic production and encourage elongation of the cultured shoots. Reculture of these etiolated shoots can then be accomplished without the "browning"problem.

The rate of shoot proliferation under these conditions is from 10- to 20-fold per month; these rates can produce a million-fold multiplication of shoots within half a year. The shoot explants usually require a month or so to become adapted to the culture environment, and to reach optimum multiplication rate.

Proliferation can be initiated by culturing explants on medium containing 1 ppm benzylamino purine; but the cytokinin concentration must be reduced to 0.1 ppm after 1-4 subcultures, according to individual clone. Otherwise the tissue becomes transparent, the leaves and stem glistening and brittle. This phenomenon has been referred to as "hyperhydry," "vitrification" or "waterlogging" (Debergh, et al., in press). Such shoots are unsuitable for rooting and acclimation to nursery conditions, but can be cultured on "elongation medium" to restore normal morphology (Table 5B, State IIB).

Monthly subcultures on State II medium with 0.1 ppm benzylamino purine yields a 10- to 20-fold increase in axillary buds formed. Cultures can be readily initiated by cutting the tissue quickly into 10 explants, each containing numerous axillary shoots. The explants will continue rapid growth on fresh medium unless the explants are cut too small. Below a critical explant size, the tissue undergoes an initial lag before resuming rapid growth. If the explants are cut smaller still, a size can be reached that never recovers from the lag

phase. The tissue grows out in all directions, including down into the medium.

(5) Stage IIB--Elongation of Shoots

To prepare shoots for rooting and subsequent acclimation to nursery conditions, they must be converted from the tightly branched, small fleshy leaf morphology (multiplication phase) to elongated shoots with lengthened leaves. This is accomplished on Stage IIB medium (elongation); similar to Stage II medium (multiplication) with 0.1 ppm benzylamino purine, plus 1 ppm gibberelic acid and 1.5% activated charcoal (Table 5B). After 15 to 20 days on this medium, 3 or 4 shoots per colony of buds will elongate with large, well-colored leaves. These can be cut and rooted.

(6) Stage III--Rooting of Shoots in Culture

The routine, reliable development of roots on cultured explants is usually the most difficult phase of any propagation program with woody shoots. The necessity of using juvenile (or rejuvenated) shoots for the initial explants has been discussed earlier (Section 3.2.B(2)). The Stage III medium necessary for rooting of in vitro, propagated, eucalypt, shoots is shown in Table 5B. The autoclave--sterilized medium is poured aseptically into presterilized EKCO aluminum tins (#788-35) and covered with sodium hypochlorite sterilized/sterile water rinsed) plastic dome lids (EKO #9088-10). Special care is required in handling these containers since the possibility of contamination is roughly proportional to their larger size, compared to tubes or baby-food jars. Cultures for collection of microcuttings should have

numerous elongated (1-3 cm) shoots. The shoots should bear more-or-less normal looking leaves. If the leaves appear succulent or otherwise abnormal, the successful establishment of viable plantlets for the nursery is doubtful.

Fifty microcuttings can be arranged in five rows of ten to fill the tins. Rooting begins after one week incubation in darkness. The plantlets are then given about 1,000-3,000 foot candles of fluorescent illumination, 16 hours per day. Roots appear within a week, and transfer to the nursery should take place soon afterwards.

(7) Stage IV--Acclimation of Cultured Plantlets to Nursery Conditions

Plantlets bearing small roots are removed from the culture vessel and collected in a solution of Benlate (Benomyl) at one-half the recommended concentration. Any adhering agar is removed and the plantlets are stuck in 128-cavity Todd planter flats (Model 150-5) containing a peat/vermiculite potting mix. The trays are held under heavy shade (85% during summer) and intermittant mist for one week. Shade can be removed gradually (e.g., 85%, 50%, 25% in weekly increments) until the plants can be transferred to full sun. After the first two weeks when roots are established, plants can be fertilized daily with a balanced nutrient solution (e.g., 20-20-20 at one lb/100 gallons). Survival rates over 80% were obtained with 1/3 of the first 33 clones tested; and even unrooted shoots have been established, although at a success rate closer to 50%.

(8) Nursery Stage--Production of Planting Stock

As expected the performance within the clonal lines can be very uniform, with striking differences noted between clones. The uniformity of the plantlets obtained from Stage III culture will largely determine the degree of uniformity in the nursery tray. A lack of uniform growth results in stunting of the weaker plants, and sometimes precocious branching among the larger plants. Under conditions of required uniform growth, the plants produce a single leader and, except for the lack of cotyledons, look exactly like a typical Eucalyptus seedling.

