

Micropropagation of Frost-Resistant *Eucalyptus*¹

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For 10 years now, AFOCEL has inscribed micro-propagation of forest trees into its research and development program of intensive silviculture.

Eucalypt is a genus which is studied for 6 years. After research on micropropagation of very juvenile trees (seedlings) our association has tried to develop the method for mature trees selected in nursery, experimental plots or plantations.

Numerous workers have shown that vegetative propagation of eucalypt was possible at the industrial level using physiologically and onto genetically mature plants. This kind of propagation needs the use of rejuvenated shoots which can be obtained by intense pruning, grafting and successive graftings (with a great attention to take cutting or graft on the precedent generation as soon as possible) or cutting back. HARTNEY published a detailed review on this topic.

Under french climate, the use of such methods as cutting back (which is very efficient in Congo or Brazil) has numerous drawbacks

- important variability (even for small climatic variations) in a few days of the rooting ability of the cuttings which have been obtained from sprouts, pruned trees or cuttings from the preceding generation. Such phenomena involve a number of complications in the organization of the supply of cuttings even if cuttings from the preceding generation are used ;

- low productivity by unit area of mother tree orchards in cuttings of good quality which increases the cost of cuttings ;

- important difficulties to overcome problems linked with rejuvenation by successive graftings ;

¹Presented at the Workshop on Eucalyptus in California, June 14-16, 1983, Sacramento, California.

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Abstract: A method for the in vitro propagation of frost resistant eucalyptus is presented. It was used for the propagation of 2-30 years old trees. This method is presently used for the fast production of mother trees from selected trees.

- very low multiplication coefficient of vegetative propagation on such material which involves a long period between the selection of the tree and the disponibility of the genotype for reforestation.

Such difficulties always present in our two nurseries which are involved in vegetative propagation are the main reason for our interest in developing an in vitro method for supplying reforestation plots or industrial nurseries.

Micropropagation of eucalypts is not new. After the first pioneer works of DURAND-CRESSWELL, numerous researchers, de FOSSARD, HARTNEY, LAKSHMI et al, developed and applied the method to different species. A recent paper by DURAND-CRESSWELL et al gives all indications on the question.

At AFOCEL, research has begun in 1976 and several communications, have been done : LODEON, FRANCKET and BOULAY, DEPOMMIER. In this paper we will present the more recent results which have been obtained on in vitro cloning of selected trees two years old or older.

PLANT MATERIAL

Taxonomic entities of eucalypts which can resist to frost to minus 18°C or 20°C or to late spring frost in France are very rare. They are essentially species from tasmanian mountains, as Eucalyptus gunnii and Eucalyptus coccifera or from australian south eastern mountains as Eucalyptus pauciflora (Eucalyptus niphophila). Species from good forest value like Eucalyptus dalrympleana or Eucalyptus delegatensis have shown in trials which have been planted earlier in France irregular success.

Actually, we have now in France three kinds of plant material

- category A : trees of good forest value which have resisted the "historic frosts" from 1956 and 1963. These trees are over 20 years old ;

- category B : trees from 5 to 15 years old which have been planted in AFOCEL or INRA³ collections. These collections are provenance trials with Eucalyptus dalrympleana or Eucalyptus delegatensis or progeny tests with off springs from category A trees. Many hybrids have been observed among these progenies. This lets us assume that the mothers may be hybrids.

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- category C : young vigorous seedlings selected in progeny or provenance trials and raised in big containers. This kind of culture allows to submit them to natural frosts for selection purpose, according to a method imagined by LACAZE and realized by DESTREMAU and CAUVIN and MARTEN. This selection for two criteria (juvenile growth and frost resistance) on seedlings 2 or 3 years old, give clones from which scions or sprouts can be taken after cutting back. Later with progress in artificial hybridization such seedlings will be replaced by controlled hybrids of the same age and selected in the same way.

For most of these selected trees, it is difficult to undertake vegetative propagation without rejuvenation. For this reason, mobilization of the selected genotype by graft or cutting (from sprout or from graft) is necessary.

Rejuvenation can be obtained totally or partially in vivo by the means of successive graftings onto young seedlings. In any cases, the in vitro primary cultivation is possible only with this kind of material (cuttings, sprouts and more frequently grafts). The mother trees obtained can be cultivated under good nutrient conditions and a great attention is paid to the sanitary status of the plant (spraying with systemic fungicides every two days specially the week before primary culture). When sprouts are used (category C), they are also treated with fungicides. In vivo and in vitro introduction is done very soon after cutting the sprouts.

IN VITRO MICROPROPAGATION

General conditions

Culture media which are used, have been established by de FOSSARD, DURAND-CRESSWELL et al, LODEON, FRANCLLET and BOULAY and DEPOMMIER.

Three kinds of medium are commonly used

- multiplication medium : M
- root induction medium : RI
- root and stem elongation medium .. : RA

For all these media the pH is 5,5 - 5,6 before sterilization. The agar concentration is 7 gl⁻¹. The sterilization occurs at 115°C-116°C during 25 minutes. After plantation, cultures are put under controlled environment with the following conditions

- photoperiod ; long day D : 16 h ; N : 8 h
- thermoperiod ; D : 24°C-25°C ; N : 18°C-20°C

Primary culture

After severance, the shoots are sealed at both ends with paraffin and treated with a disinfectant. Calcium hypochloride is commonly used (filtration of a mix containing 10 percent solid solution) but a twofold dilution of a commercial solution of sodium hypochloride is also efficient.

Once planted on agar medium, cultures (nodes + petiole + piece of leaf) are put one week in the dark. This operation is done to limit the formation of brownish compounds in the culture medium.

If we start with sprouts (explants from category C of plant material) a 30 to 40 percent infection is generally obtained with primary culture. With plant material coming from greenhouse conditions (mobilization or successive grafts or cuttings) it is necessary to decrease the time of disinfection (3 to 5 minutes instead of 8). Under such conditions, the percentage of infection varies from 10 to 50 percent.

The primary culture medium is comparable to the multiplication medium M. The composition is the following : half concentration of macronutrients of Murashige and Skoog medium and vitamins from de Fossard. The sugar is sucrose at 30 gl⁻¹.

The difference with M medium is only at the level of cytokinin concentration. B A (benzyl adenin) is used at a concentration varying from 0,1 mg l⁻¹ to 1 mg l⁻¹, depending upon clone, season of cutting and lignification of stems. In every case, auxin concentration (N A A : naphtalen acetic acid) is always 10⁻² mg l⁻¹. Other medium have also been used for this first culture, some with activated charcoal for example, give also good results.

The experimentation on primary culture with more than 25 clones, has shown that several problems arise.

- There is a very small percentage of explants which are able to react (reaction means in vitro development of the axillary buds which are present at the basis of petiole) with regard to the number of introduced uninfected explants (about 2 to 40 percent of living explants react). So, the "vitro-plants" obtained from in vitro culture are coming from a very small number of cultures (Table 1).

The limitation of experimentation at this level is due to the small number of rejuvenated explants obtained after mobilization or successive graftings or cuttings for each genotype (20 to 30 nodes are often available for this stage of culture).

- A large number of explants show also a friable brownish callusing place of budding reaction. To solve this problem frequent subculturing every 15 days from the first 2 to 3 transfers are needed.
- Hyperhydric transformation (water-logging) can also occur at this level of culture on the axillary bud developed in vitro.

These different problems are presently partially solved by frequent subculturing, decreasing cytokinin dosis, use of activated charcoal and adjustment of the time of disinfection.

Subculture and in vitro cloning

The primary culture leads when the explant reacts to elongated axillary buds. Two to four axillary buds can be obtained by reacted nodes. The separation of these axillary buds developed in vitro from the initial explant occurs progressively. Hormones (cytokinin and auxin) are necessary to obtain axillary budding. The best balance is : B A 0,2 mg l⁻¹, N A A 0,01 mg l⁻¹.

The propagation process is comparable to that of de FOSSARD et al. Cluster of buds are obtained by axillary budding. Multiplication is obtained by cutting these bushy explants in smaller pieces every three to four weeks. The time between two subcultures is a very important parameter. For maintaining a very active culture, four weeks is a maximum delay.

In subculture, during the, first transfer, all clones do not react in the same way at the multiplication level.

Figure 1 shows the evolution of six clones after five transfers. They are among the first selected clones introduced in vitro.

In transfer five, some of the clones have multiplied by 49 the initial number of introduced cultures (clone 041), some others show no evolution but stay living (clones 032, 039). With these last clones, we can obtain after eight to twelve transfers, a multiplication coefficient comparable to the first one. Depending upon the clone, there is different responses to the medium M, but this difference disappears progressively with subculturing once primary culture is successful.

An example of the delay to obtain exponential multiplication is given by the clone 16. This clone comes from a 30 year old selected mother tree. It has been introduced in vitro by taking an explant from the fourth successive grafting. On figure 2, we can see that this clone has been maintained just living to the eighth transfer. The number of copies from the ninth subculture onward from this clone is increasing, exponential multiplication is obtained for transfer 12.