Figure 1 compares four rapidly-growing clonal lines with the growth of Eucalyptus seedling crops. Within two months the plants are 20-50 cm tall with a diffuse-fibrous root system. The design of the Todd planting tray prevents root spiralling by directing root growth downward to a drain hole where roots are air-pruned. Development of finished planting stock (about 10 cm) from seed normally requires 12 weeks (Eisenhart and O'Meara, 1980; see Figure 1, open symbols); the plants from tissue culture develop more quickly. It is possible that the 12-week nursery cycle for seedling Eucalyptus could be shortened by 2-4 weeks for tissue-culture plants.

(9) Field Evaluation of Clonal Lines

Vegetative propagation can reproduce the genetic qualities of the selected adult tree. Even so, field testing is necessary to confirm the genetic superiority of the clonal propagules and to eliminate the prossibility of microenvironmental effects on the observed performance of the original select tree.

Figure 1: Growth of Eucalyptus tissue culture clones and seedlings in the nursery

Eucalyptus grandis (•, ▲) and E. Robusta (■, ▼) clonal lines from tissue culture. Open symbols are Eucalyptus grandis and robusta 1977 seedling crops grown with Osmocote (O) or liquid fertilization ( ) by Herren Nursery, Florida Department of Forestry, Lake Placid, FL.

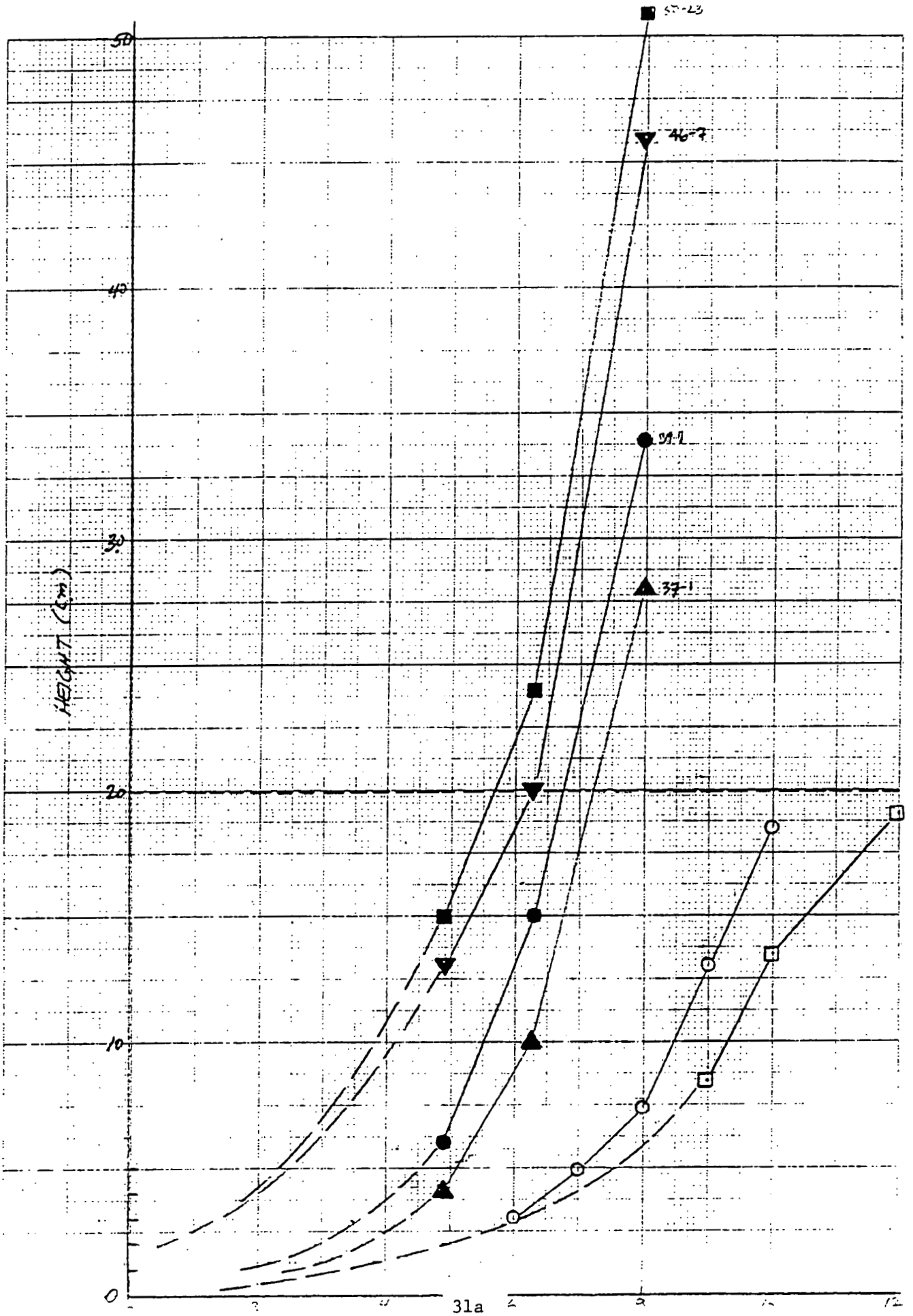

FIGURE 2: GROWTH OF *Eucalyptus* TISSUE CULTURE CLONES AND SEEDLINGS

The first estimate of biomass productivity can be obtained from evaluation of growth rates in the nursery. For example, spruce seedlings selected from the nursery bed continued to display superior growth for 18 years (Nienstaedt, 1981); and similar observations seem to hold for eucalypts showing vigorous juvenile growth (E.C. Franklin, Pers. Comm.). Predictive parameters which may lead to rapid selection techniques are being studied with Populus clones (Wray and Promnitz, 1976; Promnitz and Wray, 1976). The strongest correlation with ultimate biomass productivity is growth in the nursery or growth chamber, but these workers are attempting to refine the predictive reliability of this approach by including other biochemical, physiological and morphological measures. Ek and Dawson (1976) have described a computer simulation which allows projections of yields from very short rotation/very close spacing trails. All of these approaches have potential application to the evaluation of Eucalyptus clones.

The clones selected and propagated for field testing should be evaluated at least over the period 6-18 months. A Nelder plot will give an indication of the tree size vs spacing relationship. A second plot should be established for each clone to determine yields at the most probable spacing.

Clone failures are to be expected among vegetatively-propaged materials; but the occurrence of undesirable "c-effects" (clonal effects) demands that every clone be field-tested on the site of interest. Clonal variation will be seen in terms of optimal spacing for rotation age. That is, the effect of the transition from "free growth" to competition with adjacent trees.

Field testing also allows a determination of the range of site suitability for the superior clonal planting stock. In addition, it is possible to evaluate the following secondary criteria:

(i) phosphate sensitivity x possible counteractive treatments;

(ii) survival x planting season;

(iii) survival x herbicide treatment.

(10) Potential Improvements in the Technique

(a) Elimination of Stage III Cultures for Rooting

The basic procedure for *in vitro* propagation of *Eucalyptus* species described above includes three separate culture stages and media prior to acclimation of the propagules to nursery conditions. Under this protocol, every shoot produced in the multiplication phase (Stage II) must be transferred to a special medium for rooting (Stage III). If the need for this separate rooting step can be eliminated, the tissue culture cost per plant is reduced by almost one-half (e.g., Donnal, et al., 1978).

(b) Embryogenesis from Callus Suspension

The rapid clonal multiplication of *Eucalyptus* shoots *in vitro* yields a 10- to 20-fold increase per month by organ culture. However, a system of induced embryogenesis in callus cultures can improve this (already very good) multiplication rate another 10- to 100-fold (cf. Quan et al., 1980, 1981). Their recent report demonstrates the possibility of induced embryogenesis in *Eucalyptus* callus. The Chinese workers have succeeded in producing up to 200 embryoids per culture tube from seedling callus, and in establishing those regenerated plants in the field.

In should be pointed out that in this report seedling material was used, reemphasizing the need for juvenile or rejuvenated starting material for successful culture. New techniques are being developed for the rejuvenation of Eucalyptus (A. Franclet, Pers. Comm.). These techniques may be valuable in obtaining physiologically competent tissue for embryoid regeneration. Callus cells which regenerate via a developmental pathway nearly identical to normal sexual embryos result in complete plantlets--shoot and root. Thus the need for a separate rooting stage is eliminated, along with the associated costs. Instead the procedure is simplified to a callus proliferation medium and an embryogenesis medium.

(c) Somatic Cell Genetics

Finally, the availability of techniques to regenerate intact plants from cell culture opens the possibilities for in vitro somatic genetics. Following confirmation of plantlet regeneration from undifferentiated callus, we will initiate a series of experiments to induce and select genetically improved Eucalyptus clones. The general procedure involves (i) the establishment of cell cultures from which plantlets can latter be regenerated; (ii) mutation induction using radiation or chemical treatment of cell suspensions (cf. Howland, 1975; Howland and Hart, 1977); (iii) mutation fixation followed by culture under selective pressure to isolate the rare desired variants from the rest of the cell population (e.g., Maliga, 1978; Schieder, 1978; Carlson and Palacco, 1975); (iv) regeneration of plants from cells which survive the selection pressure; and (v) evaluation of the desired trait in the field. Plants more resistant to water stress can be obtained by culturing mutagenized cells on a high salt medium and generating plants

from the surviving cells, (e.g., tobacco--Nabors, 1976). Resistance to specific pathogen toxins would allow cells with mutation to this trait to survive on medium containing a normally lethal concentration of the toxin (e.g., Carlson, 1973). Plants able to grow on soils contaminated with toxic or inhibitory levels of specific minerals (e.g., phosphate) could also be selected in this manner. The isolation of haploid cell lines or pollen cultures (Hu Han et al., 1978; Reinert and Bajaj, 1977), would permit analysis of genetic load i.e., deleterious recessive genes usually masked by dominant alleles.