We don't know actually what are the reasons responsible of such a phenomenon : stem selection for their sanitary qualities, modification of endogenous hormonal equilibrium, habituation to the medium, none of the experiments we conducted could permit us to choose between these various hypotheses (which are not necessarily exclusive).

Under exponential multiplication conditions, with our method, the multiplication rate amounts to about 4 to 5 for each subculture and every three weeks.

Rhizogenesis, root and stem elongation, acclimatization

With the propagation medium M, we obtain stems 10 to 20 mm in height which are rooted and grown in two steps

- root induction on R I auxin containing medium ;
- root and stem elongation on R A medium with activated charcoal and no auxin.

Using an elongation medium after propagation as first recommended was leaved for the above described method.

Rhizogenesis induction

The culture medium used is the Knop medium full strength [sic] for macronutrients, micronutrients and vitamins are similar to that of M medium except I K and riboflavine which are eliminated from the medium. Sugar (sucrose) is often used at 15 gl⁻¹. Two parameters are modified according to the clone : length [sic] of dark period and auxin concentration of the medium (I B A).

A dark period is used during root initiation because it has a positive effect on root primordia formation (DRUART et al),

Just after subculturing on R I medium cultures are put for 10 to 20 days in the dark. Concerning the rhizogenesis hormone, we use I B A (indol butiric acid) added to the medium at 1,5 mg l⁻¹ or 3 mg l⁻¹. The best combinations which are used for different clones (i.e. hormone concentration, sucrose dosis, length [sic] of dark period) are indicated in table 2. The rooting percentage often exceeds 60 percent. The time on multiplication medium M before rhizogenesis has a great influence on rooting success. Stems put in induction must be taken after no more than 3 to 4 weeks in culture on medium M to get a good rooting percentage and few apical necrosis.

Figure 3 shows the rooting cinetic for the clone 047 (hybrid between gunnii and globulus). After 25 days on R I medium, about 80 percent of plants are rooted.

Root and stem elongation, acclimatization

Once stems are been induced, or when root primordia develop, rooted stems are cultivated on R A medium which has the same composition than R I but with activated charcoal added and without auxin. The sucrose concentration: is increased to 30 gl⁻¹.

Subculturing on R A medium favours the development of roots which were induced on R I medium. Within 2 to 5 days, roots reach the bottom of the test tubes. 10 to 20 days later, ramifications and secondary roots appear. After root elongation, herbaceous stems begin to grow (15 to 30 days after transplanting) and the rooted stems develop leaves

and nodes which are quite similar to those of seedlings. Rooted stems at this moment can be let during 4 to 6 weeks on this R A medium.

The medium R A has several advantages:

- At the production level, it allows the nurseryman to group the transplanting operation which he carries out once a week.
- The media allows also the regeneration of the herbaceous stem after necrosis. Such neoformed stem grows from basal axillary buds of the explant after death of the stem put in rhizogenesis induction.
- The morphology of the rooted stem obtained in vitro on R A medium makes also acclimatization easier.
- Acclimatization takes place when rooted stems have a height compatible with culture in MELFERT⁴ containers. It means 30 to 50 mm. After transfer in such containers during 10 or 30 days depending upon the season (10 days in spring or summer, 30 days in winter), the young plantlets are put under plastic film confinement. When roots appears on the side of the containers, confinement is progressively removed and plantlets are placed in greenhouse for cultivation. Percentage of success in acclimatization are variable from 30 to 100 per cent depending upon clone and season.

DISCUSSION AND PROBLEM OCCURRING WITH THIS METHOD

The scheme of this method is given in figure 4. We can see four phasis and a preparatory one which is the following

Preparatory phasis : tree selection, and collection from this one of cuttings, sprouts and grafts after mobilization or successive grafts (rejuvenation). Cultivation of this material under greenhouse conditions.

Phase I : obtention of axillary budding in vitro ;
Ia : primary culture ;
Ib : obtention of a reactive culture with experimental multiplication by axillary budding ;

Phase II : in vitro cloning ;

Phase III: obtention of rooted plantlets ;
IIIa: rhizogenesis induction ;
IIIB: root and stem elongation ;

Phase IV : acclimatization of in vitro rooted plantlets.

The preparatory phasis is very important for the success of culture and specially for reducing the delay to obtain exponential multiplication and in vitro cloning. For all the plant material we selected, no success was obtained with material collected on the tree and introduced directly in vitro. This failure is often due to infection and difficulties encountered in disinfecting the plant material without killing the explant. Successive grafting and rejuvenation seems to be a need for mature plant material. In this way, the number of subcultures needed for obtaining a reactive cluster (exponential multiplication) is reduced.

Many problems occur in vitro specially when we started the development of the method at the industrial level.