(11) Maintaining Genetic Fidelity in Culture

The principal of vegetative propagation is based on the assumption that all of the members of a clone faithfully reflect the genotype of the original select. this assumption is supported by the experiences in the Congo and Brazil with many millions of *Eucalyptus* rooted cuttings. These same data can be considered in the propagation of *Eucalyptus* shoots *in vitro*. The rational is that with organ (i.e., shoot) culture there is a constant selection from the normal pattern of elongation and axillary bud development. If callus and necrotic tissue are eliminated at each subculture, and subcultures are made frequently enough to avoid tissue senescence, then "sporting" (i.e., appearance of genetic variants) can be minimized or eliminated.

On the other hand, there are many examples in the literature of chromosomal abberrations in callus cultures. These examples however, are of callus growing on agar medium, and probably not transferred on a sufficiently frequent schedule. In any case, callus grows best at the periphery of the tissue mass and senesces at the interior.

The use of liquid suspension culture can eliminate or reduce this problem (Howland, unpubl. data).

(12) Contamination Losses

A potential risk in the tissue culture propagation is loss to microbial contamination. The presence of bacterial or fungal growth in the culture medium is often readily visible; and indicates that the vessel in question must be autoclaved and the tissue and medium discarded. More insidious is the latent contamination which is not usually visible, but can seriously reduce the rate of multiplication; and lead to losses at Stage IV (planting out). Culture lines should be indexed for the presence of contaminating microorganisms an early part of the State II multiplication. This test involves incubating tissue samples in microbial test media. These media are designed to reveal the presence of latent contamination, for example Erwinia carotovora in Dieffenbachia picta (Knauss, 1976; Knauss and Miller, 1978).

In general, the risk of contamination increases with the size of the explant (especially in Stage I) and the size of the vessel. Other sources of contamination include poor transfer technique, poor personal hygiene, ineffective sterilization of medium or dissecting instruments, dust and strong air currents, leaks in the laminar flow hood filter, escape of spores from contaminated vessels, or unregulated traffic in the laboratory area. Mites, thrips, ants or other small insects can crawl into the culture vessels, carrying with them bacterial and fungal contaminants which bloom in the enriched medium a day or so after the visit.

In the case of mites, the nearly visible animals reproduce and spread infection in a wave that appears a day or so after the mites have infected the next row of culture tubes. Mites have destroyed thousands of valuable cultures in numerous tissue culture labs. Miticide can be sprayed monthly in the culture room--on the vessels--to preclude mite infection.

Any economic analysis must acknowledge a certain loss to contamination; however, if the loss exceeds more than a few percent of the culture inventory, it is advisable to examine and perhaps modify laboratory practice.

3.3 Production and Economic Analysis

The most importants variable in evaluating the production costs for tissue culture propagation are:

(i) Rate of multiplication in Stage II

(ii) Rate of successful rooting in Stage III

(iii) Rate of successful transfer to Stage IV

(iv) Rate of loss in Stage II

(v) Rate of loss in Stage III

A. Cost Estimates for Tissue Culture Propagation

Costs associated with the preparation, transfer and incubation of cultures are largely independent of the crop being propagated, and relate most directly to the type of culture vessel being used. In this section we will consider three types of culture vessel: 25 x 150 mm culture tubes, baby food jars, and aluminum food service tins.

The cost of Stage I culture is not considered in detail here, since it does not significantly effect the cost of large-scale propagation (cf. Hartman, 1979). For example, even if a coppice culture costs two dollars and 1000 cultures must be done to establish one successfully in Stage I, this expense is amortized over the period of clonal propagation to 100,000 plants.

eg. 100 coppice node cultures @ $.....$2.00
1 successful.....$2.00/initial culture
Amortized over 100,000 plants in 6 mo....$0.002/plant

Estimates of labor time are based on the assumption that individual employees will be devoted exclusive to washing vessels, to preparing and dispensing media, or to innoculation of cultures.

(i) Stage II multiplication--25 x 150 mm culture tube basis

Cost per tube + cap use.....0.28¢/tube
(assume 12¢/tube, 5¢/cap; 60 uses)

Washing.....1.50¢/tube
(assume mechanical washing, 400 tubes + caps/hr; $6/hr labor including wages, supervision, insurance, overhead, etc.)

Medium cost.....6.00¢/tube
(assume $4/liter; 15 ml/tube)

Medium dispensing and capping.....0.50¢/tube
(assume simple automation, 20 tubes/min or 1200 tubes/hr; $6/hr labor)

Innoculation space rent.....16.22¢/tube culture cycle
(assume laboratory mortgage, insurance, maintenance, utilities, administration, and overhead expenses allocated to usable culture room shelf space-- $9.73/sq. ft./month; 1 month culture cycle for eucalyptus; 60 tubes/sq.ft.

Total cost/tube culture cycle.....32.5¢

For a 10-fold multiplication rate with Eucalyptus shoot cultures, the cost of production in culture is 32.5¢ ÷ 10 = 3.25¢/explant.

(ii) Stage II multiplication--baby food jar basis

Cost per jar + cap use.....0.58¢/jar
(assume 5¢/jar, 30¢/Magenta cap; 60 uses)

Washing.....3.33¢/jar
(assume mechanical washing; 180 jars + caps/hr; $6/hr labor)

Medium cost.....10.00¢/jar
(assume $5/ℓ; 25 ml/jar)

Medium dispensing and capping.....1.00¢/jar
(assume simple automation, 10 jars/min, 600 jars/hr; $6/hr labor)

Innoculation.....24.00¢/jar
(assume 3 explants/jar; 75 explants/hr--25 jars/hr; $6/hr labor)

Incubation.....38.924/jar culture cycle
(assume $9.73/sq.ft. shelf space/month; culture cycle for eucalypts; 25 jars/sq.ft.)

Total cost/jar culture cycle.....77.83¢

Assuming a 30% increase in yield over the multiplication rate in culture tubes, the cost of multiplication in baby food jars is: 77.83¢ ÷ (1.3 x 10 x 3 explants) = 2.00¢/explant. (The increased cost effectiveness of jars vs tubes is apparent. Culture tubes will not be considered further.)

(iii) Stage IIB elongation--baby food jar basis

Cost per jar + cap use.....0.58¢/jar

Washing.....3.33$/jar

Medium cost.....10.00¢/jar

Medium dispensing and capping.....1.00¢/jar

Innoculation.....24.00¢/jar
(assume 3 double-size
explants/jar; 25 jars/hr;
$6/hr labor)

Explant cost.....12.00¢/jar
(assume 2.00¢ x 2/double size
explant, 3/jar)

Incubation.....19.46¢/jar
(assume two week cycle for
elongation of Eucalyptus
shoots)

Total cost/jar culture cycle.....70.37¢

Assuming about 30 elongated shoots are produced/jar, the cumulative cost of elongated shoots is 70.37¢ ÷ 30 = 2.35¢/shoot.

(iv) Stage III rooting--EKCO tin basis

Cost per EKCO tin + lid.....$0.191/tin
(used only once)

Sterilizing plastic lids.....$0.10/lid
(assume 50 lids/5 min;
$6/hr labor)

Medium cost.....$0.400/tin
(assume $4/liter; 100 ml/tin)

Medium dispensing and "lid-ing".....$0.030/tin
(assume manual operation, 200
tins/hr; $6/hr labor)

Innoculation.....$2.000/tin
(assume 50 shoots/tin; 150
shoots/hr; $6/hr labor)

Cost of shoots.....$1.175/tin
(assume 50 shoots/tin; 2.35¢/shoot)

Incubation.....$1.946/tin
(assume 1 week (dark) plus
1 week (light) for rooting
of Eucalyptus shoots; 2.5
tins/sq.ft.; $9.73/2
cost/sq.ft./2 wk.)

Total cost/tin culture cycle.....$5.752

Since there is no multiplication in this culture stage, the total cost (including the previous multiplication and elongation stages) is: $5.752 ÷ 50 - $0.1150/rooted plantlet.

(v) Stage IV--Speedling planter cavity basis

Cost of sterile Speedling mix per cavity.....0.837¢/cavity
(assume $15/bag to fill 14 Model #150-5
Todd planting flats of 128 cavities each;
trays and filling labor included in
grower cost)

Cost of plantlet.....11.50¢/cavity

Transplanting.....1.750¢/cavity
(assume 300 plants/hr; $5.15/hr labor cost)

Growing.....5.00¢/cavity
(assume a 12 week nursery cycle; grower's
cost includes greenhouse rental at 90,000
plants volume, plant maintenance, watering,
fertilization, necessary pest and pathogen
controls and profit)

Total cost/field-ready Eucalyptus clone.....18.91¢

No loss estimates were included in the cost analyses above; but it is certain that losses will occur due to contamination, breakage, equipment failure and human error. Table 8 lists the cost estimates and an estimate of percent potential loss at each state of propagation.