Bacterial pollution can appear after 7 or 8 transfers, later after primary culture on selection of clean culture. ZIMMERMANN wrote about such contamination in micropropagation of fruit trees species. This problem shows that a particular attention must be paid during the phasis Ia and for industrial micropropagation. Indexation of culture must be done at the end of phasis Ib, before in vitro cloning. Indexation of culture can be done by addition of malt extract or peptone (10-100 mg l⁻¹) to the multiplication medium.

The small number of reactive cultures in phasis Ia can be bound to the disinfection method or hormonal endogenous gradients along the stem of cuttings. To obtain a clone under sterile conditions, it is therefore necessary to have a sufficient [sic] number of nodes for conducting factorials trials with combination of time of disinfection and cytokinin concentration.

Callus and brownish compounds released in the medium present also a problem at this point of the culture (phasis I) and we indicated earlier a method to decrease or avoid such problems.

During phasis II, waterlogging (hyperhydric transformation) and a too small elongation of the axillary bud are the two main problems.

Waterlogging often occurs in in vitro culture and the causes of this phenomenon have been investigated. When it occurs in our subcultures (phasis II), it is often confined to one or few buds and does not necessarily extend to the whole of the explant. Transferring the culture to medium A (elongation medium, i.e. medium M with activated charcoal added at the rate of 15 g l⁻¹) promotes the development of untransformed buds which may then be taken off for a new multiplication process. This type of activated charcoal containing medium, can also be used for the selection of stems, without callus on leaves or buds (clone 16), which can sometimes occur on multiplication medium.

A too small elongation of axillary buds is often a limitation to the obtention of a large number of stems which could be transferred on R I medium. Often, 2 to 4 buds only by culture, present the

⁴Melfert : patented containers Fertel Pot
Tour Maine Montparnasse 75755 PARIS Cedex 15

appropriate height of 10 to 20 mm. On a defined cytokinin concentration there is often a clonal response for elongation, even when decreasing the cytokinin concentration of M medium, some clones show a very small elongation. NKANKA has described [sic] similar problems with *Eucalyptus rudis* and he recommends to use vitamin E (D-L Tocopherol) to promote the elongation. Now, with this compound no or low improvement of elongation was observed in our cultures. Research to solve this problem should be oriented toward the mineral composition of the medium and also on the size of the explant in transfer. Using too small explants in transfer increase the budding but gives no elongation of the developed buds.

In phasis III, the main problems are formation of callus at the basis of the stem on R I medium and sometimes rooting of leaves in contact with the medium and also necrosis of the main stem.

Some hybrids as *gunnii* x *globulus*, root easily but with a very big and hard callus and a poor root system develops from this structure. Such callus is a problem for acclimatization because it is a source of root rot.

Decreasing sucrose concentration just limits the phenomenon but it is always present. A better definition of optimum mineral medium and the use of smaller concentration of auxins must be investigated to avoid such callus.

The ability of leaves to induce roots when they are in contact with an auxin containing medium necessitates the complete elimination of leaves at the basal part of explant before planting on R I medium. This operation is time consuming.

For necrosis of main axis at the end of rhizogenesis induction, it is possible that dark period is partially responsible for the phenomenon. R A medium corrects partially this drawback but attention must be paid to the growing time on medium M before root induction and works should be conducted to try to suppress this dark period without decreasing the rooting percentage. The substitution [sic] of agar for rockwool which gives a better aeration of the medium has begun with encouraging results.

CONCLUSION

The method presented in this paper allowed to produce about 20,000 plants from 10 to 12 clones. Most of these in vitro plantlets are used as mother trees for cuttings. But the cost of the plantlets do not yet allow to use it for reforestation. Nevertheless, the use of in vitro plantlets as mother trees for the production of cuttings is interesting and the rooting ability of cuttings taken from such plants is better than this of cuttings taken from scions or in vivo cuttings. The transfer on cytokinin medium for a long time increases the rooting potentiality. This is well demonstrated by clone 16 (old selected tree).

Another advantage of this method is to maintain the rooting ability of the clone. Some of our clones have been introduced in vitro for 3 years now and they kept their rooting potential

Most of the problems we described must be solved for "mass propagation of eucalypts" at a reasonable cost, but presently we can easily produce good mother trees, even from mature material.

It is also very interesting to see that the multiplication medium M can be used with some minor adaptation (cytokinin concentration) for various clones, species and hybrids.

We think that for obtaining the mass propagation of eucalypts by axillary budding under in vitro conditions, research should be conducted on the mechanization of the process.

The major part of the costs of a plant is labor. Some other ways must also be investigated, as somatic embryogenesis. But with this method, attention must be paid to the genetic conformity of the production.

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