TABLE 8

LOSS ESTIMATES AND REVISED COSTS

Propagation Stage	Calculated Cost	Estimated Loss	Revised Cost
II--Multiplication	2.00¢/explant	5%	2.11¢
IIB--Elongation	2.35¢/shoot	5%	2.49¢
III--Rooting	11.50¢/plantlet	10%	12.94¢
IV--Acclimation & Nursery Growth	18.91¢/plant	15%	23.94¢

Note: Revised Costs reflect the cumulative impact of loss estimates at previous stages.

B. Economic Impact of Technique Innovations

(i) Elimination of State III Culture

There are numerous points in the tissue culture production of a field-ready Eucalyptus plant which might be modified to increase productivity and/or reduce costs. These will be addressed in order of potential application and magnitude of the cost benefit. From the detailed cost analyses in the previous section, it is apparent that labor is the largest single factor in determining plant cost (Table 9). In the commercial plant tissue culture industry, labor averages about 60% of total costs (R. Strain, Pers. Comm.). Donnan et al. (1978) have illustrated the cost benefit of eliminating Stage III rooting of Ficus and Begonia in vitro by direct rooting of "microcuttings" produced in Stage II multiplication. If possible with Eucalyptus, direct rooting of elongated shoots in Stage IV would substantially reduce the estimated cost for each plant transferred to Stage IV. Additional benefits include the reduction in laboratory space and facilities needed for Stage

III cultures, and avoiding risk of contamination loss in Stage III tins. Although this latter benefit may be partially off-set by increased losses in Stage IV, failures of unrooted "microcuttings" would have to exceed 50% before a net cost disadvantage were reached. (See also Debergh and Maene, 1981).

Table 9

LABOR COSTS

Propagation Stage	Labor Cost/Item Produced	% of Cost/Item
II	0.65¢/explant	31
IIB	0.88¢/shoot	35
III	4.49¢/plantlet	35

(ii) Reduction in the Nursery Cycle

The normal 12-week nursery period required for Eucalyptus seedling development (Eisenhart and O'Meara, 1980) may not be necessary for plantlets produced in vitro. Seed germination and growth to 3 cm may take from 3-6 weeks; but Eucalyptus propagated in vitro begins the nursery cycle at that size (Section 3.2.b(8), Figure 2). In the present analysis, the growing cost (excluding plant and planting medium) is 5¢/plant/12 weeks. Reducing this period by 4 weeks would save 1.67¢, thus reducing the per plant cost from 23.94¢ to 22.27¢.

(iii) Fluid Drilling in Stage IV

A recent development in the planting of certain vegetable crops is the encapsulation of pregerminated seed in a gel matrix and

automated injection into the planting site. The gel can be formulated to include nutrients and chemicals (e.g., fungicide) to enhance the rapid establishment of the seedling (cf. Evans et al., 1981). If it is possible to apply this technique to microcuttings of Eucalyptus shoot cultures, high speed planting of nursery trays could be accomplished at a fraction of the Stage IV transplanting cost. In addition, the method would require the smallest sections of Stage II explants, reducing the plantlet cost from 12.94¢ (For Stage IIB shoot) to less than one cent (for 10-20% of a Stage II explant). This would translate into a final plant cost of less than 10¢. The availability of a somatic embryogenesis system in Eucalyptus (Quan et al., 1981) may provide the ideal source of material for fluid drilling.

(iv) Automation of Stage II Subcultures

Given the nature of Eucalyptus shoot culture, mechanical cutting of explants may be possible. The profusely branched colonies of buds are best divided for subculture by rapid cutting into ten explants; it is unnecessary to carefully dissect the shoots (Section 3.2.B(4)). By designing a machine to chop and dispense explants, Stage II culture productivity could easily increase 20-fold, reducing the cost to half of its presently estimated value.

The possibility exists that this technique could also be combined with the fluid drilling concept mentioned above, and applied to production of Stage IV explants.

C. Comparison of Cost and Value with Current or Potential Sources of Eucalyptus Planting Stock

Eucalyptus planting stock for commercial forestry is derived exclusively from seed in Florida. The Florida Division of Forestry has provided up to 1- to 2-million seedlings per year at a cost now of about $0.085 each. The production cost at the Division's Herren Nursery (Lake Placid) is very low due to the use of convict labor; and seed orchard maintenance and seed preparation costs are carried by the U.S. Forest Service and not included in the price. The seed for Herren Nursery production is provided in cooperation with the U.S. Forest Service's Eucalyptus program at Lehigh Acres, Florida. Although the select seed orchards now developed have yielded substantial improvement in seedling qualities, there is not enough improved seed to satisfy recent demand for planting stock. There would not be enough seed and seedlings to meet the rapid acceleration in demand anticipated for the installation of the BESI energy plantations. Consequently, seedlots provided by the U.S. Forest Service from "premier mother trees" have yielded up to one-third genetically deficient seedlings (Section 3.3, Table 7). Eucalyptus planting stock in California is 25¢ to 30¢ per plant (T. Geary, Pers. Comm.).

As discussed in Section 1.0, vegetative propagation of select adult trees allows a field-tested genotype to be multiplied for clonal planting. Planting stock propagated vegetatively from "elite" Eucalyptus trees (e.g., F1 hybrids) can readily double forest stand productivity in a single step. Consequently the value of clonal planting stock is closely tied to the quality of the tree supplying the vegetative propagation material. Assuming careful selection, this value is easily twice that of seedlings. Besides the increased volume,

usually a primary selection criteria, clonal plantations offer additional benefits:

improved average volume per tree (2- to 4-fold);
uniform improved form, reducing harvesting cost;
uniform response to fertilization; and
uniform tolerance to herbicides.

On the basis of these considerations, one can readily justify a cost per clonal plant which is twice that of seedling material, or more.

D. Laboratory Facilities, Equipment and Personal Requirements

The production of 7.5-million tissue cultured eucalypts, spaced over 10 months each year, involves the propagation, elongation, and rooting of shoots in the laboratory (Stages II, IIB, and III), followed by acclimation to nursery conditions and growth to planting size (Table 10). The basic facilities and equipment are listed:

(1) Glassware cleaning room, 15 m^2 (160 ft^2)

diswasher, fast-cycle restaurant type

water purifier

2 small autoclaves (for contaminated vessels)

(2) Media preparation and sterilization room, 15 m^2 (160 ft^2)

100 liter (26 gallon, stainless-steel, food-service vessel with mixing paddle and heater

pH meter and electrodes

balances, general duty and analytical

laminar-flow sterile work station

media dispensing pump

2 large capacity autoclaves and steam generator

separate ventilation and air conditioning system for media preparation and glassware rooms

(3) Storage room, 10 m^2 (108 ft^2)

(4) Cold room, (2-4°C, 36-400°F), 5 m^2 (54 ft^2)

shelving for cold storage of supplies and short-term preservation of culture lines

(5) Transfer room, 185 m^2 (2,000 ft^2)

20 laminar-flow sterile work stations

20 headphones, audio jacks with program selection

stereo microscope

(6) Culture rooms, 5 each, area 120 m^2 (1290 ft^2)

360 m^2 (3850 ft^2) shelf area, 5 levels; 80% utilization

shelf lighting with externally-mounted ballasts

shelf ventilation ducts

air-conditioning system with filtration and prositive pressure, not interconnected with other laboratory areas

temperature alarm system

gyrotory shaker

roller drum for culture tubes

(7) Research laboratory, 15 m^2 (160 ft^2)

laminar flow hood

microscope

pH meter

balances

centrifuge

incubator for indexing cultures

TABLE 10

PRODUCTION OF 6-MILLION *Eucalyptus* TREES PER YEAR

Culture Stage	Monthly Activities[1,2]	Months	Growing Space	Personnel[4]
II	468 jars			
	⇓ (1 mo) -10%[3]	Oct-Jul		0.4
II	5570 jars + 570 jars			
	(1 mo) -5%		18 m^2(195 ft^2)	
II	5292 jars			
				2.1
IIB	17,200 jars			
	(0.5 mo) 17,200 jars -5%	Nov-Aug	51 m^2(550 ft^2)	
IIB	32,682 jars			
				30.0
III	9,800 tins			
	(0.5 mo) 9,800 tins		291 m^2(3100 ft^2)	32.5 innoculators
	-10%		360 m^2(3845 ft^2) culture room area	
III	17,648 tins			
	⇓	Dec-Sep		13.6
IV	882,418 plantlets			greenhouse workers
	(3 mo) -15%		1280 m^2(13,770 ft^2)	
			1280 m^2(13,770 ft^2)	
Nursery	750,055 trees		1280 m^2(13,770 ft^2)	
	⇓	Mar-Dec	3840 m^2(41,300 ft^2)	
	861 acres		greenhouse area	

Note 1: Production of 600,000 trees/month, ten months per year.
Note 2: Single arrows (⟶), incubation steps, double arrows (⟹) transfer steps.
Note 3: Negative % associated with incubation steps indicate allowances for losses.
Note 4: Personnel figures include no supervisory or support staff.

(8) Offices and reception area, 45 m^2 (480 ft^2)

computer

viewing windows to culture room and work areas

(9) Lunch/seminar room, 22 m^2 (240 ft^2)

(10) Toilets, 2 each M/F, 25 m^2 (270 ft^2)

(11) Service and maintenance room, 15 m^2 (160 ft^2)

ballasts for culture room

air-conditioning equipment

water heater

sink

Total laboratory, 592 m^2 (6,370 ft^2), ca. 80'x80'

Equipment cost estimate, $150,000± 30%

Assuming a construction cost of $50/ft^2$ for the laboratory ($320,000) plus the equipment costs ($150,000), amortized over 10 years and allocated to an annual production of 7.5-million plants, capital investment adds about $0.01 to the cost of each plant.

A research greenhouse (111 m^2; 270 ft^2) located with access to the research laboratory and designed to facilitate sanitization, would be equipped for development of Stage IV acclimation of cultured plants, and for preparation of plants for Stage I introduction into culture.

The floor plan of the tissue culture laboratory should be designed to prevent casual traffic in the culture room area, thus reducing contamination risk. Henney, et al. (1981) recommend that access to the culture room be provided that avoids transit through the glassware cleaning, media preparation, and transfer rooms. Entrances to the work areas should be kept to the minimum required for efficiency and safety, and should each be equipped with an entry enclosed by exterior and

interior doors. The use of an adhesive mat (e.g., "tacky mat," "Rhyno mat") will help to reduce the introduction of contaminants on shoe soles; however, requiring all individuals who enter to remove his or her shoes is the safest policy. Workers should be supplied with comfortable shoes or slippers that do not collect dirt (i.e., no "fuzzy bunny" slippers); these "lab shoes" are never to be worn outside the laboratory.

Personnel required to operate a 7.5-million plant per year Eucalyptus propagation laboratory includes the following:

Glassware Technician.....1

(Assume uncapping, dumping medium, loading jar on washrack at 30 jars/min, 1800 jars/hr; fast-cycle restaurant dishwasher, 40 jars/min, 1600 jars/hr; 8 hr/day x 5 days/wk x 4.33 wk/mo = 173.2; 33,000 baby food jars/mo ÷ 1600 jars/hr - 21 hr/mo; allows 6+ hr/day for cleaning floors, changing air filters, replacing lamps, etc.)

Media Technician.....1

(33,000 jars/mo ÷ 600 jars/hr = 55 hr/mo; 15,600 tins/mo ÷ 200 tins/hr = 78.3 hr/mo; total, 133.3 hr/mo; 1.5 hr/day for clean-up, misc. duties)

Transfer Technicians (Cutters).....32.5

(see Table 10; assume 2 shifts/day; 33,000 jars/mo ÷ 75 jars/hr = 440 hr/mo; 15,660 tins/mo ÷ 3 tins/hr = 5,220 hr/mo; total, 5,660 hr/mo = 173.2 hr/mo = 32.5)

(Technicians' wages considered as "allocated cost" in analyses presented earlier--Section 3.3.A.)

Shift Supervisors.....2

Assistant Shift Supervisors.....2

Laboratory Manager.....1

Equipment Maintenance Repair.....1

Bookkeeper-Receptionist.....1

(Supervision + overhead salaries--$10,000± 20% per month, about 1.7¢ per plant.)

4.0 SUMMARY AND CONCLUSIONS

The increased productivity of clonal hybrid Eucalyptus plantations when compared to seedling stands (e.g., Martin and Quillet, 1974a; Ikemorei, 1975) is an essential requirement for the economic feasibility of a large-scale, wood fuel/feedstock industry in Central Florida. Although projections of actual yields cannot be precise, it is certain that careful selections coupled with vegetative propagation will allow the most rapid increases in productivity possible.

Vegetative propagation of select eucalypts can be accomplished at commercial scale either by the rooted cutting method (Martin and Quillet, 1974a,b,c,d; Ikemori, 1975), or by a tissue culture method (cf. this report). Comparative estimates of these two methods (Table 11) indicate the superiority of vegetative propagation by tissue culture.

Current efforts are being directed toward standardization of tissue culture production, and development of more efficient methods. Under present conditions, the plant tissue culture laboratory associated with Biomass Energy Systems, Inc. could produce up to 50,000 clonal eucalypts per month. Potential improvements in efficiency would increase our present production capacity by 10-fold. But even if none of the innovations mentioned are possible, the present study shows that high-quality, vegetatively-propagated Eucalyptus planting stock can be produced in the volumes required for large-scale plantation establishment. In addition, when the 2- to 3-fold increase in productivity is considered, tissue cultured eucalypts are comparable to the artificially low cost of seedlings currently available in Florida.

Table 11

COMPARISON OF PROPAGATION BY ROOTED CUTTINGS
VS TISSUE CULTURE

	Rooted Cuttings	Tissue Culture
cost per individual planting stock	30¢-45¢	30¢
time required to expand clone to 250,000 plants	4 years	½ year
form of planting stock	often branched, requiring pruning before planting	single axis, seedling-like form
uniformity of planted strands	excellent	excellent
availability of genetic improvement approaches (i.e., induced mutagenesis)	not feasible	yes

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